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Evolutionary history and biogeography of the genus *Scrophularia* (Scrophulariaceae) and hemiparasitic Orobanchaceae (tribe Rhinantheae) with emphasis on reticulate evolution

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vorgelegt von

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Diese Dissertation wurde angefertigt

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Eidesstattliche Versicherung und Erklärung

Eidesstattliche Versicherung

Ich, Agnes Scheunert, versichere hiermit an Eides statt, daß die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

München, den 14.12.2016 _____

Agnes Scheunert

Erklärung

Diese Dissertation wurde im Sinne von § 12 der Promotionsordnung von Prof. Dr. Günther Heubl betreut. Hiermit erkläre ich, Agnes Scheunert, dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist, und daß ich mich anderweitig einer Doktorprüfung ohne Erfolg nicht unterzogen habe.

München, den 14.12.2016 _____

Agnes Scheunert

Declaration of author contribution

In this cumulative thesis, I present the results of my doctoral research, which was conducted at the Ludwig-Maximilians-Universität of Munich under the supervision of Prof. Dr. Günther Heubl. The results of my research have been published or accepted for publication in international peer-reviewed journals. The four articles included in this dissertation are presented in chapter 4; all of them have resulted from collaboration with other scientists, and my contributions to each of them were as follows:

Article I:
Scheunert A, Fleischmann A, Olano-Marín C, Bräuchler C, Heubl G. 2012. Phylogeny of tribe Rhinantheae (Orobanchaceae) with a focus on biogeography, cytology and re-examination of generic concepts. Taxon 61(6): 1269-1285.

The study was designed by G. Heubl and C. Bräuchler. I did the conceptual design of the methodical treatment of incongruence, and conducted most of the respective analyses. I revised the manuscript, coordinated generation of complementary sequences and did their editing and alignment, re-analyzed most of the data, did the final phylogenetic analyses and interpreted the results. I wrote large parts of the Material and Methods and Results sections and created most of the figures. A. Fleischmann co-supervised the study, selected the taxon sampling, determined the plant material and interpreted the results. He helped to draft the manuscript, wrote large parts of the Introduction and Discussion including the taxonomic treatment, made the taxonomic combinations, and contributed photographs to the manuscript. C. Olano-Marín conducted the laboratory work, did most of the initial phylogenetic analyses, provided a photograph and contributed to the manuscript. C. Bräuchler helped with the selection of herbarium material, improved the manuscript in its initial phase, created some of the graphics and submitted the sequence data to NCBI's Genbank. G. Heubl supervised the work, helped with the conception of the manuscript and improved and corrected the manuscript.

Article II:
Scheunert A, Heubl G. 2011. Phylogenetic relationships among New World *Scrophularia* L. (Scrophulariaceae): new insights inferred from DNA sequence data. Plant Systematics and Evolution 291: 69-89.

G. Heubl designed the study. I selected the taxon sampling, and did the laboratory work including DNA extraction, PCR amplification and product purification, sequencing, and subsequent sequence editing, alignment and phylogenetic analyses. I also planned and performed the dating analyses and the statistical tests. I interpreted the results, wrote the manuscript and created the figures, tables and maps. G. Heubl supervised the study, the laboratory work and the phylogenetic analyses, helped to interpret the data and improved the manuscript.

Article III:
Scheunert A, Heubl G. 2014. Diversification of *Scrophularia* (Scrophulariaceae) in the Western Mediterranean and Macaronesia - phylogenetic relationships, reticulate evolution and biogeographic patterns. Molecular Phylogenetics and Evolution 70: 296-313.

The study was designed by G. Heubl and myself. I selected the taxon sampling, cultivated several of the plants in the greenhouses of the Botanical Garden in Munich, and determined the used specimens. I designed the methodical concept to address hybridization and incongruence, including the use of phylogenetic networks and the taxon duplication approach in combination with ancestral area reconstruction. I supervised the laboratory work, did editing and alignment of sequence data, conducted the statistical tests, performed the phylogenetic analyses, indel coding and biogeographic reconstruction, and created the haplotype and consensus networks. Finally I interpreted the results, wrote the manuscript and prepared the figures, tables and maps. G. Heubl supervised the study, helped to interpret the results and improved and corrected the manuscript.

Article IV:
Scheunert A, Heubl G. Against all odds: reconstructing the evolutionary history of *Scrophularia* (Scrophulariaceae) despite high levels of incongruence and reticulate evolution. Organisms Diversity and Evolution, in press.
doi: 10.1007/s13127-016-0316-0

G. Heubl and myself developed the concept of the study. I generated sequence data and supervised the laboratory work. I conducted an extensive literature search on the topics of among-dataset incongruence, possible treatments of intra-individual-polymorphism, and the evolutionary characteristics of ITS. Based on this I compiled the workflow used in the study to examine hybridization and other sources of phylogenetic incongruence and ambiguity. I conducted indel and character coding and compared the available methods. I performed data analysis (statistical tests, phylogenetic tree reconstruction, biogeography, divergence dating, consensus and Neighbor-Net networks) and interpretation, wrote the manuscript and prepared the figures, tables and the dispersal map. G. Heubl supervised the study and improved and shortened the manuscript.

Munich, 13/12/2016

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Agnes Scheunert

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Prof. Dr. Günther Heubl (supervisor)

All photographs, images and illustrations in this work were made by Agnes Scheunert unless denoted otherwise.

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Für meine Mama.

Contents

1. Summary

This dissertation presents the results of my doctoral studies, which were focussed on main phylogenetic relationships and important evolutionary processes within two families of Lamiales, Scrophulariaceae Juss. (dealing with the type genus *Scrophularia* L.) and Orobanchaceae Vent. (tribe Rhinantheae Lam. & DC.). Rhinantheae are hemiparasitic plants with a predominantly Northern Hemisphere distribution, alongside some representatives which have radiated on southern continents, e.g. the genus *Bartsia* L. sensu Molau (1990) with several Andean and two afromontane species. The figwort genus *Scrophularia* is widespread across the temperate Northern Hemisphere. It comprises about 250 species of mainly herbaceous or suffrutescent perennials, and is characterized by considerable morphological variability and chromosomal diversity. Reticulate evolution is common in *Scrophularia* as well as several genera within Rhinantheae.

The aim of this thesis was 1) to test previous phylogenetic hypotheses of Rhinantheae, also by including several small genera; 2) to investigate phylogenetic relationships within *Bartsia* sensu Molau (1990) and *Odontites* Ludw., and to re-evaluate existing taxonomic concepts; 3) to verify the monophyly of *Scrophularia* and, for the first time, establish a comprehensive phylogenetic framework including all relevant lineages; 4) to reconstruct the biogeographic history of the genus, to gain insight into colonization pathways and the processes which have formed present distribution patterns; 5) to determine possible causes for the high species diversity and endemicity in several areas of the distribution range; 6) to assess the influence of hybridization on the evolutionary history of *Scrophularia*, and to identify possible parental lineages of polyploid (and homoploid) hybrid species; 7) to examine the impact of hybridization and other processes on molecular phylogenetic reconstruction; and 8) to explore how the problems resulting from this influence can be alleviated or even utilized to elucidate the underlying processes; and to compile corresponding practical workflows.

To achieve these goals, I followed a molecular systematic approach. Nucleotide sequences from five non-coding chloroplast loci (intergenic spacers *trnQ-rps16* and *psbA-trnH*, the *trnL-trnF* region, part of the *trnK* region, and the *rps16* intron) as well as nuclear ribosomal DNA (the internal transcribed spacer region, ITS) were analyzed to infer phylogenetic relationships. This was done using Bayesian inference, Maximum Likelihood and Maximum Parsimony approaches, and statistical parsimony networking. Information from nuclear intra-individual sequence polymorphisms as well as plastid indels was coded, and selected individuals were cloned for the ITS marker. Regarding plastid and nuclear data, among-dataset incongruence and within-dataset uncertainty and conflict were assessed with statistical tests and examined using unrooted phylogenetic networks (split networks). The biogeographic history of the genus was inferred using divergence time estimation and ancestral area reconstruction. Where appropriate, a taxon duplication approach was applied to permit the incorporation of incongruent taxa into a combined analysis of markers from both compartments. Plant material for the studies was obtained from specimens on loan from several herbaria and collected during herbarium studies at Vienna (W/WU) and Harvard University (A/GH); several species were cultivated at the greenhouses at the Botanical Garden in Munich.

According to my phylogenetic analyses, Rhinantheae contain a core group of four major lineages. *Bartsia* sensu Molau (1990) is polyphyletic; most species belong to either the African genus *Hedbergia* Molau or the hitherto monotypic *Bellardia* All., with the latter also including *Parentucellia* Viv. For *Odontites*, a broader circumscription was proposed; Rhinantheae are updated to comprise app. 13 genera. Topological incongruence among plastid and nuclear trees in some cases could be the result of intergeneric hybridization; this process might also have created the genera *Nothobartsia* Bolliger & Molau and *Odontitella* Rothm. However, plastid and nuclear markers can be reconciled by pruning conflicting taxa.

By contrast, my studies on *Scrophularia* revealed far more substantial plastid - nuclear marker incongruence. In ITS, intra-individual nucleotide polymorphism is widespread, which argues for incomplete concerted evolution and causes topological ambiguity. I inferred these phenomena to be caused by a combination of reticulation (including introgression), incomplete lineage sorting ("ILS") of ancestral polymorphisms, and, regarding ITS, possibly incipient pseudogenization and inter-array heterogeneity. Species diversity in *Scrophularia* is however largely attributable to ancient and recent hybridization and (allo-)polyploidization, for which I was able to illustrate several examples. It is evident that these processes were one major driving force in the evolutionary history of the genus. The other key factor likely was allopatric speciation, promoted by the heterogeneous topography of the preferred mountain habitats. Often, these two factors have supposedly acted in combination, also under the influence of different climatic periods and associated temperature oscillations (including the Pleistocene glaciations). The emergence and diversification of *Scrophularia* are closely linked to geological and climatic events in the Irano - Turanian region and Central / Eastern Asia; the genus originated in Southwestern Asia during the Miocene. Most diversification events and further successful dispersals to its present distribution areas occurred in the Pliocene and Pleistocene. This also involved the colonization of the New World across the Bering Land Bridge and dispersal to the Macaronesian and Caribbean islands. My analyses support two main species groups in *Scrophularia*, which correspond to previously proposed major sections. The genus is monophyletic upon inclusion of the Himalayan genus *Oreosolen* Hook.f. Like in Rhinantheae, morphological synapomorphies are rarely present, and some morphological traits have presumably evolved in convergence.

Scrophularia represents a useful model for studying evolutionary history in the context of reticulation. This dissertation provides the first comprehensive phylogenetic framework for the genus, which will serve as a solid basis for subsequent research. The methodical workflows implemented in this thesis constitute valuable guidelines for researchers dealing with similarly complex plant lineages.

1. Zusammenfassung

Die vorliegende Dissertation stellt die Ergebnisse meiner Doktorarbeit vor, die die grundsätzlichen phylogenetischen Beziehungen und wichtige evolutionäre Prozesse innerhalb zweier Familien der Lamiales zum Schwerpunkt hatte, den Scrophulariaceae Juss. (und hierbei die Typusgattung *Scrophularia* L.) und den Orobanchaceae Vent. (Tribus Rhinantheae Lam. & DC.). Die Rhinantheen sind halbparasitische Pflanzen mit einer vornehmlich nordhemisphärischen Verbreitung, abgesehen von einigen Vertretern mit Radiationen auf südlichen Kontinenten, wie z.B. der Gattung *Bartsia* L. sensu Molau (1990) mit mehreren andinen sowie zwei afromontanen Arten. Die Gattung

Scrophularia, die Braunwurz, ist weitverbreitet auf der gemäßigten Nordhalbkugel. Sie umfaßt ca. 250 hauptsächlich krautige oder halbstrauchige, mehrjährige Arten, und ist durch erhebliche morphologische Variabilität sowie eine Vielfalt an Chromosomenzahlen gekennzeichnet. Vernetzte Evolution ("reticulate evolution") ist häufig, wie auch in einigen Gattungen der Rhinantheae.

Das Ziel dieser Doktorarbeit war 1) bestehende stammesgeschichtliche Hypothesen betreffend die Rhinantheae zu überprüfen, auch unter Einbeziehung mehrerer kleiner Gattungen; 2) phylogenetische Beziehungen innerhalb *Bartsia* sensu Molau (1990) und *Odontites* Ludw. zu erforschen, und bestehende taxonomische Konzepte neu zu bewerten; 3) die Monophylie von *Scrophularia* zu bestätigen und erstmalig ein umfassendes verwandtschaftliches Grundgerüst zu etablieren, das alle relevanten Linien umfaßt; 4) die biogeographische Entwicklung der Gattung zu rekonstruieren, um Einblicke in Ausbreitungswege zu erhalten sowie in die Prozesse, welche gegenwärtige Verbreitungsmuster geformt haben; 5) mögliche Gründe für die hohe Artdiversität und den Endemismus in mehreren Bereichen des Verbreitungsgebiets zu finden; 6) den Einfluß von Hybridisierung auf die Evolutionsgeschichte von *Scrophularia* einzuschätzen, und mögliche Elternlinien polyploider (und homoploider) hybridogener Arten zu identifizieren; 7) die Einwirkung von Hybridisierung und anderen Prozessen auf die molekularbasierte Rekonstruktion von Stammesgeschichte zu untersuchen; und 8) zu erkunden wie die Probleme, die sich aus diesem Einfluß ergeben, abgeschwächt oder sogar nutzbar gemacht werden können, um die zugrundeliegenden Prozesse aufzuklären; und entsprechende praktische Arbeitsabläufe zusammenzustellen.

Um diese Ziele zu erreichen, verfolgte ich einen molekularsystematischen Ansatz. Nukleotidsequenzen von fünf nicht-codierenden Chloroplasten-Loci (intergenic spacer *trnQ-rps16* und *psbA-trnH*, die *trnL-trnF* Region, ein Teil der *trnK* Region, und das *rps16* Intron), sowie ribosomale Kern-DNA (die internal transcribed spacer Region, ITS), wurden analysiert, um auf Verwandtschaftsbeziehungen rückzuschließen. Dabei wurden Bayes'sche Statistik, Maximum-Likelihood- und Maximum-Parsimony-Methoden, und Netzwerke auf Basis statistischer Parsimonie verwendet. Information aus nukleären intraindividuellen Sequenzpolymorphismen und plastidären Insertionen-Deletionen (Indels) wurde codiert, und ITS-Sequenzbereiche ausgewählter Individuen wurden kloniert. Inkongruenzen zwischen den Plastiden- und Kerndatensätzen sowie Konflikt und Unklarheit innerhalb derselben wurden mittels statistischer Tests bewertet, und unter Verwendung von nicht-gewurzelten phylogenetischen Netzwerken (Split-Netzwerken) untersucht. Aus der Datierung von Artaufspaltungen (divergence time estimation) sowie der Rekonstruktion ursprünglicher Verbreitungsgebiete (ancestral area reconstruction) wurde die historische Biogeographie der Gattung abgeleitet. Wo nötig wurde eine Methode zur Aufteilung der Sequenzinformation einzelner Akzessionen (taxon duplication approach) angewandt, um inkongruente Taxa in eine kombinierte Analyse von Markern beider Kompartimente einbeziehen zu können. Pflanzenmaterial für die Studien wurde von entliehenen Belegen diverser Herbarien entnommen, auch wurden Herbarstudien in Wien (W/WU) und an der Universität Harvard (A/GH) durchgeführt; mehrere Arten wurden in den Gewächshäusern des Botanischen Gartens München kultiviert.

Meine Abstammungsanalysen zeigen, daß die Tribus Rhinantheae eine zentrale Artengruppe aufweist, die aus vier Hauptlinien besteht. *Bartsia* sensu Molau (1990) ist polyphyletisch; die meisten Arten gehören entweder der afrikanischen Gattung *Hedbergia* Molau an oder der bis dato monotypischen *Bellardia* All.; letztere beinhaltet auch *Parentucellia* Viv. Eine breitere Umschreibung wurde für *Odontites* vorgeschlagen;

die Tribus Rhinantheae besteht nun aus ca. 13 Gattungen. Inkongruenzen bezüglich der Position von Taxa in Plastiden- versus Kernmarker-Stammbäumen könnten in einigen Fällen das Ergebnis gattungsübergreifender Hybridisierung sein; dieser Prozess könnte auch die beiden Gattungen *Nothobartsia* Bolliger & Molau und *Odontitella* Rothm. erzeugt haben. Nach Ausschluß zwiespältiger Akzessionen können plastidäre und nukleäre Marker jedoch kombiniert analysiert werden.

Im Gegensatz dazu offenbarten meine Studien an *Scrophularia* weit beträchtlichere Konflikte zwischen Chloroplasten- und Kernmarkerdaten. Zusätzlich sind innerhalb der ITS-Sequenzen häufig Nukleotid-Polymorphismen vorhanden, was zu Unsicherheiten in der Baumtopologie führt und für eine unvollständige Homogenisierung verschiedener Sequenzkopien spricht (incomplete concerted evolution). Diese Phänomene sind nach meinen Schlußfolgerungen das Ergebnis einer Kombination von retikulater Evolution (inklusive Introgression), unvollständiger Aufteilung ursprünglicher Allelvarianten (incomplete lineage sorting, "ILS"), und, im Fall von ITS, möglicherweise beginnender Pseudogenisierung und abweichender Kopien in getrennten Genbereichen. Die Artenvielfalt in *Scrophularia* ist jedoch zum großen Teil auf historische und rezente Hybridisierung und (Allo-)Polyploidisierung zurückzuführen, für die ich mehrere Beispiele aufzeigen konnte. Es ist offensichtlich, daß diese Prozesse eine der Hauptantriebskräfte in der Evolutionsgeschichte der Gattung waren. Der andere Schlüsselfaktor war wahrscheinlich geographische Artbildung, gefördert durch die ungleichmäßige Topographie der bevorzugten bergigen Habitate. Diese beiden Faktoren wirkten vermutlich des Öfteren kombiniert, auch unter dem Einfluß verschiedener Klimaperioden und der dazugehörigen Temperaturschwankungen (inklusive der Vergletscherungen während des Quartärs). Das Erscheinen und die Auffächerung von *Scrophularia* sind eng mit geologischen und klimatischen Ereignissen in der irano-turanischen Florenregion sowie in Zentral- und Ostasien verbunden; die Gattung entstand in Südwestasien, während des Miozän. Die meisten Artaufspaltungsereignisse und die weitere erfolgreiche Ausbreitung in ihre heutigen Verbreitungsgebiete fanden im Pliozän und Pleistozän statt. Dies beinhaltete auch die Kolonisierung der Neuen Welt über die Bering-Landbrücke und die Ausbreitung auf die makaronesischen und karibischen Inseln. Meine Untersuchungen stützen zwei große Artgruppen in *Scrophularia*, die bisher vorgeschlagenen Hauptsektionen entsprechen. Die Gattung ist monophyletisch nach Einbeziehung der himalayischen Gattung *Oreosolen* Hook.f. Wie in den Rhinantheen sind kaum gemeinsame abgeleitete morphologische Merkmale (Synapomorphien) vorhanden, und einige morphologische Eigenschaften haben sich vermutlich konvergent entwickelt.

Scrophularia stellt ein geeignetes Modell dar, um Evolutionsgeschichte unter dem Einfluß von Retikulation zu erforschen. Diese Dissertation legt das erste umfassende Grundgerüst der Stammesgeschichte der Gattung vor, das als solide Basis für weitergehende Forschungen dienen wird. Die methodischen Arbeitsschritte, die in dieser Arbeit angewandt wurden, bilden wertvolle Richtlinien für Forscher, die mit ähnlich komplexen Pflanzengruppen arbeiten.

2. Introduction

2.1. Biology and Systematics of Scrophulariaceae Juss.

2.1.1. Traditional circumscription of Scrophulariaceae

In his book "Genera Plantarum, secundum ordines naturales disposita", Antoine Laurent de Jussieu in 1789 validly published the scientific name Scrophulariaceae Juss., the figwort family, as an order named "Scrophulariae" (Jussieu, 1789). However, the name had been mentioned already in 1782 by Jean François Durande, who presented a French translation of de Jussieu's classification system in his "Notions élémentaires de botanique" (Durande, 1782). David Don (1835) and George Don (1838) provided a description of "Scrophularineae" containing nine and eleven tribes, respectively (see Table 1), while Verbascin(e)ae, Rhinanthaceae, Orobancheae, Cheloneae, Sibthorpiaceae and Aragoaceae were kept as separate families. At the same time, George Bentham presented a first small account on the family proposing 12 tribes (Bentham, 1835, 1836). For de Candolle's "Prodromus" (Bentham, 1846), he improved this concept and also included three subfamilies; and in "Genera Plantarum" (Bentham, 1876), he re-structured his classification, recognizing subfamilies Pseudosolaneae, Antirrhin(o)ideae and Rhinanth(o)ideae. Amongst other characters, he distinguished infrafamiliar groups based on the mode of aestivation of the corolla: in Antirrhinoideae (and Pseudosolaneae), the posterior / upper corolla lobes or the upper lip is external in bud (antirrhinoid aestivation, see Fig. 1), while in Rhinanthoideae the lower lip (respectively either the anterior / lower lobe or the lateral lobes) are positioned externally (rhinanthoid aestivation). The use of this characteristic later was adopted by many authors; although occasionally it may be hard to determine, it has long been the basis for distinguishing subfamilies in Scrophulariaceae (see Armstrong and Douglas, 1989). Indeed, the latter authors also concluded that corolla aestivation may *per se* be suitable for distinguishing higher taxonomic categories.

Based on Bentham (1876), von Wettstein presented a treatment of Scrophulariaceae in Engler and Prantl's "Die natürlichen Pflanzenfamilien" (Wettstein, 1891), which also used corolla aestivation as diagnostic character, and has been widely referred to until today. Scrophulariaceae sensu Wettstein (1891) are photosynthetic or root parasitic (including hemiparasitic) herbs, subshrubs, shrubs or trees, with alternate, opposite or whorled leaves, without stipules and with diverse inflorescences bearing axillary, zygomorphic flowers, which are characterized by a persisting calyx, mostly four stamens, and a bilocular ovary with central placentation; the fruits are capsules (or berries), and the seeds contain endosperm. The author emphasized the considerable morphological variability within the family, but remarked that anatomical characters were considered helpful in separating Scrophulariaceae from other families and in diagnosing groups of genera. However, he also stated that close relationships often hindered reliable delimitations, and emphasized the need for using combinations of vegetative and floral characters for distinguishing families. Twelve tribes were recognized (Table 1): Verbasceae and Aptosimeae (Pseudosolaneae), Calceolarieae, Hemimerideae, Antirrhineae, Cheloneae, Manuleae and Gratioleae (Antirrhinoideae), and Digitaleae, Gerardieae and Rhinantheae (Rhinanthoideae); furthermore, compared to Bentham (1876), Wettstein (1891) added tribe Selagineae to Antirrhinoideae.

The lack of distinct synapomorphic characters for Scrophulariaceae, together with the striking similarities to closely related families, encouraged Hallier (1903) to include four more tribes: Leucophylleae (already proposed within Pseudosolaneae by

Bentham, 1876), Plantagineae, Lentibularieae, and Orobancheae. Hallier (1903) was the first taxonomist to abandon the concept of delimiting subfamilies based on corolla aestivation. Further infrafamiliar concepts are listed in the comprehensive review by Thieret (1967). Francis W. Pennell, in two treatments on the Scrophulariaceae of Eastern North America (Pennell, 1919, 1935) dismissed subfamily Pseudosolaneae (which had been erected to accommodate taxa with a supposedly close relationship to Solanaceae) and instead included Verbasceae and Leucophylleae into Antirrhinoideae. Chosen tribal names refer to Bentham (1846), e.g. in resurrecting Veroniceae (which had been given subtribal rank within Digitaleae by Bentham, 1876), Buchnereae (a subtribe of Gerardieae sensu Bentham, 1876, but later used to replace the name Gerardieae which is based on a synonym) or Euphrasieae (which corresponds to Rhinantheae) of the Rhinanthoideae; three additional tribes in Pennell (1919; Paulownieae, Russelieae, Angelonieae) each contain only one, introduced or cultivated genus previously assigned to Cheloneae and Hemimerideae by Wettstein (1891).

Fig. 1. Antirrhinoid aestivation in *Scrophularia kakudensis* Franch. The reddishly marginate two upper lobes are external in bud, the two lateral and lower median lobe are positioned beneath

Since then, many authors have more or less followed Wettstein (1891) in their classifications, especially regarding more "distant" lineages: Cronquist (1981) defined Scrophulariaceae exclusive of Plantaginaceae, Orobanchaceae, Lentibulariaceae, and Globulariaceae, while additionally excluding *Paulownia* Siebold & Zucc. Takhtajan's (1980) treatment agreed with this definition, except for Globulariaceae, which together with Selaginaceae were included into Scrophulariaceae; the treatment comprised four subfamilies, Scrophularioideae, Rhinanthoideae, Globularioideae, and Selaginoideae. Later however, the author incorporated Orobanchaceae into the family as well (Takhtajan, 1987). Thorne (1992), who recognized subfamilies Scrophularioideae, Rhinanthoideae and Orobanchoideae, included Selagineae but not Globulariaceae; he agreed with Hallier (1903) in including Orobanchaceae and (again) Leucophylleae, but did not incorporate Plantaginaceae and Lentibulariaceae. Furthermore, he included Schlegelieae into the family, which he synonymized with Paulownieae.

While several families and enigmatic genera were tentatively in- or excluded from Scrophulariaceae, others were mostly regarded as distinct according to these authors, e.g. Hippuridaceae, Callitrichaceae, *Myoporum* Banks & Sol. ex G.Forst., and *Buddleja* L., although the latter had already been included into Scrophulariaceae as tribe Buddleieae/Buddlejeae (D Don, 1835; G Don, 1838; Bentham, 1835, 1836, 1846). Nonetheless until the early 1990's, Scrophulariaceae were one of the largest families within Lamiales, with app. 30 tribes recognized (according to Barringer, 1993, who himself newly described tribes Alonsoeae, Bowkerieae, Caprarieae and Freylinieae), and with app. 4000 species according to Cronquist (1981) or 3000 species within 220 genera according to Thorne (1992).

2.1.2. Current understanding of phylogenetic relationships

The classical circumscription of Scrophulariaceae was profoundly challenged when DNA-based studies became the central means of investigating phylogenetic

D. Don 1835	G. Don 1838	Bentham 1835/1836	Bentham 1846	Bentham 1876	von Wettstein 1891	Hallier 1903	Pennell 1919	Pennell 1935	Oxelman et al. 2005	APG website 2016
9	11	12	15	12 (12 subtribes)	12 (4 subtribes)	13+3	7+3	10	8	7
			Antirrhinideae	Antirrhinideae	Antirrhinoideae		Antirrhinoideae	Antirrhinoideae		
Calceolarieae	Calceolarieae		Calceolarieae	Calceolarieae	Calceolarieae	Calceolarieae				
		Hemimerideae	Hemimerideae	Hemimerideae	Hemimerideae	**Hemimerideae**			Hemimerideae	Hemimerideae
Antirrhineae	Antirrhineae	Antirrhineae	Antirrhineae	Antirrhineae	Antirrhineae	Antirrhineae	Antirrhineae	Antirrhineae		
Scrophularieae	**Scrophularieae**								**Scrophularieae**	**Scrophularieae**
			Cheloneae	**Cheloneae**	**Cheloneae**	Cheloneae	**Cheloneae**	**Cheloneae**		
			Escobedieae							
Gratioleae	Gratioleae	Gratioleae	Gratioleae	Gratioleae	Gratioleae	Gratioleae	Gratioleae	Gratioleae		
				(Limosellinae)	(Limosellinae)				Limoselleae	Limoselleae
				Manuleieae	Manuleae	Manuleeae				
					Selagineae	Selagineae				
								Collinsieae		
									Myoporeae	Myoporeae
				Pseudosolaneae	Pseudosolaneae					
				Leucophylleae		Leucophylleae		Leucophylleae	Leucophylleae	Leucophylleae
				Aptosimeae	Aptosimeae	Aptosimeae			Aptosimeae	Aptosimeae
		Verbasceae	Verbasceae	Verbasceae	Verbasceae	Verbasceae	Verbasceae	Verbasceae		
			Salpiglossideae							
		Salpiglossideae	Salpiglosseae							
			Rhinanthideae	Rhinanthideae	Rhinanthoideae		Rhinanthoideae	Rhinanthoideae		
Buddlejeae	Buddleieae	Buddle(i)eae	Buddleieae						Buddlejeae	Buddlejeae
		Digitaleae	Digitaleae	Digitaleae	Digitaleae	Digitaleae		Digitaleae		
			Sibthorpieae	(Sibthorpiinae)						
Veroniceae	Veroniceae	Veroniceae	Veroniceae	(Veronicinae)			Veroniceae	Veroniceae		
	Teedieae	Teedieae							Teedieae	
	Hallerieae									
Gerardieae	Gerardieae	Gerardieae	Gerardieae	Gerardieae	Gerardieae	Gerardieae				
Buchnereae	Buchnereae	Buchnereae	Buchnereae	(Buchnerinae)			Buchnereae	Buchnereae		
Euphrasieae	Euphrasieae		Euphrasieae	Euphrasieae				Euphrasieae		
		Rhinantheae			Rhinantheae	Rhinantheae	Rhinantheae			

Table 1. Taxonomic classification of Scrophulariaceae as provided by various authors. The number of recognized tribes is provided beneath each publication; complete references are given in the Literature section. Subfamilies are underlined; names in bold denote the tribe the generic type *Scrophularia* L. was assigned to by the respective author. Subtribes are given only if they refer to a tribe mentioned elsewhere. Hallier (1903) recognized three additional tribes not included in this table (Plantagineae, Lentibularieae, Orobancheae); three further tribes listed by Pennell (1919) each only contained one, introduced or cultivated genus (Paulownieae, Russelieae, Angelonieae)

relationships and character evolution. Olmstead and Reeves (1995) were the first to discover that Scrophulariaceae s.l. were not monophyletic, but composed of two distinct groups of taxa (named "scroph I" and "scroph II" by the authors). Thereby, it also became evident that corolla aestivation in Scrophulariaceae s.l. is a homoplastic character, as both antirrhinoid and rhinanthoid aestivation were found within "scroph II" (see also Young et al., 1999). Further work by Olmstead et al. (2001) revealed that Scrophulariaceae s.str. comprised Bentham's (1876) tribes Verbasceae, Aptosimeae, Hemimerideae without *Angelonia* Bonpl., Manuleeae and Leucophylleae, Wettstein's (1891) Selagineae, and surprisingly the small genera *Myoporum* and *Buddleja* (however included into Scrophulariaceae by Bentham, 1846). As Verbasceae also comprised the generic type *Scrophularia* L., the name of the tribe was consequently proposed to be changed to Scrophularieae (a name already used by D Don, 1835). A second clade named "Veronicaceae" (now Plantaginaceae due to nomenclatural priority reasons) included Bentham's (1876) tribes Digitaleae, Antirrhineae, Cheloneae, and Gratioleae, tribe Angelonieae (as recognized by Pennell, 1919), Globulariaceae without Selagineae, and the two aquatic families Callitrichaceae and Hippuridaceae. Most importantly, the "Veronicaceae" clade also included *Plantago* L. (Plantaginaceae), which in a way corroborated Hallier (1903). The rhinanthoid tribes Buchnereae (Gerardieae) and Rhinantheae (Euphrasieae) were found to cluster with Orobanchaceae, distant from Scrophulariaceae s.str. and Plantaginaceae (see chapter 2.2.2.). Calceolarieae, due to their distinct position, were given family rank, as well as Paulownieae and Schlegelieae. Subsequent work corroborated and expanded these findings, e.g. Beardsley and Olmstead (2002; on Phrymaceae), Müller et al. (2004; on Lentibulariaceae), Albach et al. (2005; on Plantaginaceae), Oxelman et al. (2005; on Lamiales), Rahmanzadeh et al. (2005; on Linderniaceae), Bennett and Mathews (2006; on Orobanchaceae), Schäferhoff et al. (2010; on Lamiales), Barker et al. (2012; on Phrymaceae), Refulio Rodriguez and Olmstead (2014; on Lamiidae); see also the review by Tank et al. (2006). Relationships among families within Lamiales as accepted today are illustrated in Fig. 2.

Within the previously defined Scrophulariaceae s.str., Kornhall et al. (2001) revealed Selagineae to be nested within Manuleeae. Kornhall and Bremer (2004) found that the latter clade also includes *Limosella* L., which resulted in the name of the tribe being changed to Limoselleae (which had been a subtribe of Gratioleae sensu Bentham, 1876). Oxelman et al. (2005) proposed eight tribes based on molecular results: apart from tribes in accordance with Olmstead et al. (2001), i.e. Scrophularieae, Aptosimeae, Hemimerideae, Limoselleae, Leucophylleae, Myoporeae, and Buddlejeae, Oxelman et al. (2005) included Teedieae, consisting of four taxa closely allied to Buddlejeae (and not identical in taxon arrangement to Teedieae sensu G Don, 1838 or Bentham, 1836). The APG website (Stevens, 2001 and onward; see also APG IV, 2016) largely followed this concept, but included Teedieae into Buddlejeae. A description of each tribe is available in Tank et al. (2006), their phylogenetic relationships are shown in Fig. 2.

Scrophulariaceae in their present circumscription comprise 1880 species in 59 genera (Stevens, 2001 and onward) and have a predominantly southern hemispheric distribution, with four of the seven tribes centered in South Africa (Tank et al., 2006). Diagnosis of tribes by morphological characters has remained difficult; many of the traditionally used characteristics may be plesiomorphic, which explains the large similarities among several Lamiales families. As outlined by Tank et al. (2006), Scrophulariaceae s.l. acted as a "repository" for typical Lamiales taxa lacking clear synapomorphic characters (which exist e.g. in Bignoniaceae or Lamiaceae). In consequence, Scrophulariaceae s.l. have contributed taxa to a number of other families in the course of their reduction (see Fig. 2), mainly to Plantaginaceae and Orobanchaceae but also to Phrymaceae (e.g. *Leucocarpus* D.Don from Cheloneae sensu

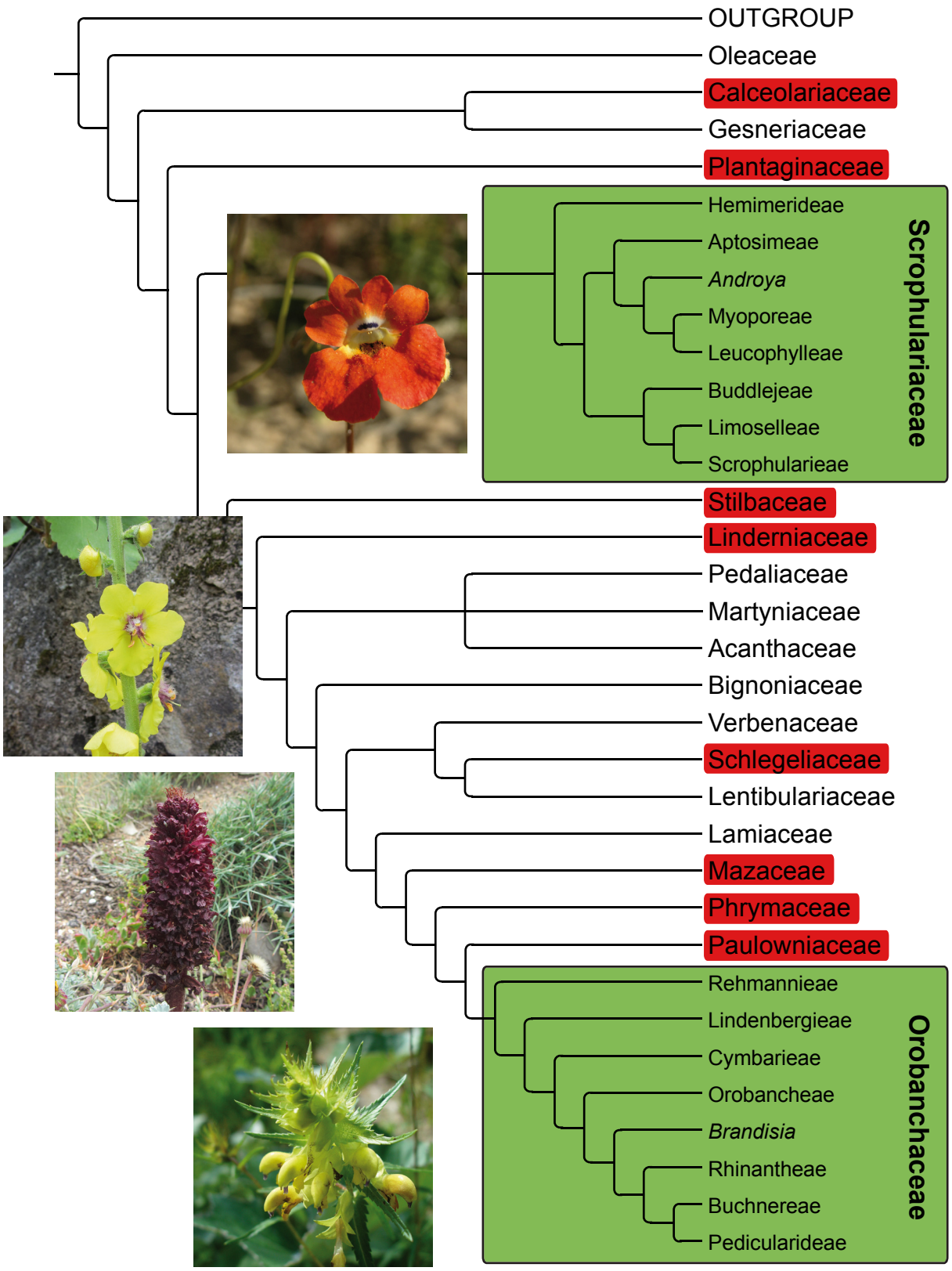

Fig. 2. Phylogenetic relationships in Lamiales. Main tree based on Refulio Rodriguez and Olmstead (2014) and the APG website (Stevens, 2001 and onward). Tribal relationships in Scrophulariaceae and Orobanchaceae are according to Oxelman et al. (2005) and McNeal et al. (2013), respectively. Families which now contain representatives of the former Scrophulariaceae s.l. are marked in red. Selected representative genera from top to bottom - Scrophulariaceae: *Nemesia* Vent., Hemimerideae (photo by Andreas Fleischmann); *Verbascum* L., Scrophularieae; Orobanchaceae: *Orobanche* L., Orobancheae; *Rhinanthus* L., Rhinantheae

Wettstein, 1891; *Mimulus* L. from Gratioleae), Calceolariaceae (from Calceolarieae),

Linderniaceae (e.g. *Torenia* L. from Gratioleae), Stilbaceae (e.g. *Halleria* L. from Cheloneae; see also tribe Hallerieae in G Don, 1838), Mazaceae (e.g. *Lancea* Hook.f. & Thomson from Gratioleae sensu Wettstein, 1891), Paulowniaceae and Schlegeliaceae (e.g. *Paulownia* and *Synapsis* Griseb., respectively, from Cheloneae).

2.2. Parasitism in plants: the example of Orobanchaceae Vent.

2.2.1. Parasitism

Parasitism is an effective strategy found throughout the tree of life (Poulin and Morand, 2000) including flowering plants (Kuijt, 1969). While myco-heterotrophic (i.e., being dependent on mycorrhizal fungi) plants rely on a fungal partner, several parasitic plants obtain other plants as hosts. The roots of the parasites are modified into special organs called haustoria (Kuijt, 1969), which are used to exploit a host by connecting to its stems or roots (stem vs. root parasites). In flowering plants, parasitism is thought to have evolved 12-13 times independently and is now known in 12 orders, with app. 1 % of all angiosperms being parasitic (Westwood et al., 2010). Hemiparasitic plants are photosynthetic and only extract water and nutrients from their host by connecting to the xylem; while some of them are dependent upon a host to mature (obligate parasites), others do not necessarily require this connection to complete their life cycle (facultative parasites). In contrast to hemiparasites, holoparasitic plants are fully heterotrophic and, apart from water and nutrients, also obtain all necessary carbon compounds from their host's phloem. The transition to holoparasitism leads to a variety of changes, including the reduction of leaves, the loss of non-haustorial roots, and in extreme cases most of the vegetative tissues (in Rafflesiaceae) as outlined by dePamphilis et al. (1997). The loss of photosynthesis and the associated pigments is accompanied by a reduction of the plastid genome and a shift towards higher substitution rates in the remaining genes (e.g. in Orobanchaceae Vent.; Wicke et al., 2013). Cusimano and Wicke (2016) found that functional losses in Orobanchaceae occur within 10 million years ("my") after transition to obligate parasitism, although many photosynthesis-related genes survive within the nuclear or mitochondrial genomes. The genomes of nonphotosynthetic species of Orobanchaceae were also shown to contain higher amounts of repetitive DNA (Piednoël et al., 2012).

2.2.2. Orobanchaceae Vent.

In Lamiales, parasitism has evolved only within Orobanchaceae. This plant family today comprises a full spectrum of (few) autotrophic (non-parasitic) plants, facultative and obligate hemiparasites, as well as holoparasites (see Westwood et al., 2010). Its members are globally distributed with a focus on temperate regions of the Northern Hemisphere and the Old World; most genera only comprise few species with limited distributions (Wolfe et al., 2005). It also includes some serious crop pests, e.g. *Striga* Lour., *Orobanche* L., or *Alectra* Thunb. (see citations in Wolfe et al., 2005).

The taxonomic boundaries of Orobanchaceae have changed considerably throughout their history. The first valid description of the family name (as "Orobanchoideae") dates back to 1799 and was provided by Etienne Pierre Ventenat in his "Tableau du Regne Végétal" (Ventenat, 1799, p. 292). G Don (1838) described the "very natural family" as distinctive by their persistent corolla, unilocular ovary (two characters which were partially refuted later, see below), and their usually parasitic life strategy with the herbaceous plants lacking chlorophyll and "proper" leaves. He also

emphasized the minute, globular, undifferentiated embryo typical for Orobanchaceae. Further characteristics of the family, also according to Beck von Mannagetta (1893), included mostly racemose terminal inflorescences, and flowers with usually two-lipped tubular corollas, four didynamous stamens and a superior ovary.

Close relationships of Orobanchaceae to Scrophulariaceae s.l. became evident early. Morphologically intermediate taxa like *Lathraea* L. were placed either in Orobanchaceae (e.g. Beck von Mannagetta, 1893) or Scrophulariaceae (e.g. Warming, 1895). Wettstein (1897) noted close relationships of Rhinantheae and other scrophulariacean genera to Orobanchaceae. Hallier (1903), who included Orobanchaceae as tribe Orobancheae into Scrophulariaceae s.l., discussed in detail the similarities among *Orobanche* and several scrophulariacean taxa from the Rhinantheae and Buchnereae. Boeshore (1920) postulated a morphological series leading from hemiparasitic genera of the "pre-Olmsteadian" Scrophulariaceae s.l. via *Lathraea* to the holoparasitic Orobanchaceae. He also stated that transitions occur between the typically unilocular ovary of Orobanchaceae and the bilocular one of Scrophulariaceae.

At the time of the first molecular evidence for Scrophulariaceae s.l. being polyphyletic (Olmstead and Reeves, 1995), other researchers aimed at revealing the origin of parasitism within Lamiales. In a study that involved both parasitic Orobanchaceae and Scrophulariaceae s.l., dePamphilis et al. (1997) found evidence for a monophyletic clade containing hemi- and holoparasitic Scrophulariaceae s.l. as well as holoparasitic Orobanchaceae. Analyses by Young et al. (1999), based on a larger sampling and more molecular markers, yielded the same results. From these two studies it has become evident that while there is only a single origin of parasitism within the group, holoparasites have evolved several times independently, a finding which refutes Boeshore's (1920) concept of a transitional series leading from hemi- to holoparasitism. Another important result was that hemiparasites of the traditional Scrophulariaceae s.l. are more closely related to Orobanchaceae than to Scrophulariaceae s.str. Young et al. (1999) consequently proposed that the parasitic clade plus the non-parasitic genus *Lindenbergia* Lehm. (Gratioleae sensu Wettstein, 1891) should be defined as Orobanchaceae. Their results were corroborated by Olmstead et al. (2001, see chapter 2.1.2.) and others (Wolfe et al., 2005; Bennett and Mathews, 2006; McNeal et al., 2013).

Thus, the former rhinanthoid tribes Buchnereae and Rhinantheae were moved to Orobanchaceae; however, neither of these tribes as traditionally defined was found to be monophyletic (Young et al., 1999). Subsequent analyses by Wolfe et al. (2005) revealed an additional lineage comprising the hemiparasites *Castilleja* Mutis ex L.f. and *Pedicularis* L., while Bennett and Mathews (2006) identified another clade at a basal position, composed of five taxa including the hemiparasitic *Cymbaria* L. and *Schwalbea* L. Relationships among the five lineages were found to differ depending on the markers used (Young et al., 1999; Wolfe et al., 2005; Bennett and Mathews, 2006). A comprehensive analysis by McNeal et al. (2013), using several molecular markers and the largest sampling to date, resulted in a new tribal classification, in which Buchnereae are sister to Pedicularideae (which includes taxa mainly from Gerardieae and Rhinantheae sensu Fischer, 2004). Sister to both are Rhinantheae; *Brandisia* Hook.f. & Thomson (which had been included into Cheloneae, or different families) is sister to this clade. The basalmost positions are occupied by a grade of Orobancheae, Cymbarieae, and *Lindenbergia*, which is sister to all other Orobanchaceae. The APG website (Stevens, 2001 and onward) followed this concept, adding Lindenbergieae and Rehmannieae. The latter contain the non-parasitic *Rehmannia* Libosch. ex Fisch. & C.A.Mey. and *Triaenophora* Soler., two genera found to be sister to *Lindenbergia* and the remaining Orobanchaceae by Xia et al. (2009). Thus, Orobanchaceae today comprise seven tribes (see Fig. 2), 99 genera and 2060 species (Stevens, 2001 and onward). Apart from those

already mentioned at the beginning of this chapter, morphological characteristics of the family include ascending = rhinanthoid petal aestivation (with very few exceptions), an often galeate upper corolla lip, anther thecae which are acuminate at apex or rounded to mucronate, and a bilocular to usually unilocular ovary (Fischer, 2004).

2.2.3. Tribe Rhinantheae Lam. & DC.

Tribe Rhinantheae Lam. & DC. as defined today has a worldwide distribution with its main diversity center in the Northern Hemisphere. It contains facultative (e.g. the annual *Rhinanthus* L., or *Euphrasia* L.) and obligate (e.g. the perennial *Tozzia* L.) hemiparasites as well as one holoparasite (*Lathraea*). Further important genera are *Odontites* Ludw. and *Bartsia* L.; the most species-rich genus within the tribe is *Euphrasia* with 170-350 species (Stevens, 2001 and onward). Uribe-Convers and Tank (2015) estimated the Rhinantheae clade to be app. 31 million years old, based on secondary calibration using an ITS age estimate by Wolfe et al. (2005), or alternatively a geological constraint.

Morphological synapomorphies for Rhinantheae are largely missing; the typical mode of corolla aestivation in the tribe, rhinanthoid with the two lateral lobes of the corolla clasping the lower median one in bud, is not exclusive within Orobanchaceae (also found in Pedicularideae). Rhinantheae are annual or perennial herbs or subshrubs; the racemose inflorescences feature scale-like, leaf-like or showy bracts. The corolla is bilabiate; the two lobes of the upper lip are usually fused into a helmet-like or rostrate galea. However, the lobes can also be bifid or variously bilobate; in few genera, the upper lip lobes are even more or less free and expanded, resulting in almost actinomorphic corollas, e.g. in *Hedbergia abyssinica* (Benth.) Molau (Fischer, 2004).

Molecular phylogenetic relationships were first revealed by Bennett and Mathews (2006). The authors found *Melampyrum* L. to be sister to the remainder of Rhinantheae, and a clade of *Rhinanthus* and *Lathraea* plus *Rhynchocorys* Griseb. placed in a basal position. The remaining taxa (e.g. *Euphrasia*, *Tozzia*, *Bartsia*, *Odontites*) formed a clade which is here referred to as the "core group of Rhinantheae". It also became clear that *Bartsia* in its traditional circumscription was polyphyletic, with *B. alpina* L. placed distant from the New World species, which grouped with *Parentucellia* Viv. Těšitel et al. (2010) showed *B. alpina* to be sister to the rest of the core group of Rhinantheae. The taxonomically difficult *Odontites* seemed to be paraphyletic with respect to one (*Bornmuellerantha* Rothm.) of the four genera that had been segregated from it by Rothmaler (1943) and Bolliger (1996). Phylogenetic positions of the other three taxa (*Macrosyringion* Rothm., *Bartsiella* Bolliger, *Odontitella* Rothm.) remained however uncertain, as well as the taxonomy of *Bartsia*; *Bartsia trixago* L. is now regarded (again) as distinct genus (*Bellardia* All.), but African *Bartsia* (two species distributed in alpine regions of Eastern Africa) had never been sampled.

Within Rhinantheae, Těšitel et al. (2010) found multiple independent transitions from perennity to annuality in several unrelated lineages and concluded that typical features of annuals must thus be analogous. Reconstructions of seed size evolution revealed large seeds to be an ancestral feature; these are primarily present in basal lineages of Rhinantheae. Furthermore, regarding the group of their Rhinanthus-Lathraea, Rhynchocorys and Melampyrum clades, the largest seeds are found in annual (compared to perennial) species. According to Těšitel et al. (2010), this is possibly due to enhanced light competition pressure on the seedlings in their typical habitats. Smaller, less competitive seeds in *Odontites*, *Euphrasia* and New World *Bartsia* were correlated by the authors with their occurrence in stressful environments characterized by open communities. The combination of reduced seed size together with a preference

for low-competition habitats might have promoted long distance dispersal in the latter two genera (Těšitel et al., 2010).

The biogeographic origin of family Orobanchaceae was hypothesized north of the Tethys Sea by Wolfe et al. (2005). Rhinantheae originated in temperate Western Eurasia according to Těšitel et al., (2010), possibly including the Caucasian region, where *Rhynchocorys* was inferred to have its origin by the authors. Other lineages have arisen in the Mediterranean according to this analysis (e.g. *Odontites*, *Nothobartsia* Bolliger & Molau, *Bellardia*). Uribe-Convers and Tank (2015) found Europe to be the ancestral range for Rhinantheae and several of the backbone node ancestors, as well as *Rhynchocorys* and *Odontites*. The range of the *Bellardia* ancestor could not be unequivocally determined by their analyses: possible ranges included Europe, South America and/or Eurasia.

2.3. The genus Scrophularia L.

2.3.1. Taxonomic history

The first mention of plants with this name is attributed to Dioscorides. Matthaeus Silvaticus, in his famous pharmacopoeia "Liber / Opus pandectarum medicinae", which was finished in 1317 and first printed in 1474, included "Scrofularia" because of its use as a remedy for "scrofula" (Silvaticus, 1498), a disease characterized by infectional swelling of the lymph nodes (see Mann, 2009). According to the Doctrine of Signatures, the use of the plants against scrofula, as well as other "knots" like haemorrhoids, anogenital warts or ulcers continued through medieval times and was based on the knots found on the roots of *S. nodosa* L. (Ehrlich, 1720; Mann, 2009). The Latin genus designation and the English name "knotted figwort" still refer to that use, as do old german names like "Groß Feigwarzen-Kraut" or "Wurm-Kraut" (see Ehrlich, 1720; Mann, 2009). Years before Linné published his master work "Species Plantarum" with formal descriptions of several *Scrophularia* species (Linné, 1753), the Saxon-Thuringian physician Heinrich Christian Ehrlich presented a dissertation on *Scrophularia* (Ehrlich, 1720), in which he compiled ancient and contemporary information on the genus. He provided a morphological characterization himself, while also reproducing several descriptions by former botanists (e.g. Johann Bauhin, Robert Morison, John Ray, Joseph Pitton de Tournefort and August Bachmann/Rivinus), amongst those possibly one of the oldest descriptions of *Scrophularia*, which he attributes to Adam Lonicer, Hieronymus Bock/Tragus and Jacob Theodor/Tabernaemontanus, dating back to the 16th century:

"RADICEM ALBAM, &, FABARIAE IN MODUM, NODOSAM,
CAULES QUADRANGULOS, FUSCOS, FOLIA BASILICO AC URTICAE
CONFORMIA, FLORES COCHLEARUM TESTAS REFERENTES,
VASCULA SEMINALIA EX ROTUNDO ACUMINATA,
SEMINAQUE HYOSCYAMO SIMILIA"

[root pale, and, as in the Fabariae, knotty, shoots quadrangular, dark brownish, leaves like those of basil and *Urtica*, flowers resembling snail shells, seed capsules acuminate from a globe, and seeds similar to those of *Hyoscyamus*]

Apart from characterizing *Scrophularia* morphologically, Ehrlich (1720) mainly concentrated on medicinal aspects. Wydler (1828) was the first to give a detailed account on all species known at the time, and accepted 47 of them. He also cited historic contributions on the genus (also compare Stiefelhagen, 1910), e.g. by C Bauhin (1623), Willdenow (1800), Persoon (1806) and Sprengel (1825), who already knew eight to 48 species. His two proposed main sections were named by G Don (1838) as *Scrophularia* sects. *Venilia* G.Don and *Scorodonia* G.Don (= *S.* sect. *Scrophularia*) and complemented by a third, *Canina* G.Don (Table 2). The genus was placed in tribe Scrophularieae by the author, while Bentham, after first associating it with *Verbascum* L. in Verbasceae (Bentham, 1835), later sorted it into Cheloneae of Antirrhinoideae (Bentham, 1846, 1876). Bentham (1846) recognized *Scrophularia* sects. *Venilia* and *Scorodonia*, but incorporated parts of the latter together with members from *S*. sect. *Canina* into a section which he called *Tomiophyllum* Benth. Boissier (1879), who provided a treatment mainly on the Asian species in his "Flora orientalis", apart from *S.* sects. *Scorodonia* and *Tomiophyllum* recognized *S.* sect. *Ceramanthe* Rchb. (which had been elevated to genus rank by Dumortier, 1834) and added *S.* sects. *Mimulopsis* Boiss. and *Pycnanthium* Boiss., each with one species only. The genus itself was kept in tribe Cheloneae by Boissier (1879) and also by Wettstein (1891), a fact that was criticized by Hallier (1903), who classified it into Hemimerideae while emphasizing its affinities to Verbasceae, i.e., *Celsia* L. / *Verbascum*.

Wydler 1828	G Don 1838	Bentham 1846	Boissier 1879	Stiefelhagen 1910
47	60	79	78	143
"I" (2)	**Venilia G.Don (3)**	**Venilia G.Don (8)**	**Ceramanthe Rchb. (11)**	
			Pycnanthium Boiss. (1)	
			Mimulopsis Boiss. (1)	
"II" (45) "A" (23) "B" (11) "C" (11)	**Scorodonia G.Don (44)**	**Scorodonia G.Don (36)**	**Scorodonia G.Don (16)**	**Anastomosantes Stiefelh. (76)** Vernales Stiefelh. (9) Scorodoniae (Benth.)Stiefelh. (67)
	Canina G.Don (13)	**Tomiophyllum Benth. (35)** Lucidae Benth. (23) Caninae Benth. (12)	**Tomiophyllum Benth. (49)** Oppositifoliae Boiss. (44) Sparsifoliae Boiss. (5)	**Tomiophyllum Benth. (67)** Farinosae Stiefelh. (1) Orientales Stiefelh. (3) Lucidae Stiefelh. (63)

Table 2. Intrageneric classification in *Scrophularia*. (Sub)sections as erected by previous authors (for publication details refer to the Literature section). The number of recognized species is given beneath each publication. Names in bold are main sections, subsections are listed using regular font. Numbers in brackets after (sub)section names represent corresponding species numbers

The most recent classical monographic treatment was done by Stiefelhagen (1910). He recognized 143 species and divided the genus into two main sections and five subsections which are based mainly on leaf vein characteristics (clearly anastomosing or not): *Scrophularia* sects. *Tomiophyllum* Benth. (= *S.* sect. *Canina*) and *Anastomosantes* Stiefelh. (= *S.* sect. *Scrophularia*). In this thesis, to avoid confusion with other taxonomic entities or clades, Stiefelhagen's (1910) names will be used to refer to the two main sections in *Scrophularia*, instead of the nomenclaturally correct designations.

2.3.2. Morphology, distribution and phylogenetic relationships

Today, the genus *Scrophularia* comprises app. 250 species of mainly herbaceous or suffrutescent perennials, and more rarely biennial or annual herbs (some examples

Fig. 3. Selected species of *Scrophularia*, from left to right and top to bottom: *S. kakudensis* Franch., *S. vernalis* L., *S. grandiflora* DC., *S. calliantha* Webb. & Berthel., *S. canina* L. ssp. *hoppii* (W.D.J.Koch) P.Fourn., *S. villosa* Pennell, *S. lowei* Dalgaard, *S. nodosa* L., *S. smithii* Hornem. ssp. *smithii*

are shown in Fig. 3). The plants are glabrous to densely villose, sometimes foetid, and characterized by mostly quandrangular stems with opposite leaves (the upper ones sometimes alternate) of various shapes and margin types. The inflorescence is typically a thyrse, can be bracteolate or frondose, and wears few- or many-flowered cymes

arranged in mainly simple or compound dichasia. The flowers are generally zygomorphic (now rather an exception within Scrophulariaceae, see Tank et al., 2006), having a five-lobed calyx with or without a scarious margin, a sympetalous, bilabiate, tubular to subglobose, typically ventricose corolla of greenish, reddish and/or brownish color, and four fertile stamens which are more or less didynamous and have unilocular, reniform anthers. The fifth (adaxial) stamen is generally sterile, forming a staminode consisting of a basal part that is normally adnate to the upper corolla lip (the former filament) and a free, scale-like part (the former anther), which can have various shapes and sizes (clearly visible in the flower in Fig. 3i). The superior ovary is bilocular and connected to a capitate to weakly bilobed stigma; a fleshy, nectariferous disc is found at the base of the ovary. The fruit is a septicidal, globose to subconical, often apiculate capsule containing numerous small seeds; these have an alveolated endosperm with elongated alveoles (Fischer, 2004).

Similarities to the sister genus *Verbascum* include the general floral morphology and leaf architecture, septicidal many-seeded capsules, often cymose inflorescences, tricolporate pollen and single-celled subepidermal idioblasts. Further analogies are found in habitat preferences and the temperate northern hemispheric distribution with a Southwestern Asian center of diversity (see below), which also is unusual within Scrophulariaceae s.str. (Hallier, 1903; Lersten and Curtis, 2001; Fischer, 2004; Oxelman et al., 2005). Plesiomorphic characters, which are shared with other lineages, are typical for Scrophulariaceae s.l. as described above, and are also present in *Scrophularia* and *Verbascum*. Examples are the longitudinally ridged seeds also found in *Limosella* (Scrophulariaceae s.str.), *Linaria* Mill. (Plantaginaceae), *Lindernia* All. (Linderniaceae), Orobanchaceae and others (Hallier, 1903), or the presence of iridoids which cause the typical blackening of plant material upon drying, and which as iridoid glycosides may deter herbivores by their bitter taste (Fischer, 2004). In *Scrophularia*, morphological characteristics in general are considered extremely variable among and within species (Stiefelhagen, 1910; Grau, 1981a, b) and are not necessarily useful for delimitation of species or species groups (Wydler, 1828; Grau, 1981b). The high morphological plasticity found in the genus is also correlated with a surprisingly high tendency to hybridization and polyploid formation (chromosome numbers range from 2n = 18 to 2n = 96; Shaw, 1962; Goldblatt and Johnson, 1979 and onward); successful artificial crossings were made by e.g. Goddijn and Goethart (1913), Shaw (1962), or Dalgaard (1979), and several authors mentioned natural hybrids, e.g. Stiefelhagen (1910), Pennell (1943) or Grau (1981a).

Species of *Scrophularia* occur in forests, on river banks, in scrubs and grassland, in rock crevices or on gravelly substrates, on mountain slopes or cliffs, and also along roadsides and in disturbed places. Several species are found in shady or moist habitats; others are xerophytic and occur also in dry environments, while the genus comprises only very few desert plants. *Scrophularia* occurs from coasts and lowlands to alpine regions, with the majority of species inhabiting mountainous regions and highland plateaus (Stiefelhagen, 1910; Shaw, 1962). The distribution of the genus extends throughout the temperate zone of the Northern Hemisphere (Fig. 4), with very few species expanding into tropical regions (these are mostly limited to higher altitudes, e.g. in the Greater Antilles). The primary diversity center is situated in the Irano - Turanian floristic region sensu Takhtajan (1986), with emphasis on Iran and Turkey (42 and 59 species; Flora of Turkey, Lall and Mill, 1978; Flora Iranica, Grau, 1981a; Flora of Turkey Supplement, Davis et al., 1988), Afghanistan (Grau, 1981a), and, within the area of the Flora of the USSR, the Caucasian and Central Asian region (Gorschkova, 1997). New species are continuously described (e.g. Attar, 2006; Attar and Hamzeh'ee, 2006; Attar et

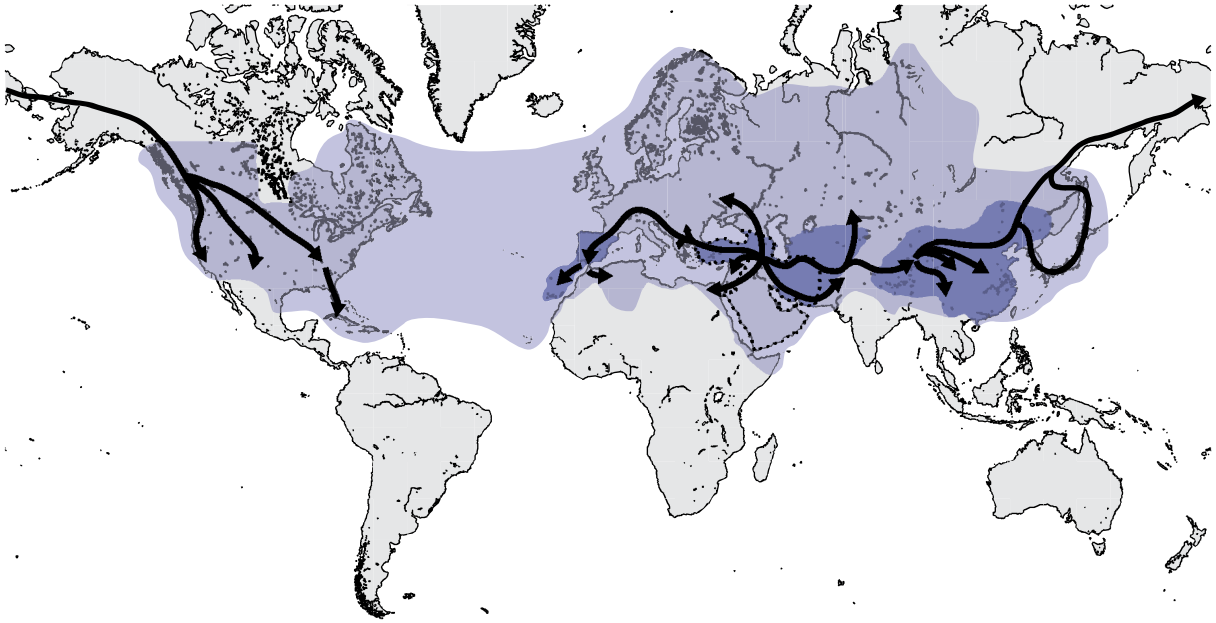

Fig. 4. Distribution of *Scrophularia* and assumed migration routes. The ancestral region of the genus as inferred by biogeographic reconstructions is marked by a dashed line. The present-day distribution of the genus is shown in light blue, centers of species diversity are highlighted in dark blue. The primary diversity center is located close to the ancestral region of the genus. Black arrows indicate main dispersal routes as inferred from plastid DNA data. Note that arrows in some cases may illustrate more than one dispersal

al., 2006; Mozaffarian, 2010; Ahmad, 2014; Kandemir et al., 2014; Uzunhisarcıklı et al., 2015). Secondary centers of species richness are located in China (36 species; Flora of China, Hong et al., 1998) and southwards in the Himalayan region and Pakistan, as well as the Iberian Peninsula and Macaronesia (28 species; Dalgaard, 1979; Flora Iberica, Ortega Olivencia, 2009) and adjacent areas. African species are mostly restricted to the Mediterranean realm (Hartl, 1965). Apart from treatments for the floras of the most species-rich regions, morphological surveys and / or taxonomic revisions have been provided for e.g. the Western Himalayas (Pennell, 1943), the Middle East (Eig, 1944), North America (Pennell, 1935, 1947; Shaw, 1962), the Balkan Peninsula (Grau, 1981b), Pakistan (Qaiser et al., 1988), Iran (Attar, 2006), and Korea (Jang and Oh, 2013).

Phylogenetic relationships had been only rarely addressed. The close relationship of *Scrophularia* and *Verbascum*, proposed as early as 1835 by Bentham (see chapter 2.3.1.) and later also suggested by Thieret (1967) and others, was confirmed first by Olmstead and Reeves (1995), who found *Scrophularia* being sister to *Verbascum* including *Celsia*. Kornhall et al. (2001) identified a sister relationship of the Scrophularia-Verbascum clade to the South African *Antherothamnus* N.E.Br. The closest relative of *Scrophularia* known to date is the genus *Oreosolen* Hook.f. as discovered by Albach et al. (2005) and Oxelman et al. (2005). *Oreosolen* is endemic to the Himalayas and the Tibetan Plateau and is used as a traditional Tibetan medicine (Rosendal Jensen et al., 2008). Oxelman et al. (2005) pointed out its close relation to *Scrophularia* in terms of floral morphology and leaf architecture, and emphasized the unusual Northern Hemisphere distributions of both genera, which are shared with *Verbascum* (see above). Altogether, tribe Scrophularieae thus now comprises four genera in the APG system (Stevens, 2001 and onward). Wang et al. (2015) sequenced accessions of few Eastern Asian species to elucidate the taxonomic status of *S. koraiensis* Nakai. Attar et al. (2011) provided a preliminary molecular phylogeny, which was however limited to only 20 Iranian taxa. Navarro Pérez et al. (2013) included 77-108 accessions into a time-calibrated phylogeny. The authors confirmed the monophyly of the genus and

postulated its divergence in the Miocene. However, their sampling did not comprise samples from all distribution areas and proposed subsections; their main focus was the reconstruction of pollination system shifts in the history of the genus.

2.3.3. Pollination biology and evolution of the staminode

The pollination biology of *Scrophularia* offers several interesting aspects as well: *Scrophularia* flowers are self-compatible but protogynous, thus favoring cross-pollination. In part of the species, centrifugal incurvation of the style additionally separates the sexual organs before the stamens open (see e.g. Shaw, 1962; Ortega Olivencia and Devesa Alcaraz, 1993a). The flowers present sucrose-rich nectar; the nectar composition of individual species (with one exception) is largely independent of their respective pollinator group (Rodríguez Riaño et al., 2014). Ortega Olivencia and Devesa Alcaraz (1993b), in a study on 24 representatives, found greater nectar and pollen production in taxa from *S.* sect. *Anastomosantes* compared to *S.* sect. *Tomiophyllum*, putatively due to larger corolla and anther sizes in the former. In most species, the flowers match the characteristics of wasp-flowers; Brodmann et al. (2012) revealed that they also contain green leaf volatiles in their odour, which are highly attractive to wasps. It seems intriguing that in many plants, these volatiles are also indicators of an infestation with herbivores, which constitute suitable prey for wasps.

Consequently, the main pollinators are traditionally considered wasps and syrphid flies, with further *Diptera*, bees, and bumblebees complementing the pollinator spectrum (see Shaw, 1962, Ortega Olivencia and Devesa Alcaraz, 1993b,c; Fateryga, 2011; Valtueña et al., 2013; and references therein). Close pollinator-plant relationships do not seem to exist, as most pollinators are generalists unable to distinguish among different *Scrophularia* species. In some species with large and showy flowers, birds also act as pollinators (e.g. Ortega Olivencia et al., 2012; see chapter 5.5.). Within the inflorescences, the main pollinator groups follow a pattern of rather upward than downward vertical movements, accompanied by horizontal movements whose proportion is apparently positively correlated to flower size (Valtueña et al., 2013). The authors conclude that this, together with the fact that dichogamy is not synchronized throughout the inflorescence, implies that geitonogamy (i.e., self-pollination accomplished by the transfer of pollen from one flower to the stigma of another flower on the same plant) is not avoided in *Scrophularia*.

The importance of its pollinators for *Scrophularia* is illustrated by results from Ortega Olivencia and Devesa Alcaraz (1993c), where plants in a study on 22 taxa showed both reduced fruit-set and seed-set per capsule when pollinators were excluded. An exception was *S. peregrina* L., which readily set full fruit following self-pollination according to Shaw (1962). Altogether, intrafloral self-pollination does not seem to play a major role in the genus. From the different groups of pollinators, *Syrphidae* seemed to be important only in *S.* sect. *Tomiophyllum* as noted by Ortega Olivencia and Devesa Alcaraz (1993c), although these insects visit *Anastomosantes* flowers as well (Shaw, 1962). The significance of hoverflies for flowers of *S.* sect. *Tomiophyllum* was confirmed by Valtueña et al. (2013) and also in a study on five Crimean species by Fateryga (2011), who proposed a shift towards hoverfly pollination in this section in response to the open landscapes inhabited by its species. The author also speculated on wasp-pollination to be the ancestral condition in *Scrophularia*, which was later corroborated by Navarro Pérez et al. (2013).

Most species of *Scrophularia* are characterized by an adaxial staminode adnate to the upper part of the corolla (see chapter 2.3.2. and Fig. 3i). Staminodes, i.e. sterile,

modified stamens, usually emerge during an evolutionary reduction of the androecium (Walker-Larsen and Harder, 2000). Single stamens within a whorl can be modified into staminodes during the development of zygomorphic (instead of actinomorphic) flowers. Staminodes thus represent a transitional phase: they can be present as vestigial organs, which might get lost during subsequent evolution, or can acquire secondary floral functions and even become essential floral components (Walker-Larsen and Harder, 2000; Ronse De Craene and Smets, 2001). These functions may be attractive (e.g. display of color, odor), nutritional (e.g. nectar production) or structural (e.g. nectar recipients or obstacles to selfing or nectar theft) according to Ronse De Craene and Smets (2001) and connected to pollination in various ways (e.g. also by mediating pollinator contact to the stigma or anthers). In vestigial, little altered staminodes as in most *Scrophularia* species, functions are often difficult to discern. The scale-like distal part of the *Scrophularia* staminode obtains various shapes and also may be reduced to a small, awl-shaped structure like in *S. canina* L. Rodríguez Riaño et al. (2015b) found that the function of the staminode is not connected to nectar secretion. Larger staminodes also do not hinder access to the flower; instead, they act as barriers to reduce the dilution of nectar by the entry of rainwater (Rodríguez Riaño et al., 2015a). According to López et al. (2016), larger staminodes act as attraction units to pollinators during multiple visits. However, they do not improve pollinator contact to the reproductive organs by decreasing the opening of the corolla or by forcing them into a correct position. Small staminodes do not seem to fulfil a special role; the function of the exceptionally large staminodes in some species of *S.* sect. *Tomiophyllum* yet remains to be revealed.

2.3.4. Anatomy and phytochemistry

Anatomical studies were conducted by several authors. *Scrophularia* has anatropous, unitegmic and tenuinucellate ovules according to investigations in *S. himalensis* Royle ex. Benth. by Natesh and Bhandari (1974), who also found two coexisting types of embryo sac development (polygonum plus allium type) in this species. Bhandari and Natesh (1985) reported on endosperm development in *Scrophularia*. Lersten and Curtis (1997) investigated foliar idioblast occurrence in *Scrophularia* and *Verbascum*; single-celled idioblasts are apparently restricted to these two genera (Lersten and Curtis, 2001; see chapter 2.3.2.). Within *Scrophularia*, idioblasts are more frequent in species of *S.* sect. *Tomiophyllum* than *S.* sect. *Anastomosantes*, however they are abundantly present in North American species (Lersten and Curtis, 1997). Makbul and Beyazoğlu (2009) emphasized the taxonomic value both of idioblasts and the distribution and dimension of sclerenchymatic tissue in cortex and phloem, and presented stem and leaf sections. Makbul et al. (2006) stated that these anatomical characters, plus the mean number of stomata, epidermal cell characteristics, and the diameter of the vascular bundle, might be more important for species identification than morphological traits.

Typical secondary metabolites found in root and leaf tissues of *Scrophularia* include iridoid glycosides, phenylpropanoids, phenolic acids, flavonoids and saponins. These agents account for the medicinal properties of several species (e.g. anti-inflammatory, antibacterial, fungicidal, cardiovascular, or diuretic activities, see de Santos Galíndez et al., 2002). According to Potter's Herbal Cyclopedia (Williamson, 2003), *S. nodosa* can be used for wound healing, as a laxative, and is diuretic and anti-inflammatory. In Traditional Chinese Medicine (TCM), several species are used for therapeutic purposes, most notably *S. ningpoensis* Hemsl. and *S. buergeriana* Miq., which are listed in the Encyclopedia of Traditional Chinese Medicines (Zhou et al., 2011) as

remedies for various disorders including insomnia and dry eyes. Indications of pharmaceutic effects were also found in studies on e.g. *S. scorodonia* L., *S. deserti* Delile, *S. striata* Boiss., *S. hypericifolia* Wydler, *S. orientalis* L., or *S. oxysepala* Boiss. (Diaz et al., 2004; Stavri et al., 2006; Azadmehr et al., 2013; Kosari-Nasab et al., 2013; Alqasoumi, 2014; Lange et al., 2016; Orangi et al., 2016). In this respect, the ancient reputation of *Scrophularia* as a medicinal plant is still valid today.

3. Methodology

3.1. Testing for incongruence among phylogenetic trees

When the analysis of two or more molecular markers during phylogenetic reconstruction yields different trees, the question arises on whether their topologies are congruent, i.e. support the same phylogenetic hypothesis. Incongruence can result from a variety of sources (see Discussion, chapter 5.4.), but irrespective of its origin, it can distort a combined analysis of different markers, especially if datasets lead to well-supported, but conflicting topologies. Therefore, the first step is to assess if statistically significant incongruence is present at all. Three methods have been used here (several more are reviewed in detail in Johnson and Soltis, 1998):

1) Visual inspection for taxa and clades displaying hard incongruence

Two trees are compared and examined for "hard", i.e. well-supported incongruence of single accessions or entire clades (for example, taxon A receives high support as sister to taxon B in one tree, but as sister to taxon C in another tree). Congruence is rejected if the support values for conflicting placements in both trees equal or exceed 70 % bootstrap support (BS), a widely used threshold established by Mason-Gamer and Kellogg (1996). Bayesian posterior probabilities (PP) of ≥ 0.95 have also been used; cut-offs may be decreased in cases where reduced supports, due to high levels of homoplasy or sequence polymorphism (see chapter 3.3.), are to be expected. By obscuring phylogenetic relationships, these would prevent taxa from being recognized as hard incongruent, which is by definition dependent on the support of associated nodes. In this case, additional examination of phylogenetic networks based on coded polymorphisms (see chapter 3.4.) is advisable; accessions displaying "soft" incongruence should be further examined. Identification of hard incongruence taxa was done for all articles of this dissertation.

2) Testing for incongruence with the Incongruence Length Difference (ILD) test

This statistical test (Farris et al., 1995) evaluates the likeliness of two datasets supporting the same phylogenetic hypothesis. In principle, a combined analysis of congruent datasets should result in a tree whose length is equal to the sum of both lengths of trees yielded by individual analysis of each marker alone. If datasets are incongruent, combination will result in greater homoplasy and consequently higher tree length - the null hypothesis is rejected. This is tested by comparing the sum of lengths of the two single marker trees against that of trees obtained from two datasets which are made up of randomly chosen characters from both markers (i.e., combined datasets). The procedure is repeated several times to ensure statistical significance; a p-value of < 0.05 suggests that the data are incongruent. Chloroplast markers thus can be tested against nuclear markers; if the result is significant, all accessions which putatively cause the conflict (e.g. already identified hard incongruence taxa) are pruned from the datasets and the test is repeated. If now congruence is supported, stepwise re-addition of single taxa helps to assess the individual amount of incongruence introduced by each respective taxon, and which taxa should be finally excluded from a combined analysis (see chapter 3.2.). The ILD test was used in Articles I-IV.

3) Templeton's significantly less parsimonious (SLP) test

The SLP test (Templeton, 1983) investigates whether the data of one dataset are in significant conflict with the topology supported by another dataset. To test this, a heuristic search with dataset A is constrained by one (or more) topologies representing relationships supported by dataset B. The trees from this search are compared to those of an unconstrained heuristic search (or to an associated consensus tree) of dataset A. If

the constrained trees are significantly longer ($p < 0.05$), the null hypothesis is rejected, i.e., the "strange" topology is in conflict with the data. The SLP test might be used in addition to the ILD test, as the latter has been criticized for putative weaknesses, e.g. a high rate of false positives, by Barker and Lutzoni (2002) and Darlu and Lecointre (2002). The SLP test was applied in Articles II and III.

3.2. Combining incongruent datasets for phylogenetic tree construction

The question whether significantly incongruent datasets should be combined has been discussed extensively (see Huelsenbeck et al., 1996). Some authors advocate a concatenation approach which ignores the incongruence altogether (e.g. Gadagkar et al., 2005), while others have shown that this might produce misleading results (Kubatko and Degnan, 2007; Weisrock et al., 2012). A widely used alternative is the "conditional combination approach" (reviewed in Huelsenbeck et al., 1996; Johnson and Soltis, 1998), where taxa which display significant topological incongruence (see chapter 3.1.), or more generally introduce conflict into a combined dataset, are excluded prior to the analysis. Where the incongruence is not too widespread and clearly detectable (see Articles I and II of this dissertation), this yields reliable and satisfying results.

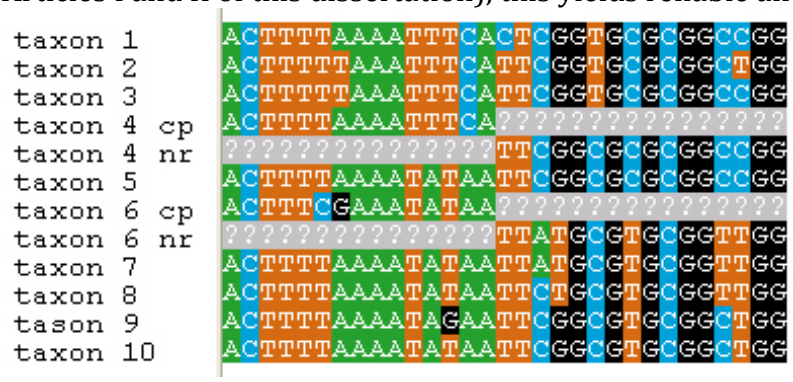

Fig. 5. Exemplary taxon duplication in an alignment with two conflicting taxa (4 and 6). The incongruent marker information is split, by duplication of the respective taxon into one "plastid-only" (cp) and one "nuclear-only" (nr) accession. For more details see Pirie et al. (2008, 2009)

However, in groups where reticulation and/or incomplete lineage sorting (ILS) is frequent, pruning of hard incongruent taxa will disregard large portions of the available data. An elegant solution to this problem was presented by Pirie et al. (2008, 2009). Their approach allows the inclusion of conflicting data into the alignment, by duplication of the respective taxa into one "plastid-only" and one "nuclear-only" accession ("taxon duplication approach"; Fig. 5). This way, analyses will not be impeded by incongruence issues, tree resolution is improved, and the placement of nuclear and plastid representations in the tree possibly allows conclusions on the complex evolutionary history of these taxa (Pirie et al., 2008).

While the taxon duplication approach can be very useful for phylogenetic and biogeographic reconstructions of medium-sized datasets (see Article III of this thesis), the method becomes increasingly unfeasible for larger samplings in species-rich genera with many conflicting taxa. In these cases, pruning taxa consequently is no alternative either; plastid and nuclear data are thus often analyzed separately and then interpreted individually and compared (as done in Article IV).

3.3. Character and indel coding

When molecular marker datasets are to be analyzed separately, sufficient resolution in the resulting single marker trees is required for significant interpretation and conclusions. Unfortunately, some processes which produce among-dataset

incongruence (e.g. ILS or hybridization) are often also responsible for lowered supports and polytomies in the respective phylogenies. In the biparentally inherited nuclear DNA, markers of which are frequently used for reconstruction, hybridization will result in (at least) two different copies in the genome of the offspring. Also without any reticulation, retained polymorphisms from a common ancestor, which persist in a population, can produce the same patterns, especially in young lineages (where sequence divergence among species is low anyway). These differences among single copies of a gene lead to intra-individual site polymorphism in direct sequences (i.e., generated by direct Sanger sequencing); some examples are illustrated in Fig. 6a. A variety of other processes can also produce intra-individual variability (see chapter 5.4.); this is especially true for multi-copy nuclear markers like ITS.

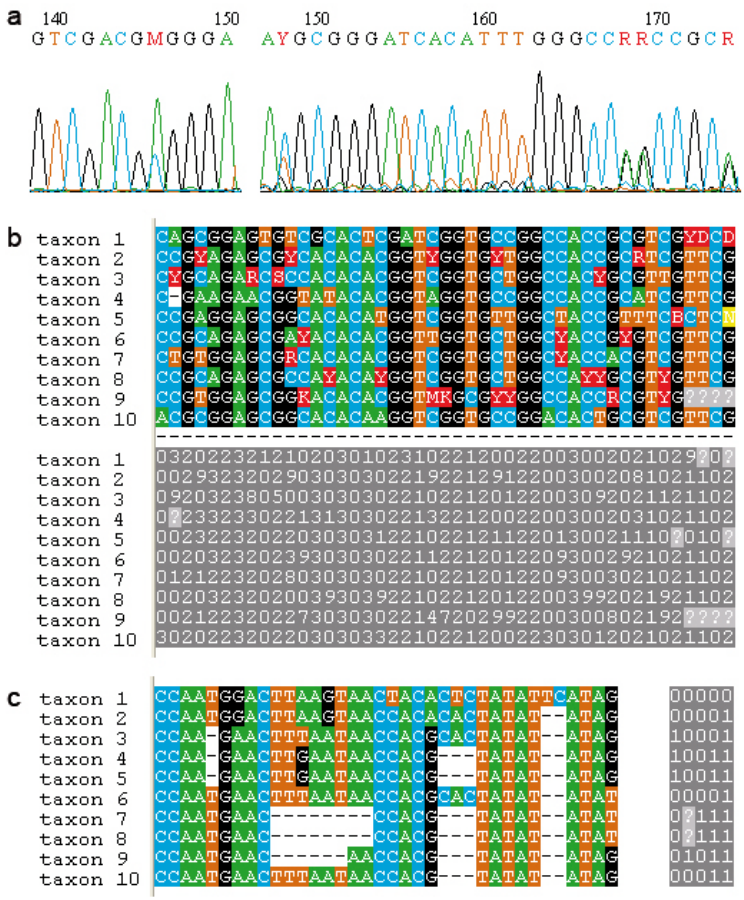

Fig. 6. Sequence polymorphisms and coding methods. Intra-individual site polymorphism in two exemplary pherograms obtained from *Scrophularia* (a), with corresponding IUPAC characters. These ambiguities can be treated as informative, by recoding all characters of the alignment as a standard matrix (b); thereby, each IUPAC code is given a distinct character state. (c), exemplary coding of plastid indels using the simple indel coding procedure (Simmons and Ochoterena, 2000); the binary matrix is added to the alignment

Large amounts of polymorphisms in sequences hamper phylogenetic reconstruction by obscuring important relationships, particularly if they are present at synapomorphic sites of an alignment. Many algorithms used for tree reconstruction treat the respective sites as missing data or ambiguous information, so that unequivocal assignment of the respective taxa to a certain phylogenetic group will fail. This leads to polytomies and weakly supported nodes and hinders correct interpretation of the data (see e.g. Campbell et al., 1997; Fuertes Aguilar and Nieto Feliner, 2003; Fehrer et al., 2009).

In species-poor lineages, the problem can be solved by cloning the marker regions of the respective accessions (Nieto Feliner and Rosselló, 2007). In large genera, where cloning of all taxa cannot be accomplished (for example, only exemplary cloning was performed in Article IV), the information content drawn from direct sequences can be enhanced by explicitly treating intra-individual polymorphisms as informative. For example, this can be done by the use of step matrices during calculations (Potts et al., 2014), which directly model the change from e.g. nucleotide character "C" to "T" via "Y" ("C" and "T"). Alternatively, single site variabilities, oligonucleotide motives or the whole alignment can be recoded into a character matrix, which is then analyzed using step matrices or, more straightforward, as categorical dataset (Grimm et al., 2007; Potts et al., 2014). For analyses of infrageneric relationships in *Scrophularia* (see Article IV), I followed the latter approach:

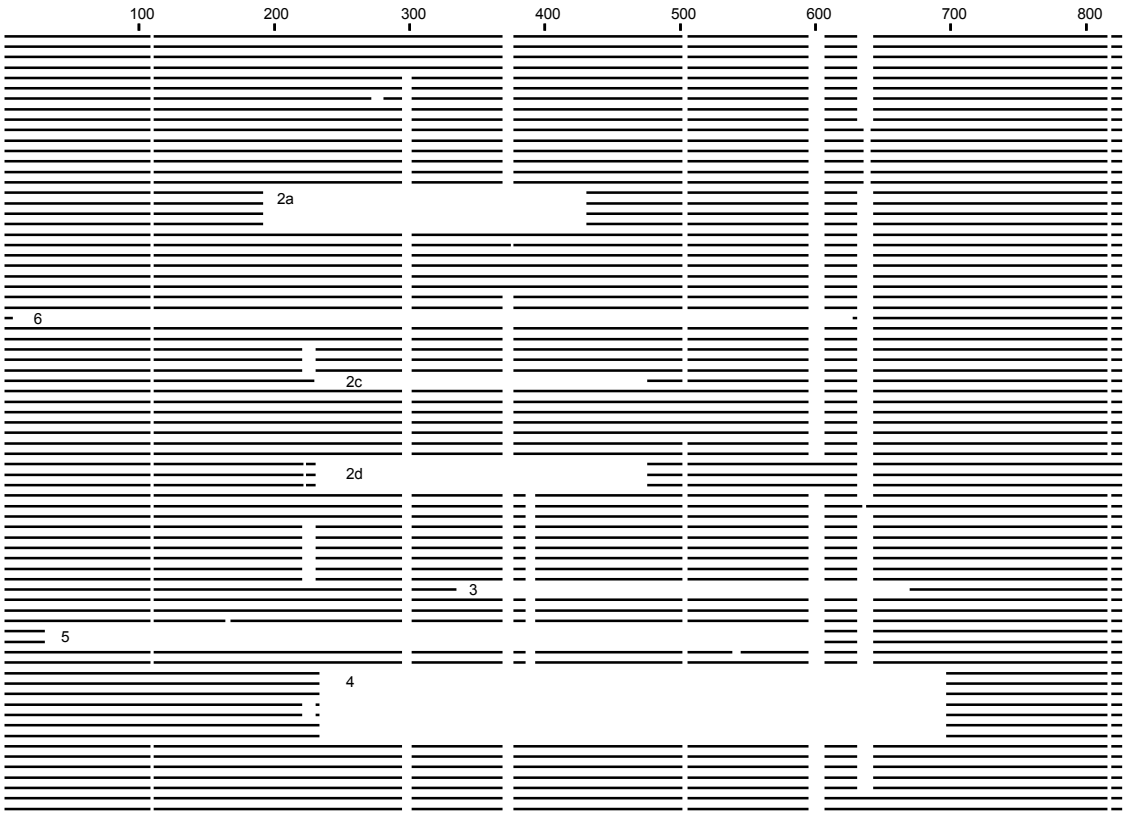

Fig. 7. Indel patterns in a representative sample of 75 *Scrophularia* accessions. Length type numbers of indels correspond to those in Table 2 of Article IV. Type 2a and 2d are characteristic for the Polyantha and Scopolii clades, respectively. However, indels of similar length also occur spontaneously (type 2c). The longest indels are found in the Orientalis clade and *S. rubricaulis* Boiss. (types 5 and 6); all 25 analyzed accessions from the Nodosa and NW / Japan clades feature a characteristic 597-bp indel (type 4)

the complete alignment was recoded as a standard matrix, with each IUPAC code receiving a distinct number ("0-9" or "?"; Fig. 6b). This way, all nucleotide character states, simple or ambiguous, are treated as distinct characters; analyses are done using settings analogous to those for categorical (e.g. morphological) data. This approach does not discriminate between polymorphisms derived from different processes (which may be difficult to ascertain), but incorporates all available sites; the amount of additional information obtained can be considerable depending on the ambiguity present in the dataset.

Uniparentally inherited markers are usually free from intra-individual polymorphism. Here, limited resolution on the species level is often caused by a lack of informative characters in slow-evolving regions. Additional phylogenetic signal may be contained in insertions-deletions (indel) patterns in the alignment of the ingroup. This information can be extracted (as done in Articles I-IV) by coding indels as a binary matrix of present-absent states (1-0) for every accession, which is generated sequentially for each indel (an example is given in Fig. 6c). This method, known as "simple indel coding", was presented by Simmons and Ochoterena (2000). The resulting binary matrix is then added to the alignment and analyzed accordingly; the occurrence of every indel is thus treated as a single mutation event independent of indel length. Particular indels may be diagnostic for species groups (as in *Scrophularia*; Fig. 7) and, if large enough, might be recognized directly by characteristic length variations of the amplified PCR products (reduced or increased length of the fragment on the PCR gel, compared to those of congeners; see Articles II and IV); this constitutes an efficient distinguishing tool for the respective species groups.

3.4. Network methods

To infer the evolutionary history of a group and reveal phylogenetic relationships among its members, the standard approach is to compute a bifurcating phylogenetic tree from a given set of sequences. This relies on the tacit assumption that the underlying processes also follow this bifurcating principle. However, this assumption is often violated, e.g. during adaptive radiation where multiple new lineages are generated from one common ancestor; in cases where ancestors and descendants coexist (this would require labelling internal nodes); or, importantly, when lineages interact with each other in a "horizontal" way, e.g. by hybridization or introgression (Moulton and Huber, 2009). Evolutionary mechanisms often are considerably non-treelike, and even if a single molecular marker supports one bifurcating phylogenetic tree, other genes are likely to support different phylogenetic hypotheses, due to processes like ILS and other phenomena which create incongruence as outlined above (Huson and Scornavacca, 2011).

All of these processes are better depicted by a phylogenetic network, rather than by a bifurcating tree. Thereby, one important type are "split networks". A split represents a bipartition of a given set of taxa which is based on some kind of information (e.g. sequence similarity); the split divides the sample into two groups. Generally, each branch (or edge) in an unrooted phylogenetic tree also represents a split, in a way that its removal results in two subtrees (groups) supported by the split. A collection of "compatible" splits makes up a bifurcating tree; if incompatible splits are present, these can only be visualized in a network (Huson and Bryant, 2006), with each split represented by a set of parallel edges (for an example see Fig. 8). Split networks can be created from a variety of data types, including sequences, distances, quartets, or phylogenetic trees. As they are unrooted, they are not appropriate to explicitly trace a putative evolutionary history (in contrast to rooted, explicit phylogenetic networks like hybridization networks); the function of these implicit networks is to visualize connections and / or incompatibility in a dataset (Huson and Scornavacca, 2011). The latter is important for exploratory data analysis, a concept advocated by Morrison (2010). By evaluating the properties of the present data before analyzing them, the suitability of the intended methods can be estimated and data-inherent pitfalls, which might lead to erroneous conclusions, can be avoided. For this dissertation, no explicit networks were constructed to model hybridization and polyploidization, as incongruence in *Scrophularia* is also due to other processes (see Discussion, chapter 5.4.). Three types of unrooted phylogenetic networks were used:

1) consensus networks (CN)

If incompatibility / incongruence is present within or among different datasets, computing a consensus tree from all trees obtained during an analysis will often conceal part of the results in favor of other - sometimes only slightly better supported - relationships. A consensus network (CN; Holland and Moulton, 2003; Holland et al., 2004) will display all signal above a certain threshold (e.g., "present in 30 % of trees"). Edge lengths of these networks correspond to the split frequency within the sampled topologies. If trees based on two different markers are used (like in Fig. 8), the consensus network enables the examination of phylogenetic among-dataset incongruence, its extent and possible causes (i.e., accessions causing the conflict, which are usually found close to heavily networked regions). If such conflicting accessions have already been identified (by the methods described above), these can be excluded and the network re-generated to see how far the problem is alleviated by pruning taxa.

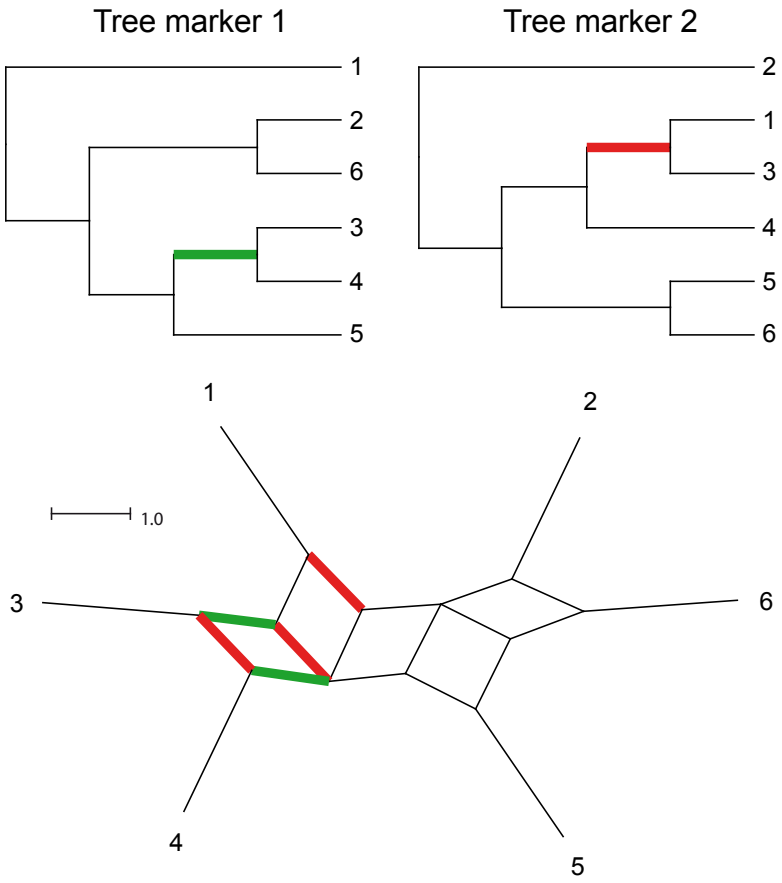

Fig. 8. Example for a splits graph: a consensus network representing compatible and incompatible splits from two trees yielded by analysis of different markers. The set of parallel edges in the network colored in green represents the bipartition {3,4}|{1,2,5,6}; the corresponding branch in the unrooted tree from marker 1 is marked. The respective split is not compatible with the red one in the tree from marker 2. The phylogenetic network illustrates both relationships (using a threshold ≤ 50 % for displaying splits, here 33 %). Edge lengths are proportional to the number of trees supporting a split. Drawing adapted from Morrison (2010)

Extremely entangled relationships, depicted by high numbers of parallel edges, suggest that combination of the data might not be reasonable. (Filtered) Super Networks can be constructed in cases where trees differ with respect to the sampled accessions (Huson et al., 2004, 2006). Consensus networks or filtered super networks were computed in Articles I, III and IV.

2) Neighbor-Net (NN) splits graphs

In a nuclear dataset which is confounded by reticulation and/or ILS effects, a split network based on the raw data can provide important insights about conflicting signals which might lead to problems in phylogenetic tree reconstruction (Morrison, 2010). The Neighbor-Net (Bryant and Moulton, 2004) is based on pairwise distances; information contained in polymorphic sequence sites can be incorporated by basing the network on polymorphism p-distances (Schliep, 2011; Potts et al., 2014). However, it is important to notice that the NN, just like the CN, will display all signal contained in the data irrespective of its source (Bryant and Moulton, 2004; Morrison, 2010), including conflicts based on methodical artifacts or, in the case of CNs, lack of information. A Neighbor-Net was used to examine the ITS dataset in Article IV of this dissertation.

3) Haplotype networks

When taxa are too closely related (i.e., of very low sequence divergence) to be resolved in a phylogenetic tree by the markers used for reconstruction, their relationships can be displayed in a haplotype network. This type of unrooted phylogenetic network is different from the split networks discussed above. Here, different haplotypes (i.e., sequence "configurations" inherited as a single unit, present in one or more taxa) are represented by nodes and are joined by edges which denote the difference among the two connected haplotypes, e.g. the nucleotide position at which they differ (Huson and Scornavacca, 2011). Missing intermediate states are inferred during calculation and correspond to assumed unsampled or extinct haplotypes. Haplotype networks can be

constructed with various methods; the "TCS approach" (Templeton et al., 1992; Clement et al., 2000), which was used here, is based on statistical parsimony (definition see Templeton et al., 1992). Normally, plastid sequences (which are collapsed into haplotypes by the program) at population level are used for these networks; however they are also helpful when sequence divergence among species is low. Shared haplotypes across species boundaries in the same geographical region can point towards introgression/hybridization; common ancestry (but also ancient hybridization events) might result in haplotypes shared among geographically distant species. Radial patterns of change from a central haplotype are consistent with radiation processes, while divergent haplotypes are found in isolated and hence possibly older lineages. As in split networks, too low diversity of the sequences will result in uncertainties in the network (there represented by loops). A haplotype network was constructed in Article III of this dissertation to elucidate the relationships among closely related *Scrophularia* species from the Iberian Peninsula and Macaronesia.

4. Scientific manuscripts

The present dissertation is based on the following four publications:

Scheunert A, Fleischmann A, Olano-Marín C, Bräuchler C, Heubl G. 2012. Phylogeny of tribe Rhinantheae (Orobanchaceae) with a focus on biogeography, cytology and re-examination of generic concepts.
Taxon 61(6): 1269-1285. Article I

Scheunert A, Heubl G. 2011. Phylogenetic relationships among New World *Scrophularia* L. (Scrophulariaceae): new insights inferred from DNA sequence data.
Plant Systematics and Evolution 291: 69-89.
doi:10.1007/s00606-010-0369-z Article II

Scheunert A, Heubl G. 2014. Diversification of *Scrophularia* (Scrophulariaceae) in the Western Mediterranean and Macaronesia - Phylogenetic relationships, reticulate evolution and biogeographic patterns.
Molecular Phylogenetics and Evolution 70: 296-313.
doi: 10.1016/j.ympev.2013.09.023 Article III

Scheunert A, Heubl G. 2017. Against all odds: reconstructing the evolutionary history of *Scrophularia* (Scrophulariaceae) despite high levels of incongruence and reticulate evolution.
Organisms Diversity and Evolution, Online First.
doi: 10.1007/s13127-016-0316-0 Article IV

The articles are provided in the following chapters.

4.1. Article I

Phylogeny of tribe Rhinantheae (Orobanchaceae) with a focus on biogeography, cytology and re-examination of generic concepts.

by Agnes Scheunert, Andreas Fleischmann, Catalina Olano-Marín, Christian Bräuchler & Günther Heubl

Taxon 61(6): 1269-1285 (2012)

I

The final publication is available on Ingenta Connect at
http://www.ingentaconnect.com/content/iapt/tax/
2012/00000061/00000006/art00008

Phylogeny of tribe Rhinantheae (Orobanchaceae) with a focus on biogeography, cytology and re-examination of generic concepts

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Abstract A molecular systematic approach using DNA sequences of two non-coding chloroplast loci (*trnK*, *rps16*) and the nuclear ITS region was applied to reconstruct phylogenetic relationships within the tribe Rhinantheae (Orobanchaceae). This tribe includes approximately 19 genera of hemiparasitic plants predominantly occurring in the Old World. An exception is the genus *Bartsia* which, according to previous taxonomic treatments, includes a remarkable radiation (ca. 45 species) in the Andes, two species distributed in Afromontane regions, and only one species (*Bartsia alpina*) ranging from the alpine mountains of northern and central Europe to northeastern North America. The present phylogenetic study includes the most comprehensive taxon sampling of Rhinantheae to date, with main focus on the relationships of the Mediterranean genera. Both nuclear and plastid datasets reveal a core group of Rhinantheae comprising four major lineages. Our analyses suggest that (1) the northern temperate *Bartsia alpina* is sister to the rest of the core group; (2) African *Bartsia* are more closely related to the monotypic African genus *Hedbergia* than to other congeneric taxa; (3) South American *Bartsia* are nested within a highly supported clade including *Parentucellia* and *Bellardia*; (4) *Nothobartsia* and *Odontitella* are likely to be the results of at least one intergeneric hybridization event. Despite topological conflicts regarding some taxa, the polyphyly of *Bartsia* and a broadly circumscribed *Odontites* are unambiguously supported by our results. Our tree topologies indicate that the importance of certain morphological characters traditionally used for generic delimitation (such as shape and indumentum of corolla, anthers, and capsules) has been overestimated, and that some of these characters are presumably convergent. Available information on chromosome numbers corroborates the results presented here.

Keywords hemiparasitic plants; nrITS; Orobanchaceae; Rhinantheae; *rps16*; *trnK*

Supplementary Material The alignment is available in the Supplementary Data section of the online version of this article (http://www.ingentaconnect.com/content/iapt/tax).

■ INTRODUCTION

The cosmopolitan angiosperm family Orobanchaceae (broomrape family) is a morphologically diverse group of almost exclusively parasitic plants, which form a well-supported monophyletic lineage in the Eudicot order Lamiales (APG III, 2009). Except for the non-parasitic East Asian genus *Lindenbergia* Lehm., which is sister to all remaining genera (Young & al., 1999), members of the family are either holoparasites lacking chlorophyll, or green, photosynthetic hemiparasitic plants (either obligate hemiparasites, which means they require a host plant for successful growth, or facultative hemiparasites, which are able to complete their life cycle independent of a host). Orobanchaceae form a monophyletic group (e.g., Young & al., 1999; Olmstead & al., 2001; Wolfe & al., 2005; Bennett & Mathews, 2006); hence it can be assumed that parasitism evolved only once in this lineage (dePamphilis & al., 1997; Nickrent & al., 1998; Young & al., 1999).

Circumscription of Rhinantheae. — Rhinantheae were traditionally recognized as comprising hemiparasitic plants of former Scrophulariaceae s.l. (i.e., subfamily Rhinanthoideae sensu Wettstein, 1891), based on Bentham (1846, 1876). After the disintegration of Scrophulariaceae based on molecular data (Young & al., 1999; Olmstead & al., 2001; Oxelman & al., 2005), the parasitic members of this family were transferred to Orobanchaceae s.l. (Young & al., 1999; Wolfe & al., 2005; Bennett & Mathews, 2006; Tank & al., 2006; APG III, 2009). These taxa had previously been placed in the two large tribes Rhinantheae Benth. and Gerardieae Benth. (= Buchnereae Benth.) while the third tribe, Digitaleae Benth., completing the Rhinanthoideae according to Wettstein's treatment (1891), consists of non-parasitic plants and was recently transferred to Plantaginaceae (Olmstead & al., 2001). Members of the two parasitic tribes were distinguished based on a different pattern of the imbricate ascending corolla aestivation. This so-called "rhinanthoid aestivation" is a synapomorphy of all Orobanchaceae, however with some variation concerning the arrangement of the corolla lobes in bud: in flowers of Buchnereae, the central lobe of the three lobes of the lower corolla lip is folding over the two lateral ones, whereas in all Rhinantheae, the two lateral lobes clasp the median one (Thieret, 1967; Armstrong & Douglas, 1989). The morphology-based assignment of the hemiparasitic taxa to

This publication is dedicated to *Dr. Markus Bolliger* on the occasion of his 60th birthday.

these two tribes has not changed since the first proposal of this taxonomic concept by Wettstein (1891); however it is not fully supported by molecular data (Young & al., 1999; Wolfe & al. 2005; Bennett & Mathews, 2006; Tank & al., 2006). None of the tribes is monophyletic, and in phylogenetic reconstructions several members of each tribe are part of the respective other, rather following biogeographic patterns than the classical taxonomic concept (Young & al., 1999; Bennett & Mathews, 2006; Tank & al., 2006). Genera such as *Pedicularis* L., *Castilleja* Mutis ex L. f. (both previously Rhinantheae), and *Agalinis* Raf. (previously Buchnereae) are now placed in a common clade, which is referred to as tribe Pedicularideae Duby (Bennett & Mathews, 2006; Tank & al., 2009). However, as no modern phylogeny-based taxonomic concept for all taxa of Rhinantheae has been proposed yet, the traditional system of Wettstein (1891) is followed here, with the exception of *Pedicularis* as a member of Pedicularideae following Tank & al. (2009).

Morphological characters, generic concepts, and distribution of Rhinantheae. — Apart from petal aestivation and parasitic habit, Rhinantheae share no other generative or vegetative synapomorphy (Fischer, 2004). The plants are annual or perennial herbs, sometimes even small subshrubs with a woody base, and have racemose inflorescences in which the flowers are subtended by scale-like, leaf-like, or showy bracts. The corolla is bilabiate and consists of five connate petals, two forming the upper lip and three forming the lower lip. The two lobes of the upper lip are usually fused into a helmet-like or rostrate galea in which the anthers are inserted (most notably in *Rhynchocorys* Griseb., *Odontites* Ludw. and *Bartsia* L. (Fig. 1). However, bifid or to various degree bilobate lobes can also be found (e.g., *Euphrasia* L.), and the lobes of the upper lip are even free and expanded in *Hedbergia* Molau, *Bornmuellerantha* Rothm. and *Tozzia* L. (Fischer, 2004). For generic delimitation in Rhinantheae, mainly corolla morphology, but also palynological characters (pollen size, shape, exine ornamentation) were often used (Rothmaler, 1943; Inceoğlu, 1982; Molau, 1988; Bolliger & Wick, 1990; Bolliger, 1996; Lu & al., 2007).

Some of the recently segregated genera of the tribe, such as *Macrosyringion* Rothm., *Odontitella* Rothm., *Bartsiella* Bolliger, *Bornmuellerantha*, and *Nothobartsia* Bolliger & Molau have been questioned by taxonomists, but—with the exception of *Nothobartsia* (Těšitel & al., 2010)—have not been included in preceding molecular studies. All have been separated from *Odontites* (or *Bartsia* in case of *Nothobartsia*) based on rather minor morphological and palynological characters (Rothmaler, 1943; Bolliger & Molau, 1992; Bolliger, 1996), yet are still classified as *Odontites* (viz. *Bartsia*) in a broader circumscription in several flora treatments (e.g., Webb & Camarasa, 1972; Davis, 1978; Valdés & al., 1987; Jahn & Schönfelder, 1995; Mabberley, 2008).

Rhinantheae are of worldwide distribution, but the highest generic and species diversity is found in the Northern Hemisphere. Main centers of species richness are located in the Mediterranean area (*Odontites*) and the holarctic region (*Melampyrum* L., *Rhinanthus* L.). Some genera also have their center of alpha diversity in South America (*Bartsia*), Asia and Oceania (*Euphrasia*; Fischer, 2004; Bennett & Mathews, 2006).

Preceding molecular studies on Rhinantheae. — Rather few phylogenetic studies have addressed generic-level relationships within Rhinantheae exclusively; most of the work has focused on the phylogenetic framework and evolution of holoparasitism in Orobanchaceae (dePamphilis & al., 1997; Young & al., 1999; Olmstead & al., 2001; Schneeweiss & al., 2004; Wolfe & al., 2005; Bennett & Mathews, 2006; Morawetz & al., 2010). These studies provide good insight into intergeneric relationships in the family, however, some of the taxonomically difficult groups of Rhinantheae remained underrepresented. A recent phylogenetic study on Rhinanthoid Orobanchaceae (Těšitel & al., 2010) corroborated this group as monophyletic and identified certain major lineages within the tribe. Though based on a larger taxon sampling than any previous study, several key taxa such as *Macrosyringion*, *Bornmuellerantha*, *Odontitella*, *Bartsiella* and the African representatives of *Bartsia* were not included, thus leaving important questions unanswered.

The present study is based on an extensive taxonomic sampling of Rhinantheae to comprise taxa absent in previous studies. Based on an enlarged dataset, our goals are to test the previously published phylogenetic hypotheses in a more comprehensive context, particularly investigating the impact of narrowly endemic and poorly studied genera (*Bartsiella*, *Bornmuellerantha*, *Macrosyringion*, *Nothobartsia*, *Odontitella*) on phylogenetic reconstruction, and to infer phylogenetic relationships within and between taxa of *Bartsia* and *Odontites* to test existing taxonomic concepts.

■ MATERIALS AND METHODS

Plant material. — The taxon sampling follows the treatment of Fischer (2004), who recognized Orobanchaceae within Scrophulariaceae, and covers a representative number of species from 16 of the 20 genera included in tribe Rhinantheae. For the ingroup (tribe Rhinantheae), a total of 34 accessions representing 29 species from 16 genera were included in the analyses. Selection of outgroup taxa was based on the comprehensive molecular phylogeny of Orobanchaceae by Bennett & Mathews (2006) and the phylogeny of Rhinanthoid Orobanchaceae by Těšitel & al. (2010). *Striga* Lour. (tribe Buchnereae) and *Pedicularis* (tribe Pedicularideae) were chosen as outgroups. Voucher specimen data, including sources and accession numbers, are provided in the Appendix. For sequences obtained from NCBI's GenBank, references to the place of original publication are given. Herbarium specimens used for DNA extraction were identified with the keys provided by Molau (1990) for *Bartsia*, and Bolliger (1996) for *Odontites* s.l. A single *Bartsia* voucher representing sterile specimens from Peru used in the present study ("*Bartsia* sp. Peru") could not be fully determined to species level due to the lack of flowers. Nevertheless, it was included in the study so as to increase the number of South American *Bartsia* species.

DNA extraction and amplification. — Total genomic DNA was extracted either from fresh leaf material (three taxa) or from herbarium specimens (30 taxa) using the NucleoSpin Plant

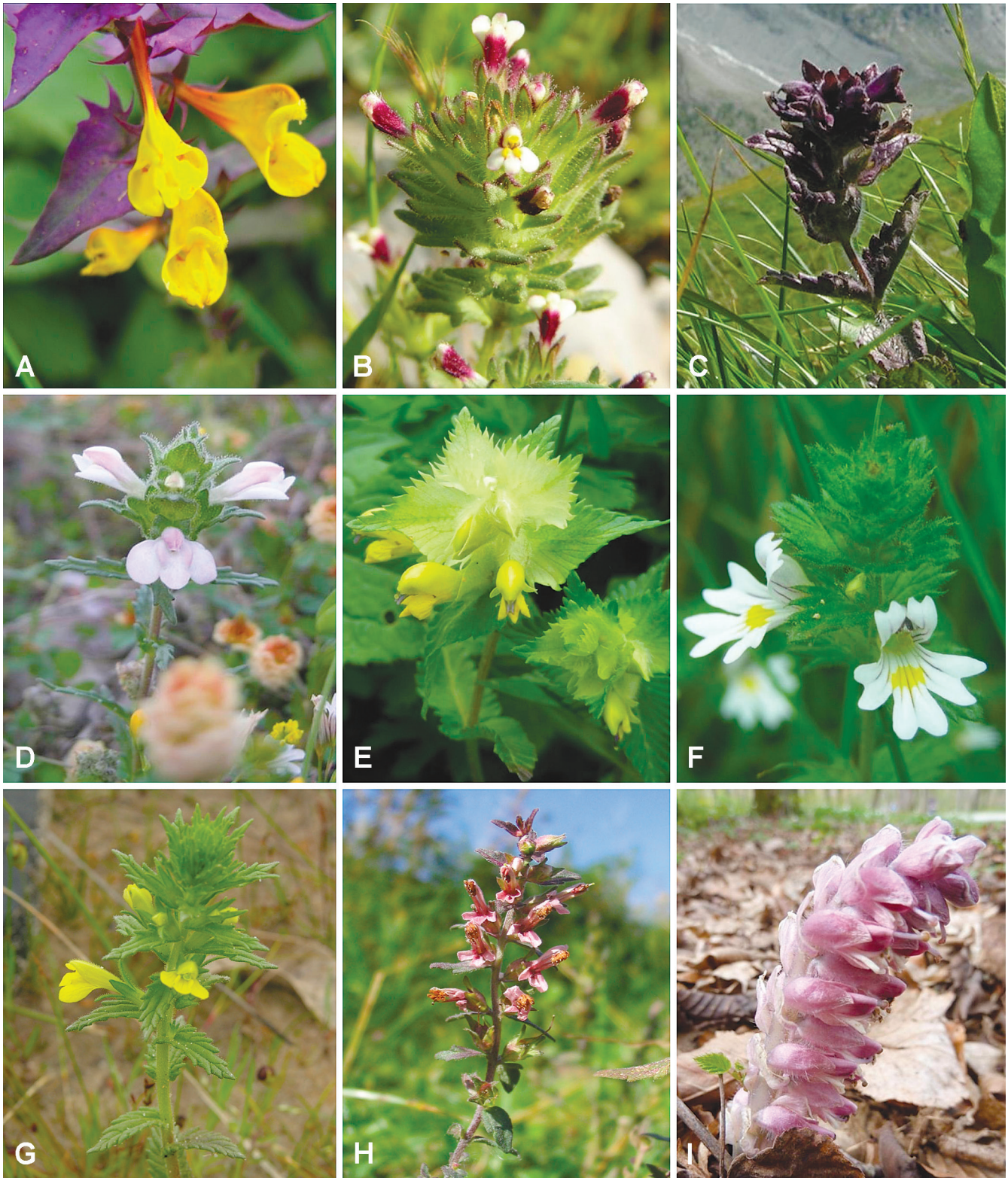

Fig. 1. Selected species of representative genera of Rhinantheae (Orobanchaceae). **A,** *Melampyrum nemorosum* L.; **B,** *Parentucellia latifolia* (L.) Caruel; **C,** *Bartsia alpina* L.; **D,** *Bellardia trixago* (L.) All.; **E,** *Rhinanthus alectorolophus* (Scop.) Pollich; **F,** *Euphrasia officinalis* L.; **G,** *Parentucellia viscosa* (L.) Caruel; **H,** *Odontites vernus* Dumort.; **I,** *Lathraea squamaria* L. — Photographs A, E & F, F. Brambach; B–D, G–H, A. Fleischmann; I, C. Olano-Marín.

Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's standard protocol for genomic DNA extraction. An additional phenol/chloroform extraction step was performed to remove proteins and potentially interfering secondary metabolites. The DNA was dissolved in 50 µl elution buffer (10 mM Tris/HCl) and checked for quality on a 1.4% agarose gel. A standard amount of 2 µl DNA template was used for PCR.

Two non-coding chloroplast regions (part of the *trnK* region, comprising the partial *matK* gene and the 3′-terminal end of the *trnK* intron, and the *rps16* intron) plus one nuclear ribosomal region (the internal transcribed spacer region, ITS) were chosen for phylogenetic analyses. These markers have been previously used in Rhinantheae and closely related lamialean groups (Schäferhoff & al., 2010; Těšitel & al., 2010). PCRs were performed with total genomic DNA using *Taq*-polymerase (Hybaid, AGS, Heidelberg, Germany) and primers LEU1 (Vargas & al., 1998) and ITS4 (White & al., 1990) for ITS; trnK-2R (Johnson & Soltis, 1994) and Sat2-1200F (Bräuchler & al., 2010) for partial *trnK*; and rps-F and rps-R2 (Oxelman & al., 1997) for *rps16*.

The cycling profile for ITS and the *trnK* region consisted of an initial denaturation step at 94°C (2 min 30 s) followed by 40 cycles of 30 s (ITS) or 1 min (*trnK*) denaturation at 94°C, 30 s (ITS) or 1 min (*trnK*) annealing at 53°C and 1 min 15 s (ITS) or 1 min 30 s (*trnK*) elongation at 72°C, and a 10 min final extension step at 72°C. The PCR amplification profile used for the *rps16* intron consisted of an initial denaturation step at 94°C (5 min) followed by 40 cycles of 30 s denaturation at 94°C, 1 min annealing at 50°C and 1 min 30 s elongation at 72°C, and a 7 min final extension step at 72°C. PCR products were purified using the NucleoSpin Extract II Kit (Macherey-Nagel) following the manufacturer's protocol.

Sequencing. — Direct sequencing, employing the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Freiburg, Germany) followed the manufacturer's protocol. Products were purified by Sephadex filtration (G50-Superfine, Amersham Biosciences) and were run on an ABI 3730 DNA analyzer (Applied Bio Systems, Foster City, California, U.S.A.). All markers were sequenced bidirectionally using the same primer pairs as for amplification. For the *trnK* region, the internal primers Sat2-1780F/Sat16-1780R (Bräuchler & al., 2010) and Sat16-2150R (Bräuchler & al., 2005) were used in addition to cover sequence gaps.

Phylogenetic analysis. — All sequences generated in the study were assembled and aligned automatically with the MUSCLE v.3.8.31 software (Edgar, 2004) and adjusted manually using BioEdit v.7.0.5.1 (Hall, 1999); mononucleotide repeats and ambiguously aligned regions were excluded from further analysis. Before incorporating nuclear and chloroplast indels in the analyses, their phylogenetic information content was assessed. This is regarded essential because within the parasitic lineages of Lamiales (which contain fast-evolving groups), nuclear indel data in particular are suspected to be highly homoplasious and thus could distort the inferred results (Schäferhoff & al., 2010). Ingroup indels were coded according to the simple indel-coding method (Simmons & Ochoterena, 2000), as implemented in SeqState v.1.4.1 (Müller, 2005) and added to the data as a binary matrix. Including indels into the combined chloroplast dataset increased support values in all but one case, while ITS indels improved node support values in only six cases but weakened them in 14 cases, especially in the basal part of the tree; an alternative topology was suggested in one case when using indels, but this received almost no support (results not shown). As this suggests a perturbingly high level of homoplasy in the nuclear indel dataset, these were excluded from further analyses, while chloroplast indels were coded as single mutation events.

The three markers were analyzed in a combined matrix as well as in two separate datasets (ITS and chloroplast). All analyses were conducted using both a Bayesian and maximum likelihood (ML) approach. Bayesian analyses were performed with MrBayes v.3.2 for 64bit systems (Ronquist & al., 2012), applying a GTR+Γ substitution model with four rate categories to the chloroplast partition and a SYM+Γ substitution model to the ITS partition, as suggested by MrModelTest v.2.3 (Nylander, 2004) as best fit to the DNA data. The binary indel data were analyzed separately using the model settings recommended by Ronquist & al. (2009); for chloroplast and combined analyses, a mixed dataset was defined (one/two DNA partitions, one binary partition), using the best-fit model settings for each partition. Two Markov chain Monte Carlo (MCMC) runs with four chains each (one cold, three hot chains with default temperature t = 0.2) were started from independent random trees and computed 10 million generations, with trees sampled every 2000th generation. After discarding a burn-in of 500 trees (1/10 of all sampled trees) from each run, a consensus tree was calculated.

ML analyses were performed with RAxML v.7.2.8 (Stamatakis & al., 2008) using raxmlGUI v.0.95 (Silvestro & Michalak, 2011). Ten thousand rapid bootstrap replicates were computed using the GTR+Γ substitution model (GTRGAMMA, replaced automatically by BINGAMMA for indel characters); these were subjected to a thorough ML search with *Striga asiatica* (L.) Kuntze as outgroup, and without a constraint tree defined. Each analysis provided one fully resolved best-scoring ML tree.

Assessing incongruence. — Before combining the nuclear and chloroplast markers, these were tested for incongruence following Bull & al. (1993). Whether or not datasets with a potentially different phylogenetic history should be combined has been the issue of extensive debates (see reviews by Miyamoto & Fitch, 1995; Queiroz & al., 1995; Huelsenbeck & al., 1996). In Rhinantheae, several examples of intrageneric reticulate evolution caused by introgression and hybridization have been reported, e.g., in *Euphrasia*, *Rhinanthus*, *Melampyrum*, and *Odontites* (e.g., Yeo, 1968; Kwak, 1978; Bolliger & al., 1990; Wesselingh & Van Groenendael, 2005; Liebst, 2008; Těšitel & al., 2010). As the amount of heterogeneity present on the intergeneric level cannot be assumed to be neglectible, assessment of incongruence prior to any combined analysis must be considered particularly important in this group. Following the "conditional combination approach" (Huelsenbeck & al., 1996; Johnson & Soltis, 1998), taxa displaying considerable incongruence between nuclear and chloroplast data should be excluded from a combined dataset.

We applied several methods to assess levels of data heterogeneity: first, phylograms obtained from the chloroplast and nuclear datasets alone were visually examined and compared for well-supported discrepancies ("hard incongruence"; Mason-Gamer & Kellogg, 1996) using a cut-off of 70% ML bootstrap support. As reticulate relationships were to be expected and support values might be artificially lowered in fast-evolving groups due to raised levels of homoplasy (especially in the ITS dataset, as pointed out, e.g., by Albach & Chase, 2004), we employed an additional, more liberal threshold of 85% Bayesian support, to reliably identify all cases of incongruence. The respective taxa were then further analyzed using both a statistical and a network approach. The incongruence length difference (ILD) test, implemented in PAUP* v.4.0b10 (Swofford, 2003) as partition homogeneity test, was performed with 1000 replicates and the MAXTREES option set to 100. The chloroplast markers were tested against each other, and the combined chloroplast dataset against ITS, always including outgroups. Previously identified taxa showing hard incongruence were then successively excluded, the ILD tests were repeated and the results compared to those of the complete dataset. Employing again a conservative approach, we decided to assess the degree of incongruence introduced by a single taxon based on the increase of the ILD *P*-value when excluding that taxon. In addition, a split network was constructed in order to visualize contradicting signals contained in the Bayesian chloroplast and ITS trees. This was done with SplitsTree v.4.12.3 (Huson & Bryant, 2006) and by using the trees from the first of two runs each of the chloroplast and ITS analysis (discarding 1/10 burn-in trees). A consensus network (CN) applying a threshold of 0.25 (which presents branches appearing with a frequency of 25% or higher of all trees obtained) was generated using mean edge weights; splits were transformed with the equal angle transformation method followed by a convex hull optimization (Dress & Huson, 2004), using weights, and no filter for the resulting splits. From this network, one or more taxa were then removed using the "exclude selected taxa" option of SplitsTree, and the resulting CNs were compared. Finally, only taxa showing hard incongruence in their placements as well as significant results in the ILD test were excluded from the combined analysis, to avoid loss of valuable information due to false positives.

■ RESULTS

Sequencing and alignment. — A total of 98 sequences were generated for this study, 33 each for the *trnK* region and the *rps16* intron, and 32 for ITS. As the sequence pherograms for the ITS marker region provided a clear, unambiguous signal without any signs of polymorphisms, no cloning was performed. In a few taxa where sequencing failed, sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov) to complete the taxon sampling: these include *Melampyrum nemorosum* L. (ITS), *Euphrasia stricta* J.P. Wolff ex J.F. Lehm. (*trnK*, *rps16*, ITS), *Pedicularis sylvatica* L./*P. attollens* A. Gray (*trnK*, *rps16*, ITS) and *Striga asiatica* (*trnK*, ITS). For *Striga*, no *rps16* intron sequence was available in GenBank; however, as the outgroups were intended to be in accordance with those in Bennett & Mathews (2006) and Těšitel & al. (2010), the genus was nevertheless used, and *rps16* was coded as missing data for the combined analysis. For the same reason, we used GenBank sequences for *Pedicularis* originating from two species (*P. attollens* for *rps16* and ITS and *P. sylvatica* for *trnK*, as this marker sequence was not available for *P. attollens*). The combined DNA data matrix of *trnK*, *rps16*, and ITS contained 2800 aligned characters. The average sequence length was 1032 basepairs (bp) for *trnK*, 784 bp for *rps16*, and 689 bp for ITS. Detailed information on alignment statistics for all markers including the proportions of parsimony-informative characters is provided in Table 1.

Table 1. Alignment characteristics and statistics for ITS, *trnK* region, *rps16* intron, combined chloroplast dataset, and combined dataset. Number of constant characters, parsimony-informative characters and % parsimony-informative characters refers to non-excluded characters; number of excluded characters includes peripheral regions of the alignment not suitable for analysis, mononucleotide repeats and regions which could not be aligned unambiguously; proportion of unknown characters calculated without peripheral regions of the alignment.

	ITS	*trnK*	*rps16*	Comb. Chloroplast	Combined
Number of taxa	36	36	34	36	30
Sequence length (average)	537–737 bp (689 bp)	813–1082 bp (1032 bp)	701–848 bp (784 bp)	1044–1898 bp (1772 bp)	1588–2594 bp (2461 bp)
Aligned length	767 bp	1147 bp	886 bp	2033 bp	2800 bp
Excluded characters	197 bp	108 bp	153 bp	261 bp	458 bp
Constant characters	275 bp	708 bp	561 bp	1269 bp	1544 bp
Parsimony-informative characters	191 bp	133 bp	74 bp	207 bp	398 bp
% parsimony-informative characters	33.51%	12.80%	10.10%	11.68%	16.99%
Unknown characters within alignment (average)	0–14.63% (0.96%)	0–22.81% (1.05%)	0–6.76% (0.84%)	0–44.20% (3.40%)	0–35.65% (2.79%)
Average G+C content	55.24%	34.24%	34.54%	34.39%	40.27%

Chloroplast and ITS analyses. — The combined chloroplast dataset—including coded indels—showed a standard deviation of split frequencies of 0.002 after the Bayes runs. ML optimization resulted in a final likelihood of −7324.158667, the length of the best tree found was 0.571629, and the alpha parameters were estimated at 0.754251 for the DNA data partition, and at 2.602716 for the binary indel data partition. The ITS dataset, at the end of the Bayes analysis, also had a standard deviation of split frequencies of 0.002. ML optimization resulted in a final likelihood of −4653.464574, with a best tree length of 2.041682, and an alpha parameter of 0.459579. Bayesian and ML analyses resulted in highly similar topologies; therefore the ML bootstrap support values (BS) were plotted onto the respective Bayesian 80% consensus tree. Nodes from the ML tree not supported by Bayesian analysis were added to the consensus tree only if their support equalled or exceeded 75%. Individual phylogenetic reconstructions from the combined chloroplast (*trnK*, *rps16*) and the single nuclear marker (ITS) are shown in Figs. 2 and 3.

The topology of the ITS tree is largely similar to that of the combined chloroplast tree. ML bootstrap support values are considerably lower than Bayesian posterior probabilities

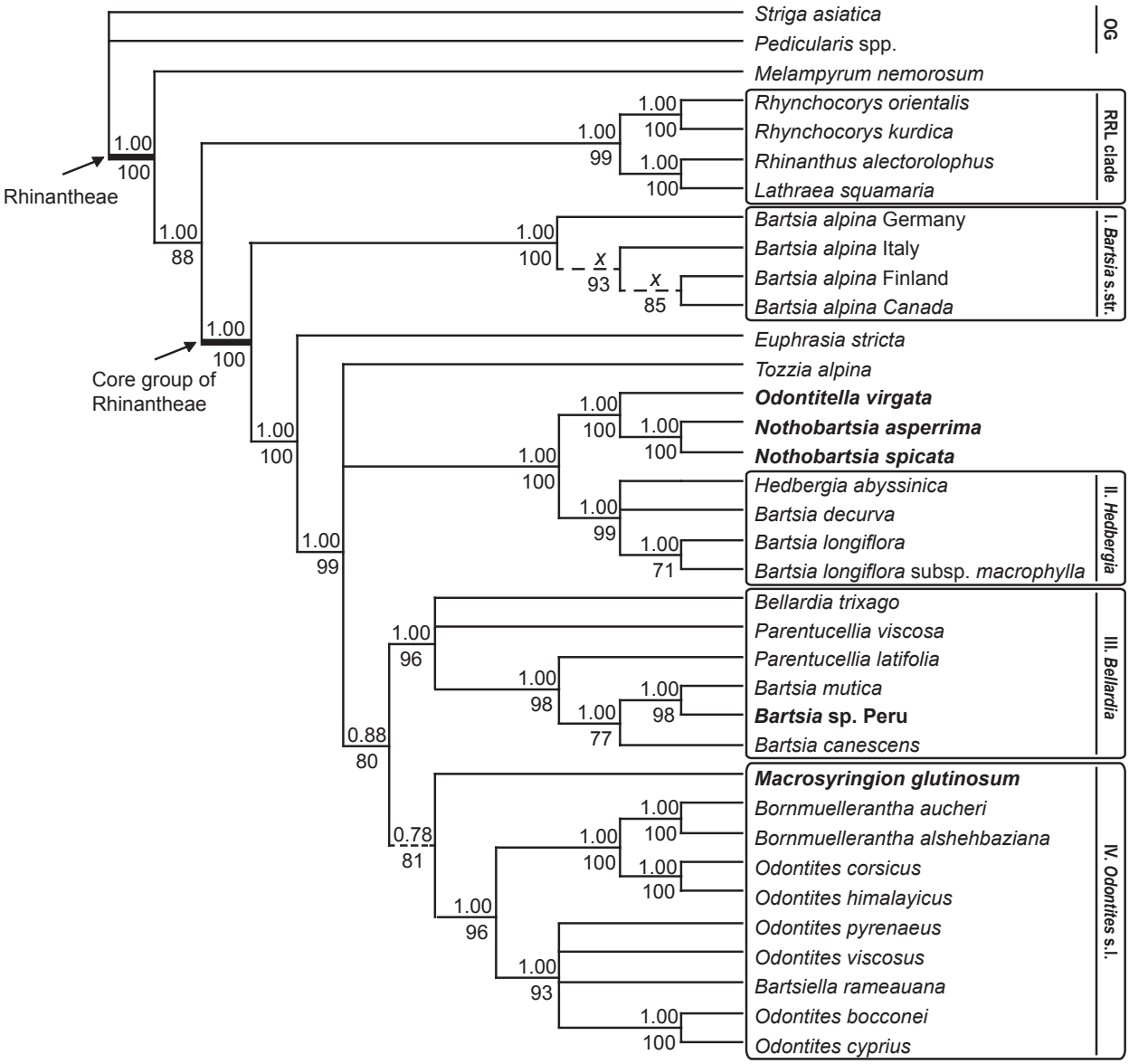

Fig. 2. Bayesian consensus tree (cladogram) from the combined chloroplast dataset (*rps16* intron and *trnK* region). Posterior probabilities (PP) are given above each node, maximum likelihood (ML) bootstrap support values (BS) for the corresponding node are indicated below. Only nodes equalling or exceeding either 80% in Bayesian or 75% in ML analysis are shown. Branches sufficiently supported by ML only are represented by dashed lines (corresponding PP values are given, no support in Bayesian inference is denoted by an "x"). PP values obtained from 9002 trees, BS values obtained from a best-scoring ML tree from 10,000 bootstrap replicates with subsequent maximum likelihood optimization (not shown). Divergence of the "Rhinantheae" and "core group of Rhinantheae" (as defined in the text) is marked by arrows. Taxa which were excluded in the combined analysis are highlighted in bold. OG, outgroup; RRL clade, *Rhynchocorys*-*Rhinanthus*-*Lathraea* clade.

(PP). *Melampyrum* is revealed as highly supported sister to an equally supported clade which comprises all remaining ingroup taxa in both analyses; however, support for these two nodes is weak in the nuclear ML tree (BS: 66 and 64, respectively). A clade comprising *Rhynchocorys*, *Rhinanthus* and *Lathraea* L. (referred to as the "RRL" clade) receives high support in the chloroplast topology (PP: 1.00, BS: 99), but is only moderately to weakly supported in the nuclear tree (PP: 0.81, BS: 50). The remaining genera of the ingroup constitute the "core group of Rhinantheae", a clade with high support in almost all analyses (chloroplast PP: 1.00, BS: 100; ITS PP: 1.00, BS: 68). Within this group, four main clades can be identified: "*Bartsia* s.str." (clade I), "*Hedbergia*" (clade II), "*Bellardia*" (clade III) and "*Odontites* s.l." (clade IV), see Figs. 2 and 3. In both datasets, clade I is sister to a clade containing clades II–IV in addition to *Euphrasia stricta* and *Tozzia alpina* L. as well

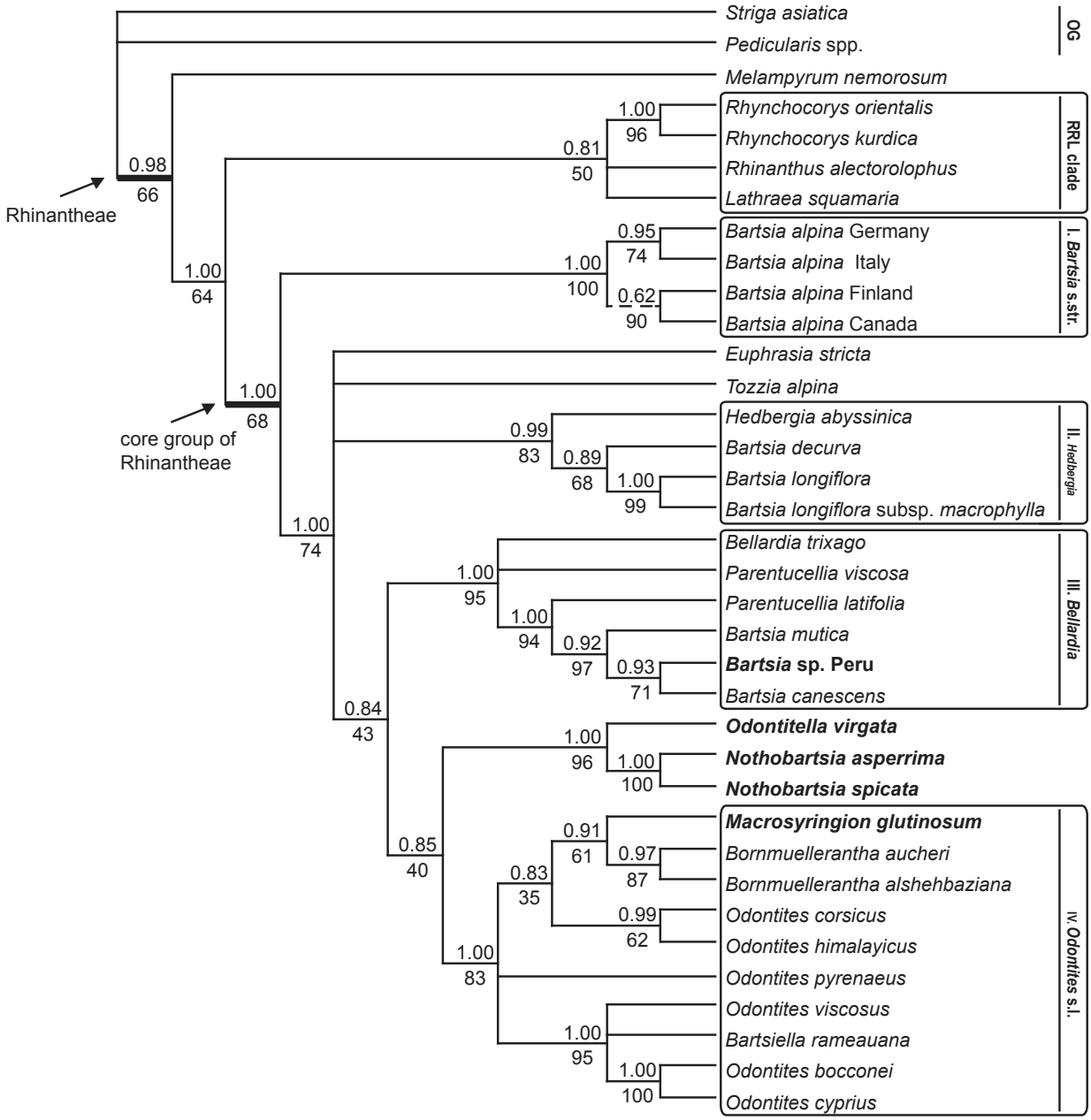

Fig. 3. Bayesian consensus tree (cladogram) from the nuclear internal transcribed spacer region (ITS). Posterior probabilities (PP) are given above each node, maximum likelihood (ML) bootstrap support values (BS) for the corresponding node are indicated below. Only nodes equalling or exceeding either 80% in Bayesian or 75% in ML analysis are shown. Branches sufficiently supported by ML only are represented by dashed lines. PP values obtained from 9002 trees, BS values obtained from a best-scoring ML tree from 10,000 bootstrap replicates with subsequent maximum likelihood optimization (not shown). Divergence of the "Rhinantheae" and "core group of Rhinantheae" (as defined in the text) is marked by arrows. Taxa which were excluded in the combined analysis are highlighted in bold. OG, outgroup; RRL clade, *Rhynchocorys-Rhinanthus-Lathraea* clade.

as a group of *Odontitella virgata* (Link) Rothm., *Nothobartsia asperrima* (Link) Benedí & Herrero and *N. spicata* (Ramond) Bolliger & Molau. In the chloroplast tree, *Euphrasia* is highly supported as sister to the remaining taxa, which in turn are part of an unresolved polytomy; the latter consists of clade II plus a clade comprising *Nothobartsia* and *Odontitella*; clades III–IV; and *Tozzia*. In the ITS tree, the position of *Euphrasia* remains unresolved. Information about the relationships among clades II–IV is more easily obtained from the combined analyses of all three markers (see below), and detailed information on each clade is also given there.

While the monophyly of clades II, III and IV is highly supported in most reconstructions, several taxa within the clades show contradicting positions in the chloroplast and nuclear phylogenies (hard incongruence as defined above): *Macrosyringion glutinosum* (M. Bieb.) Rothm. is moderately supported as sister to the remaining taxa of clade IV in the chloroplast tree (PP: 0.78, BS: 81), while it is deeply nested within the clade in the ITS tree, forming a clade with the two species of *Bornmuellerantha* (PP: 0.91, BS: 61). *Bartsia* sp. Peru is sister to *B. mutica* (Kunth) Benth. in the chloroplast analysis (PP: 1.00, BS: 98), but sister to *B. canescens* Wedd. in the ITS analysis (PP: 0.93, BS: 71). *Bartsia alpina* L. from Italy is sister to the accessions from Finland and Canada in the chloroplast tree (PP: –, BS: 93), but groups with the specimen from Germany in the ITS tree (PP: 0.95, BS: 74). However, the most obvious contradictory placement concerns *Nothobartsia*, the two species of which are revealed as monophyletic with maximum support. *Nothobartsia* is part of a highly supported clade with *Odontitella virgata* in both analyses, but this clade is sister to the *Hedbergia* clade in the chloroplast analysis (PP: 1.00, BS: 100), while it is sister to the *Odontites* s.l. clade in the ITS analysis with moderate support (PP: 0.85, BS: 40).

Incongruence tests and phylogenetic reconstruction. — The ILD test showed the two chloroplast datasets (*trnK*, *rps16*) to be congruent for all taxa ($P = 0.442$), so these data were concatenated in all analyses and analyzed as one combined chloroplast dataset. In contrast, when testing the ITS marker against the combined chloroplast matrix, the test displayed significant heterogeneity ($P = 0.013$, with values <0.05 considered significant following Farris & al., 1995 and Cunningham, 1997), implying conflicting signal within the data.

Sequential exclusion of the taxa showing hard incongruence as defined above (the group of *Nothobartsia asperrima*/*N. picata*/*Odontitella*; *Macrosyringion*; *Bartsia* sp. Peru; and *B. alpina* [Italy]) resulted in a noticeable increase in *P*-values in five cases ($P + 0.064$ without *Nothobartsia* and *Odontitella*, $P + 0.065$ when excluding *Macrosyringion*, and $P + 0.122$ when additionally removing *Bartsia* sp. Peru, compared to $P = 0.013$ when including all taxa). Excluding *Bartsia alpina* did not improve congruence in the ILD test (decrease in *P* by 0.003). Consequently, *Bartsia alpina* was maintained in the sampling for the combined analysis of all markers, while the other five taxa were excluded.

This is in accordance with the consensus network (CN) constructed from 9002 Bayesian chloroplast and nuclear trees. The CN shows tree-like as well as network-like relationships, illustrated by sets of parallel edges (branches) which indicate differing signals within the data (Fig. 4A). Almost no reticulations are present in the *Hedbergia* and *Bellardia* clades (with few exceptions in the latter, due to the variable positions of *Bartsia* sp. Peru and *Bellardia trixago*), while relationships in the *Odontites* s.l. clade are revealed to be complex. This is largely attributable to *Macrosyringion*, while the conflicts in the placement of the *Nothobartsia*/*Odontitella* group result in the highly network-like central part. When the five taxa showing hard incongruence are removed from the network, it takes on a much more tree-like structure (Fig. 4B), greatly simplifying from 78 splits and 18 sets of parallel edges to 63 splits and 8 sets of parallel edges. Confidence values for each remaining set of parallel edges are given at the respective branches in Fig. 4B. The ILD test showed this reduced dataset to be congruent ($P = 0.264$) and thus suitable for the combined analyses.

Combined analysis. — A combined analysis was conducted using all three markers and a reduced set of 29 ingroup taxa. The Bayesian 80%/ML 75% consensus tree is shown in Fig. 5. The standard deviation of split frequencies at the end of the Bayes analysis was 0.002. ML optimization resulted in a final likelihood of −11241.558677, a best tree length of 0.837353 and an alpha parameter estimated at 0.430033 for the DNA data partition and at 4.327658 for the binary indel data partition. As in the chloroplast and nuclear trees, Bayesian and ML topologies computed from the combined dataset were very similar, so the ML bootstrap supports could be plotted onto the Bayesian consensus tree.

Within the combined phylogeny, *Melampyrum*, here represented by *M. nemorosum*, is again revealed as sister to the rest of the Rhinantheae (PP: 1.00, BS: 99). The RRL clade comprising *Rhynchocorys orientalis* Benth. and *Rhynchocorys kurdica* Nábělek (PP: 1.00, BS: 100) and a clade of *Rhinanthus alectorolophus* (Scop.) Pollich and *Lathraea squamaria* L. (PP: 1.00, BS: 100) now receives maximum support (PP: 1.00, BS: 100). This RRL clade is sister to the highly supported "core group of Rhinantheae" (PP: 1.00, BS: 95), which comprises four clades, each with unambiguous support for their monophyly.

Clade I ("*Bartsia* s.str.") is composed of all included accessions of *Bartsia alpina*, covering the whole geographic range of the species. This clade is inferred as sister to the remaining Rhinanthoid taxa (PP: 1.00, BS: 95), in accordance with the separate chloroplast and nuclear results. Within clade I, there is varying support for a sister relationship between accessions of *B. alpina* from Finland and Canada (PP: 0.61, BS: 95), and Germany and Italy (PP: 0.96, BS: 77).

Euphrasia is indicated as sister to the remaining clades plus *Tozzia alpina* of the monotypic *Tozzia* with high support (PP: 1.00, BS: 97). As in the single-marker analyses, the latter genus remains unresolved, reflecting its two, almost equally probable positions in the CN (Fig. 4B; confidence values 41.6 vs. 39.5).

Clade II ("*Hedbergia*") is composed of the monotypic *Hedbergia* and the African accessions of *Bartsia* (*B. decurva* Hochst. ex Benth., *B. longiflora* Hochst. ex Benth. and *B. longiflora* subsp. *macrophylla* (Hedberg) Hedberg), with the latter two taxa highly supported as sisters (PP: 1.00, BS: 99).

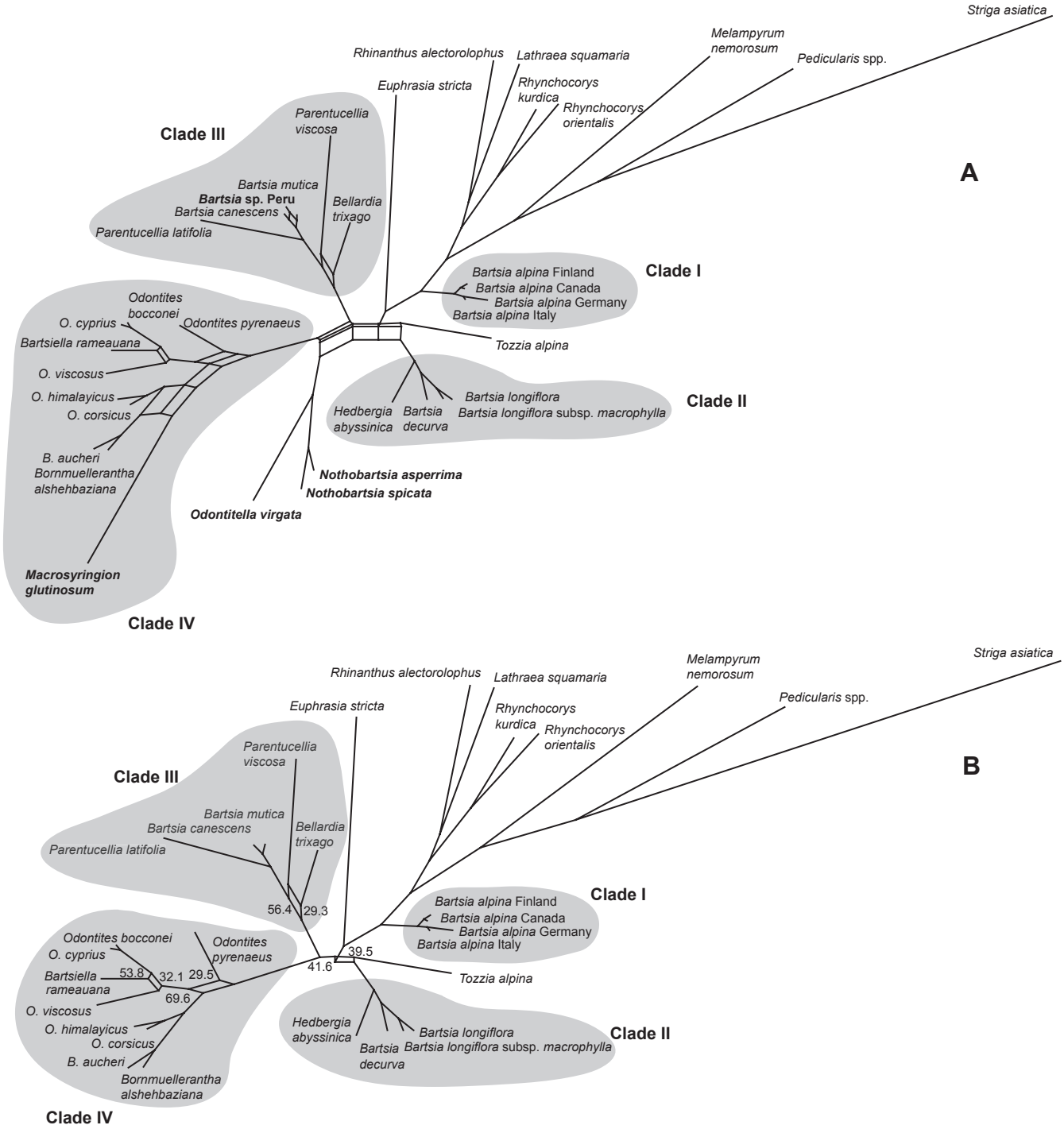

Fig. 4. Split consensus networks for the combined chloroplast and ITS data, using a 25% threshold, obtained from the collections of trees produced by the separate Bayesian analyses of the combined chloroplast dataset and the ITS dataset, respectively (yielding the consensus trees shown in Figs. 2 and 3). Trees from the first run of each analysis (discarding a 10% burn-in) were analyzed (9002 trees). Depicted edge lengths are proportional to mean branch lengths, values attached to edges denote corresponding confidence values. Composition of the consensus network based on **A)** all 36 taxa included in the study, and **B)** with five taxa displaying high levels of incongruence (*Macrosyringion glutinosum*, *Bartsia* sp. Peru, *Nothobartsia asperrima*, *Nothobartsia spicata* and *Odontitella virgata*, shown in bold italics in A), removed using the "exclude selected taxa" command (see text). The tree from the Bayesian analysis with all markers combined and based on the reduced sampling presented in B) is shown in Fig. 5. Clades I–IV are shaded grey: I, *Bartsia* s.str.; II, *Hedbergia*; III, *Bellardia*; IV, *Odontites* s.l. — *O.*, *Odontites*; *B.*, *Bornmuellerantha*.

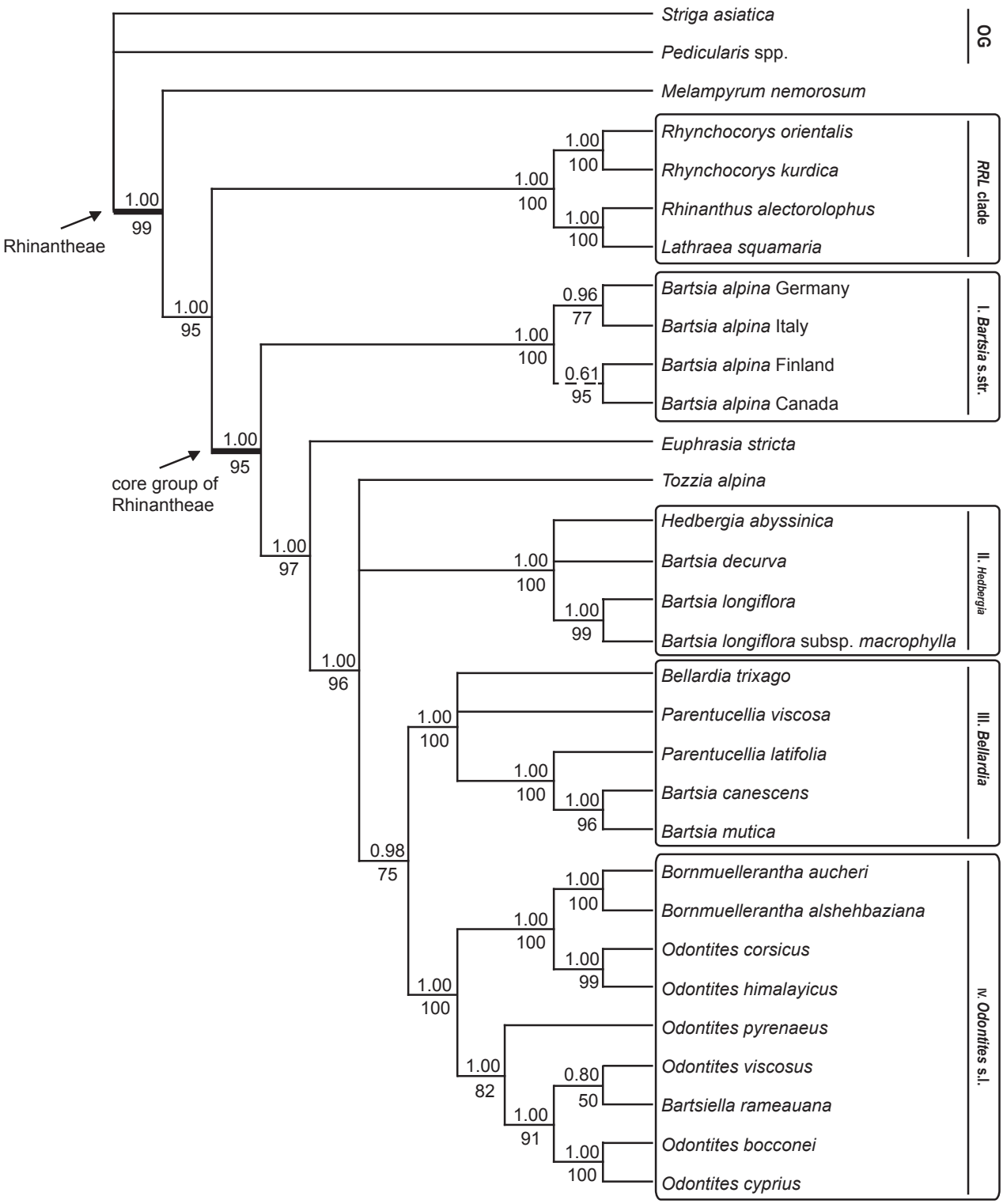

Fig. 5. Bayesian consensus tree (cladogram) from the combined dataset (ITS, *rps16* intron and *trnK* region). Posterior probabilities (PP) are given above each node, maximum likelihood (ML) bootstrap support values (BS) for the corresponding node are indicated below. Only nodes equalling or exceeding either 80% in Bayesian or 75% in ML analysis are shown. Branches sufficiently supported by ML only are represented by dashed lines. PP values obtained from 9002 trees, BS values obtained from a best-scoring ML tree from 10,000 bootstrap replicates with subsequent Maximum Likelihood optimization (not shown). Divergence of the "Rhinantheae" and "core group of Rhinantheae" (as defined in the text) is marked by arrows. OG, outgroup, RRL clade, *Rhynchocorys-Rhinanthus-Lathraea* clade.

The sister relationship between clade III and IV is highly supported in the Bayesian analysis and receives moderate support in the ML analysis (PP: 0.98, BS: 75).

Clade III ("*Bellardia*") comprises the two included species of Neotropical *Bartsia*, two accessions of *Parentucellia* Viv., and *Bellardia trixago* (L.) All. The monotypic *Bellardia* All. and *Parentucellia viscosa* (L.) Caruel are shown in a polytomy, while *P. latifolia* (L.) Caruel is sister to the South American species of *Bartsia* (PP: 1.00, BS: 100). A close relationship of *Parentucellia* and the Neotropical *Bartsia* species to *Bellardia* is strongly indicated in all analyses; furthermore, *Parentucellia* is clearly paraphyletic, with the South American *Bartsia* species nested within it. Neotropical *Bartsia* itself (here represented by *B. canescens* from Peru and *B. mutica* from Argentina) is supported as monophyletic (PP: 1.00, BS: 96).

Clade IV ("*Odontites* s.l.") contains six species of *Odontites* being part of two subclades which receive maximum to moderate support: one subclade (PP: 1.00, BS: 82) is composed of four *Odontites* species and the monotypic *Bartsiella* (*Bartsiella rameauana* (Emb.) Bolliger) nested within it, being sister to *O. viscosus* (L.) Clairv. with weak support (PP: 0.80, BS: 50). A second group comprises *O. bocconei* (Guss.) Walp. and *O. cyprius* Boiss.; *O. pyrenaeus* (Bubani) Rothm. is indicated as sister to all taxa of the subclade. The other subclade (PP: 1.00, BS: 100) consists of the sister species *O. corsicus* G. Don and *O. himalayicus* Pennell, and of the monophyletic *Bornmuellerantha*, comprising *B. aucheri* (Boiss.) Rothm. and the recently described *B. alshehbaziana* (Dönmez & Mutlu, 2010). Given the positions of *Bornmuellerantha* and *Bartsiella*, *Odontites* is rendered paraphyletic in this analysis.

■ DISCUSSION

Biogeography of Rhinantheae. — Těšitel & al. (2010) conducted a dispersal-vicariance analysis and identified temperate western Eurasia as the origin of the Rhinanthoid Orobanchaceae, in accordance with the Laurasian origin north of the Tethyan Sea as assumed by Wolfe & al. (2005) and the Eurasian origin inferred for the cosmopolitan hemiparasitic *Euphrasia* by Gussarova & al. (2008). Most genera of the core group of Rhinantheae sampled here have a distinct center of diversity in the Mediterranean area. An exception are the frutescent perennial Afromontane species of *Bartsia* sect. *Longiflorae* Molau (Molau, 1990) and the perennial hemiparasitic monotypic *Hedbergia* from East Africa, which form a highly supported group in all analyses (Figs. 2, 3 & 5). Molau (1988, 1990) considered *Hedbergia* to represent an ancestral lineage of Rhinantheae, based on its distinctive corolla morphology. However, the contrary is evident from our molecular phylogenetic reconstructions, as *Hedbergia* is clearly revealed as member of core Rhinantheae, closely associated with the Afromontane species of *Bartsia*. Regarding their disjunct distribution pattern, the core Rhinantheae agree with some other typical elements of the European alpine flora, representatives of which can often be found in Afromontane habitats (Hedberg, 1970; Gehrke & Linder, 2009; Emadzade & Hörandl, 2011).

The most interesting biogeographic pattern of the *Bellardia* clade is the position of the Neotropical species of *Bartsia* s.l. as a lineage derived from the Mediterranean taxa. The New World *Bartsia* species are confined to Andean montane habitats of Colombia, Bolivia, Peru, and Chile to northern Argentina, and seem to descend from a Mediterranean lineage represented by the extant *Parentucellia latifolia*, which is widespread across the Mediterranean area and beyond, ranging from the Canary Islands to eastern Asia. As this species is revealed as sister to all Neotropical species of *Bartsia* s.l., it can be assumed that the ancestor of the Neotropical lineage arrived from the Mediterranean via long-distance dispersal, and diversified in an adaptive radiation to occupy the new habitats. Examples for disjunct Mediterranean-Andean taxa with an Old World origin are found in several other plant groups, such as *Eryngium* L., *Lupinus* L., Menthinae, *Pericallis* D. Don, and *Senecio* L. (Panero & al., 1999; Coleman & al., 2003; Hughes & Eastwood, 2006; Calviño & al., 2008; Bräuchler & al., 2010; Kadereit & Baldwin, 2012).

Taxonomic implications. — Although the sampling of this study is limited, our results allow some taxonomic conclusions regarding the generic placement of several taxa. This is especially true for *Bartsia* s.l. (in the circumscription of Molau, 1990), and the small genera *Bartsiella* and *Bornmuellerantha*. The latter two genera were previously included within *Odontites* (Wettstein, 1891), but were later recognized as distinct based on corolla shape, anther indumentum, and pollen types (Rothmaler, 1943; Bolliger & Wick, 1990; Bolliger & Molau, 1992; Bolliger, 1996).

The type of *Bartsia* (*Bartsia alpina* L.; typ. cons.) is unambiguously dissociated from the remaining members of the genus in both tree and network analyses, and it is shown as sister to the core group of Rhinantheae. As a result, *Bartsia* L. (nom. cons.) should be redefined as a monotypic genus including only *B. alpina* (equalling *Bartsia* sect. *Bartsia* in Molau, 1990), which is geographically restricted to Arctic-alpine (montane) Europe and northeastern North America. Thus circumscribed, the genus consists of one hemiparasitic, perennial rhizomatous geophyte with a perennial woody rhizome and annual aerial shoots, whereas all other species included in *Bartsia* sensu Molau (1990) are either annuals, or perennial hemicryptophytes (with a woody base and monopodial growth). Reliable chromosome counts for *B. alpina* show a range of cytotypes including $2n = 24$ (Löve & Löve, 1956; Löve, 1982; Dalgaard, 1988; Molau, 1990; Dobeš & Vitek, 2000), the odd number of 36 (which might perhaps be from a triploid specimen), tetraploid $4x = 48$ (Dobeš & Vitek, 2000) and hexaploid $6x = 72$ (Taylor & Rumsey, 2003), with $2n = 24$ being the most common and predominant number recorded throughout the European range of the species (Molau, 1990; Dobeš & Vitek, 2000). This suggests a polyploid series with a consistent base number of $x = 12$ in the genus. However, the report of 36 chromosomes might also imply a base number of $x = 6$.

The two yellow-flowered, hemiparasitic, frutescent Afromontane species of *Bartsia* (*Bartsia* sect. *Longiflorae*), *B. decurva* and *B. longiflora* (including *B. longiflora* subsp. *macrophylla*; Hedberg & al., 1979; Molau, 1990) are associated with

the monotypic Afromontane *Hedbergia* (*H. abyssinica* (Benth.) Molau) in all phylogenetic analyses, as well as in the consensus network (Figs. 2–5). Since vegetative morphology and palynology provide strong evidence for this clade to form a natural unit, we propose to transfer the respective African taxa of *Bartsia* to *Hedbergia*. In consequence, the genus requires an updated circumscription to include *Bartsia* sect. *Longiflorae* (see "New Generic Circumscriptions"), and thus is no more exclusively characterized by a rotate corolla symmetry (Hedberg & al., 1980; Molau, 1988). The unique actinomorphic symmetry of the flowers observed in *Hedbergia* (Molau, 1988) might have evolved in adaptation to a different pollinator. Unfortunately, there is not much cytological data published for members of this clade: for the African *Bartsia* species only a single chromosome count of $2n$ = app. 28 for *B. decurva* (as *B. macrocalyx* R.E. Fr.) was reported by Hedberg (Hedberg, 1957; Hedberg & Hedberg, 1977; Hedberg & al., 1979), and no count for *Hedbergia* is available so far.

The sister relationship of the *Odontites* s.l. clade and the *Bellardia* clade is highly supported in the consensus tree of the combined dataset (Fig. 5). This corroborates the results of Těšitel & al. (2010) who found a clade containing *Parentucellia* species which was sister to several *Odontites* taxa. Furthermore, both groups are well-circumscribed by several morphological characters. All members of the *Odontites* s.l. clade are characterized by one-sided racemes, whereas the racemose inflorescences of the members of the *Bellardia* clade are predominantly multilateral (partially unilateral in the Neotropical *Bartsia* sect. *Diffusae* Molau; Molau, 1990). Furthermore, members of the *Bellardia* clade have upright ovules (Molau, 1990), whereas members of the *Odontites* s.l. clade share the synapomorphy of having pendulous ovules (Rothmaler, 1943; Bolliger, 1996).

In accordance with preceding studies (Wolfe & al., 2005; Bennett & Mathews, 2006; Těšitel & al., 2010), our phylogeny supports the monophyly of the New World *Bartsia* species and their placement embedded in a grade of *Bellardia* and *Parentucellia*. However, the results of the present study were obtained using a sample designed to avoid misleading results caused by the incorporation of incongruent data, a fact that was not accounted for by Těšitel & al. (2010). While the monotypic *Bellardia* recently has been re-included in *Bartsia* in several treatments (e.g., Molau, 1990; López-Sáez & al., 2002), it is clearly not supported as part of *Bartsia* s.str. here. Instead, it is part of a highly supported clade together with *Parentucellia* and the New World species of *Bartsia* s.l. Its phylogenetic position underlines the close affinity to *Parentucellia*: *Bellardia trixago*, especially the yellow-flowered variant (Benedí, 2002), is not only frequently confused with *Parentucellia viscosa* due to morphological similarity and overlapping distribution ranges (Benedí, 1998), but apparently is also naturally hybridizing with the latter (Valdés & al., 1987; Benedí, 1998)—however, the identity of the putative hybrids is somewhat dubious. Considering morphological, molecular and biogeographical evidence, including *Parentucellia* in *Bellardia* seems reasonable; in doing so, the generic name *Bellardia* All. has nomenclatural priority over the younger name *Parentucellia* Viv. As the New World species of *Bartsia* s.l. are nested within *Parentucellia* and the *Bellardia* clade, these species also should be included in *Bellardia*. The cytological data available for the group give some additional support, as both *Bellardia* and *Parentucellia*, as well as New World *Bartsia* share a chromosome base number of $x = 12$ (although this is a common base number in Rhinantheae): *Bellardia trixago* is a diploid with $2n = 24$ (Speta, 1971; Molau, 1990), while *Parentucellia* is tetraploid ($2n = 48$; Speta, 1971; Molau, 1990), except for one report of a diploid karyotype in an introduced invasive population of *P. viscosa* in California (Chuang & Heckard, 1992). This count, however, could have resulted from misidentification of a *Bellardia trixago* specimen. The Neotropical *Bartsia* species studied by Molau (1990) were either diploids with $2n = 24$, or tetraploids with $2n = 48$, the latter restricted to the Andean sections.

The *Odontites* s.l. clade, irrespective of internal topological conflicts, is supported in the chloroplast, ITS and combined analyses as well as in the consensus network, arguing for a broader definition of the genus. Our results strongly support *Odontites* to include *Bornmuellerantha* and *Bartsiella*, while the position of *Macrosyringion* remains doubtful in this respect (see below). The divergent corolla and anther morphology observed in *Bornmuellerantha* (Rothmaler, 1943; Bolliger, 1996; Dönmez & Mutlu, 2010) is a synapomorphy of its two species. However, both are deeply nested within *Odontites* s.l. in all analyses. The karyotype of *Bornmuellerantha aucheri* ($2n = 24$; Bolliger & Molau, 1992; Bolliger, 1996) fits the common base number of $x = 12$ shared by most taxa of *Odontites*. Therefore, we propose to reclassify *Bornmuellerantha* as *Odontites*, applying a broad definition of the genus as mentioned above. The small monotypic *Bartsiella* has been segregated from *Odontites* s.l. based mainly on palynological evidence (Bolliger & Wick, 1990; Bolliger, 1996). However, here it is also revealed as nested in *Odontites* s.l. Thus, recognizing *Bartsiella* as distinct does not seem justified, and the name should be transferred back to *Odontites*, as originally envisioned by Emberger (1932). To date, no chromosome counts are available for *Bartsiella*.

Macrosyringion shows considerable incongruence regarding its placement in the chloroplast and ITS phylogenies: it is nested within the *Odontites* s.l. clade in the ITS topology, but is sister to all other taxa of clade IV in the chloroplast tree. Although ancient hybridization could possibly produce a pattern like this (Joly & al., 2006), the process which created the incongruence cannot be clearly elucidated here. Furthermore, support values for a clade IV including *Macrosyringion* are low (PP: 0.78, BS: 81) in the chloroplast phylogeny, raising the question whether the genus should be included into a more broadly defined *Odontites*. Chromosome numbers cannot provide additional evidence as the reported number of $2n = 24$ for *Macrosyringion* (with the dysploid number $2n = 26$ occasionally observed in *M. longiflorum*; Bolliger & Molau, 1992; Bolliger, 1996) is found in *Odontites* as well as other taxa of Rhinantheae. Morphological characteristics are ambiguous as well: the prolonged corolla tubes, which could possibly represent a synapomorphy for the genus, were considered not significant enough to support it as distinct (Rothmaler, 1943). The shortly bifid corolla lip, however, additionally used by

Rothmaler to exclude *Macrosyringion* from *Odontites*, could also represent a transitional link to the deeply bilobate upper lip of *Bornmuellerantha*, which is sister to *Macrosyringion* in the ITS topology (Fig. 3) and clearly part of *Odontites* s.l. Given the available evidence, a formal inclusion of the genus in *Odontites* is not advisable without further evidence by analyses applying a broader sample of *Odontites* taxa as well as additional markers.

The taxonomic placement of the two species of *Nothobartsia* has frequently changed in the past, as the plants unite morphological characters of both *Bartsia* and *Odontites* (Molau, 1990; Bolliger & Molau, 1992). Both taxa were originally described as *Bartsia* (e.g., Wettstein, 1891) before a separate genus, *Nothobartsia*, was proposed by Bolliger & Molau (1992). In the present study, the genus is found as sister to *Odontitella virgata*, with which it forms a highly supported clade in all analyses.

This clade, like *Macrosyringion*, features conspicuous incongruence in the single-marker reconstructions and almost exclusively accounts for the highly networked central part of the CN in Fig. 4A. In the chloroplast tree, it is sister to *Hedbergia* and the Afromontane *Bartsia* species with maximum support. This relationship is supported palynologically by a highly similar retipilate exine sculpture, which differs from the reticulate pollen of *Odontites* and the majority of Rhinantheae (Hedberg & al., 1979, 1980; Bolliger & Wick, 1990; Molau, 1990; Bolliger & Molau, 1992; Bolliger, 1996). However, a retipilate pollen sculpture is also found in the only distantly related *Macrosyringion*. In the ITS phylogeny, the *Nothobartsia*/*Odontitella* group is sister to the *Odontites* s.l. clade, yet with moderate to weak support (PP: 0.85, BS: 40). This position is supported by the common synapomorphy of a pendulous ovule position (Rothmaler, 1943; Molau, 1990; Bolliger & Molau, 1992; Bolliger, 1996) which is not found in the remainder of core Rhinantheae. The latter character could be considered derived within Rhinantheae, and thus is more likely to represent a synapomorphy of the *Odontites* s.l. clade and the *Nothobartsia*/*Odontitella* group (as suggested by the ITS topology), rather than having evolved in parallel as indicated by the chloroplast topology. The affinity of *Nothobartsia* and *Odontitella* to *Odontites* s.l. is further confirmed by the fact that seeds and capsules of the two genera closely resemble those of *Odontites* s.l. (Molau, 1990; Bolliger & Molau, 1992; Bolliger, 1996).

The incongruent single-marker phylogenies, and the highly supported relationship to the *Hedbergia* clade in the chloroplast tree, as opposed to strong morphological similarities with *Odontites* s.l., argue for the presence of a reticulate pattern in the origin of the *Nothobartsia*/*Odontitella* group. Assuming ancient hybridization between ancestral lineages leading to the *Odontites* s.l. clade and the *Hedbergia* clade would provide a possible explanation for the origin of the putative ancestor of *Nothobartsia* and *Odontitella*. A hybrid origin could explain the morphological characteristics of the two perennial species of *Nothobartsia*, which are intermediate between *Bartsia* and *Odontites* s.l. (Bolliger & Molau, 1992). Equally, the annual *Odontitella* shares characters with both *Odontites* s.l. and *Bartsia* s.l. (Rothmaler, 1943; Bolliger, 1996), the latter suggesting a possible relationship to the *Hedbergia* clade.

■ NEW GENERIC CIRCUMSCRIPTIONS

As evident from our molecular phylogenetic reconstructions and as already discussed above, some combinations are required to maintain monophyly of certain genera. The paraphyly of *Bartsia* L., which has been known since Bennett & Mathews (2006) and is evident from our tree topologies, requires to circumscribe it as monotypic genus, comprising only the type species *Bartsia alpina* L. Therefore, we propose the following new combinations for the African and Neotropical taxa which have hitherto been assigned to *Bartsia*:

Hedbergia longiflora (Hochst. ex Benth.) A. Fleischm. & Heubl, **comb. nov.** ≡ *Bartsia longiflora* Hochst. ex Benth. in Candolle, Prodr. 10: 545. 1846 – Holotype: [Ethiopia], inter frutices in rupium rimis medio regionis ad latus septentrionale montis Kubbi, 12 Dec 1837, *Schimper 418* (K photo!; isotype: M!).

Hedbergia longiflora subsp. ***macrophylla*** (Hedberg) A. Fleischm. & Heubl, **comb. nov.** ≡ *Bartsia macrophylla* Hedberg in Symb. Bot. Upsal. 15: 174. 1957 ≡ *Bartsia longiflora* subsp. *macrophylla* (Hedberg) Hedberg in Norweg. J. Bot. 26: 7. 1979 – Holotype: Uganda, Ruwenzori, Bujuku Valley near Bigo camp, at small steam, 3400 m, 21 Mar 1948, *Hedberg 349* (UPS; isotype: K photo!).

Hedbergia decurva (Hochst. ex Benth.) A. Fleischm. & Heubl, **comb. nov.** ≡ *Bartsia decurva* Hochst. ex Benth. in Candolle, Prodr. 10: 545. 1846 – Holotype: [Ethiopia], in latere boreali montis Silke [Mt. Selki], 22 Feb 1840, *Schimper 1329* (K photo!; isotype: M!).

For *Parentucellia latifolia* (L.) Caruel and *P. viscosa* (L.) Caruel used in the present study, there are prior combinations to include them in *Bellardia* All. (this generic name has nomenclatural priority over *Parentucellia* Viv.), which should be used for these taxa in order to avoid paraphyly of *Bellardia*.

Bellardia latifolia (L.) Cuatrec. in Trab. Mus. Ci. Nat. Barcelona 12: 428. 1929 ≡ *Euphrasia latifolia* L., Sp. Pl. 2: 604. 1753 – Lectotype (designated by Sutton in Jarvis, Order Out Of Chaos: 514. 2007): [icon.] *Euphrasia pratensis Italica latifolia* in Morison, Pl. Hist. Univ. 3: 431, s. 11, t. 24, f. 8. 1699.

Bellardia viscosa (L.) Fisch. & C.A. Mey in Index Seminum [St. Petersburg] 2: 4. 1836 ≡ *Bartsia viscosa* L., Sp. Pl. 2: 602. 1753 – Lectotype (designated by Fischer in Feddes Repert. 108: 113. 1997): [icon.] *Euphrasia lutea latifolia palustris* in Plukenet, Phytographia: t. 27, f. 5. 1691.

A third, yet taxonomically doubtful species of *Parentucellia* (*P. floribunda* Viv., representing the generic type), endemic to Libya, is accepted in numerous treatments (e.g. Qaiser, 1982). Since we were neither able to consult the type nor to obtain recently collected material for molecular analysis, we refrain from making a new combination here.

Although it is very likely that the Neotropical species of *Bartsia* (sensu Molau, 1990) are monophyletic, we hesitate to propose new combinations for the unsampled taxa, and exemplarily refer to the two representatives used in our study:

Bellardia canescens (Wedd.) A. Fleischm. & Heubl, **comb. nov.** ≡ *Bartsia canescens* Wedd., Chlor. Andina 2: 123. 1860 – Lectotype (designated by Molau in Opera Bot. 102: 76. 1990): Peru, Lima, without date, *Dombey s.n.* (P; isotype: PH photo!).

Bellardia mutica (Kunth) A. Fleischm. & Heubl, **comb. nov.** ≡ *Euphrasia mutica* Kunth, Nov. Gen. Sp. [quarto] 2: 334. 1818 ≡ *Bartsia mutica* (Kunth) Benth. in Candolle, Prodr. 10: 548. 1846 – Lectotype (designated by Molau in Opera Bot. 102: 58. 1990): Peru, crescit locis siccis Peruviae inter Lucarque et Ayavaca, 1300 hex [ca. 2400 m], without date, *Bonpland 3466* (P photo!; isotype: H photo!).

A broad circumscription of *Odontites* Ludw., including the small genera *Bornmuellerantha* Rothm. and *Bartsiella* Bolliger, is in agreement with the phylogenetic results, and requires the following new combination:

Odontites alshehbazianus (Dönmez & Mutlu) A. Fleischm. & Heubl, **comb. nov.** ≡ *Bornmuellerantha alshehbaziana* Dönmez & Mutlu in Novon 20: 265. 2010 – Holotype: Turkey, Antalya, Gazipaşa, 1760 m, 23 Sep 2006, *Dönmez & Mutlu AAD 14036* (HUB; isotypes: E, INU, M!, MO).

■ CONCLUSION

The Rhinantheae as studied here are characterized by some obvious topological incongruences between the plastid and nuclear datasets. *Nothobartsia* constitutes the most conspicuous example of reticulation in the evolutionary history of Rhinantheae, explaining part of the incongruent patterns observed. Hybridization is likely to play a major role in Rhinantheae, especially in *Odontites* s.l. However, incongruence in general may have several other reasons, including sampling artefacts, incomplete lineage sorting, or low sequence divergence in closely related groups with ongoing speciation (which is likely to apply in case of the incongruent placement of the South American *Bartsia* sp. Peru). The sample and focus of this study do not allow us to discriminate unequivocally among the different processes and to determine those actually involved here. Nevertheless, our findings allow some reliable conclusions concerning the circumscription of *Bartsia*, *Bellardia*, and *Hedbergia*, and corroborate a broader circumscription of *Odontites*. Further extension of the results presented here seems advisable, using a comprehensive sample including several specimens of all taxa known to account for overall genetic diversity within the group. Analysis of several nuclear DNA sequence regions and a thorough assessment of hybridization on the inter- and intrageneric level (applying cloning techniques where necessary) can surely complement our understanding of this important tribe of Orobanchaceae.

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Appendix. List of taxa used in the phylogenetic analyses with voucher information. Previously published sequences of ITS, *trnK* and *rps16* are provided with reference citations. Key to references: (1): Těšitel & al., 2010; (2): Schäferhoff & al., 2010; (3): Tank & Olmstead, 2008; (4): Müller & al., 2004; (5): Morawetz & Wolfe, 2009; (6): Young & al., 1999.

Taxon name; region of origin, coll. date, collector, coll. no. (voucher location); acc. no. ITS; acc. no. *trnK*; acc. no. *rps16*

Bartsia alpina L.; Germany, 2000, *Förther, H., 10816* (M); JF900502; JF900567; JF900535; – ***Bartsia alpina*** L.; Canada, 1965, *Doppelbaur, H., 203* (M) JF900505; JF900570; JF900538; – ***Bartsia alpina*** L.; Finland, 1971, *Federley, B., s.n.*; JF900504; JF900569; JF900537; – ***Bartsia alpina*** L.; Italy, 1972, *Lippert, W. & Podlech, D., 11758* (M); JF900503; JF900568; JF900536; – ***Bartsia canescens*** Wedd.; Peru, 1998, *Beenken, L., 1033* (M); JF900518; JF900582; JF900550; – ***Bartsia decurva*** Hochst. ex Benth.; Kenya, 1983, *Gebauer, G., s.n.* (M); JF900511; JX629749; JF900543; – ***Bartsia longiflora*** Hochst. ex Benth.; Kenya, 1978, *Grau, J., 1899* (M); JF900510; JF900575; JX629747; – ***Bartsia longiflora*** subsp. ***macrophylla*** (Hedberg) Hedberg; Rwanda, 2010, *Fischer & Thiel, 20415* (private herb. E. Fischer); JF900519; JF900583; JF900551; – ***Bartsia mutica*** (Kunth) Benth.; Argentina, 2008, *Bräuchler, C., 5170* (M); JF900517; JF900581; JF900549; – ***Bartsia* sp.;** Peru, 2001, *Henning, T. & Schneider, C., 18* (M); JF900516; JF900580; JF900548; – ***Bartsiella rameauana*** (Emb.) Bolliger; Morocco, 1951, Rauh, W., 393 (M); JF900523; JF900587; JF900555; – ***Bellardia trixago*** (L.) All.; Spain, 2010, *Schuhwerk, F., 27* (M); JF900513; JF900577; JF900545; – ***Bornmuellerantha alshehbaziana*** Dönmez & Mutlu; Turkey, 2006, *Dönmez, A. & Mutlu, B., 14036* (HUB); JF900522; JF900586; JF900554; – ***Bornmuellerantha aucheri*** (Boiss.) Rothm.; Iran, 2001, *Podlech, D. & Zarre, Sh., 55243* (M); JF900521; JF900585; JF900553; – ***Euphrasia stricta*** J.P. Wolff ex J.F. Lehm.; Czech Republic, 2007, *Svobodova, S., 5091* (CBFS); FJ790051(1); FJ790111(1); n/a; – ***Euphrasia stricta*** J.P. Wolff ex J.F. Lehm.; n/a, n/a, *Borsch, T., 3785* (BONN) n/a; n/a; FN794093(2); – ***Hedbergia abyssinica*** (Benth.) Molau; Ethiopia, 1973, *Ash, J.W., 2054* (M); JF900509; JF900574; JF900542; – ***Lathraea squamaria*** L.; Germany, 2010, *Olano-Marín, C., 1* (M); JF900500; JF900565; JF900533; – ***Macrosyringion glutinosum*** (M. Bieb.) Rothm.; France, 1997,

Appendix. Continued.

Dutartre, G., 18417 (M); JF900520; JF900584; JF900552; – ***Melampyrum nemorosum*** L.; Germany, 1998, *Lippert, W., 27890* (M); FJ797592 [(1)]; JF900562; JF900530; – ***Nothobartsia asperrima*** (Link) Benedí & Herrero; Portugal, 1977, *Malato-Beliz, J. & Guerra, J. A., 14115* (M) JF900508; JF900573; JF900541; – ***Nothobartsia spicata*** (Ramond) Bolliger & Molau; France, n/a, *Bordère, 5908* (M); JX629746; JX629748; JX629750; – ***Odontitella virgata*** (Link) Rothm.; Spain, 1988, *Montserrat, P. & al., 15513* (M); JF900507; JF900572; JF900540; – ***Odontites bocconei*** (Guss.) Walp.; Italy, 1996, *Certa, G., 18421* (M); JF900528; JF900592; JF900560; – ***Odontites corsicus*** G. Don; France, 1998, *Lambinon, J., 98/765* (M); JF900525; JF900589; JF900557; – ***Odontites cyprius*** Boiss.; Cyprus, 2004, *Vitek, E., Abr-61* (M); JF900529; JF900593; JF900561; – ***Odontites himalayicus*** Pennell; Pakistan, 1955, *Webster, L. & Nasir, E., 6290* (M) JF900526; JF900590; JF900558; – ***Odontites pyrenaeus*** (Bubani) Rothm.; Spain, 1996, *Nydegger, M., 35183* (M); JF900527; JF900591; JF900559; – ***Odontites viscosus*** (L.) Clairv.; France, 1985, *Perrin, F., 12515* (M); JF900524; JF900588; JF900556; – ***Parentucellia latifolia*** (L.) Caruel; Greece, 2007, *Tillich, H.J., 5333* (M); JF900515; JF900579; JF900547; – ***Parentucellia viscosa*** (L.) Caruel; Greece, 2003, *Tillich, H.J., 4488* (M); JF900514; JF900578; JF900546; – ***Pedicularis attollens*** A. Gray; n/a, n/a, *Tank, D., 01-50* (WTU); EF103743[(3)]; n/a; EF103821[(3)]; – ***Pedicularis sylvatica*** L.; n/a, n/a, *Müller, K., 744* (BONN); n/a; AF531781[(4)]; n/a; – ***Rhinanthus alectorolophus*** (Scop.) Pollich; Germany, 2010, *Olano-Marín, C., 3* (M); JF900501; JF900566; JF900534; – ***Rhynchocorys kurdica*** Nábělek; Iraq, 1957, *Rechninger, K.H., 11069* (M); JF900499; JF900564; JF900532; – ***Rhynchocorys orientalis*** Benth.; Armenia, 2003, *Fayvush, G. & al., 03-1382* (M); JF900498; JF900563; JF900531; – ***Striga asiatica*** (L.) Kuntze; n/a, n/a, *Morawetz, J. 116* (OS); EU253604[(5)]; AF052000[(6)]; n/a; – ***Tozzia alpina*** L.; Austria, 1998, *Panzer, R., s.n.* (M); JF900512; JF900576; JF900544.

4.2. Article II

Phylogenetic relationships among New World *Scrophularia* L. (Scrophulariaceae): new insights inferred from DNA sequence data.

by Agnes Scheunert & Günther Heubl

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ORIGINAL ARTICLE

Phylogenetic relationships among New World *Scrophularia* L. (Scrophulariaceae): new insights inferred from DNA sequence data

Agnes Scheunert · Günther Heubl

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Abstract The genus *Scrophularia* L. (Scrophulariaceae) comprises 200–300 species, of which approximately 19 are distributed in North America and the Greater Antilles. To investigate phylogenetic and biogeographic relationships of the New World species, two intergenic spacers (*trnQ-rps16* and *psbA-trnH*) of chloroplast DNA and nuclear ribosomal ITS were sequenced. Phylogenetic analyses revealed three distinct New World clades that correspond to their geographical distribution and are corroborated by morphological characters. Phylogenetic inference indicates the eastern American *S. marilandica* L. as sister to all Antillean species; for colonization of the Caribbean archipelago, a late Miocene dispersal event from the North American mainland is assumed. There is evidence for a hybrid origin of the most widespread North American species, *S. lanceolata* Pursh. The results further suggest that *S. nodosa* L. is sister to all New World and three Japanese species of *Scrophularia*. The latter form an Eastern Asian–Eastern North American (EA-ENA) disjunction with six New World species. We propose an eastern Asian origin for the New World taxa of *Scrophularia*. Divergence times estimated using a relaxed molecular clock model suggest one or more Miocene migration events from eastern Asia to the New World via the Bering Land Bridge followed by diversification in North America.

Keywords *Scrophularia* · North America · Greater Antilles · Molecular phylogeny · *trnQ-rps16* intergenic spacer · *psbA-trnH* intergenic spacer

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Introduction

Scrophularia L. (Scrophulariaceae), commonly known as figwort, is a widespread genus of mainly holarctic distribution (Hong 1983) and comprises about 200 (Fischer 2004) to more than 300 species (Willis 1973). Its origin is assumed to be the Himalayan region (Stiefelhagen 1910) while its primary diversity center is found in Iran and adjacent areas, where 64 species occur (Grau 1981). Fifty-seven species are reported for Turkey (Lall and Mill 1978) and nearly as many for Russia/Middle Asia, and 36 species are listed for China (Shu 1998). Species numbers decrease in Europe [only six species in Central Europe according to Hartl (1965)], but another secondary diversity center is found on the Iberian Peninsula with 22 species (Ortega Olivencia 2009). The number of species of *Scrophularia* is considerably lower in the New World: about 18–20 species are known from North America and the Caribbean (Greater Antilles) (USDA 1982; Liogier 1994).

The Scrophulariaceae, as traditionally defined by morphological characters, is not supported by molecular analyses. Phylogenetic studies based on DNA sequencing have radically changed their circumscription since reconstructions indicated that the family does not represent a monophyletic entity. Olmstead and Reeves (1995) identified two monophyletic clades named Scroph I and Scroph II, the former including *Buddleja* L., *Selago* L., *Verbascum* L., and the type genus *Scrophularia*. The clade Scroph I was named Scrophulariaceae s.str. by Olmstead et al. (2001), while Scroph II is equivalent to Plantaginaceae. Subsequent molecular phylogenetic studies on the delimitation of the Scrophulariaceae (e.g., Oxelman et al. 1999, 2005; Rahmanzadeh et al. 2004; Albach et al. 2005) have generally confirmed the pattern revealed by Olmstead and Reeves (1995) and Olmstead et al. (2001).

Despite the large number of infrafamilial studies on Scrophulariaceae (e.g., Albach and Chase 2001; Kornhall et al. 2001; Gebrehewit et al. 2000), there is a considerable lack of knowledge concerning phylogenetic relationships within the familiar type genus *Scrophularia*. Major revisions of the genus were provided by Wydler (1828), Bentham (1846), and Boissier (1879). The most comprehensive treatment of the genus was done by Stiefelhagen (1910). He arranged 145 species into two sections and five subsections, using leaf anastomosis, petal length, shape of the corolla tube, and life form as distinguishing features. As no extensive studies have been conducted on the genus since then, a systematic revision using methods from molecular biology is of great interest. The present study focusing on the New World taxa of *Scrophularia* is a first step in a series of treatments for major geographical areas towards the goal of a comprehensive revision of the genus.

Stiefelhagen (1910), in his treatment of the genus, argued that all New World species described at that time (except *S. macrantha* Greene ex Stiefelhagen, a distinctive species native to New Mexico) were conspecific with *S. nodosa* L. Actually, the widespread species do share a very similar morphology. However, this assumption was rejected by Pennell (1935; 1947). In his treatment of the eastern (Pennell 1935) and far western (Pennell 1947) North American species, he recognized two species for eastern North America (*S. marilandic*a L. and *S. lanceolata* Pursh) and five species for the westernmost area (*S. villosa* Pennell, *S. atrata* Pennell, *S. californica* Cham. & Schltdl., *S. multiflora* Pennell, *S. lanceolata*). Regional studies were published for Arizona and New Mexico (Tidestrom and Kittell 1941), Arizona (Kearney and Peebles 1951), California (Munz and Keck 1959), and Nevada (Edwin 1959). A comprehensive study on western North American *Scrophularia* was provided by Shaw (1962), but no phylogenetic study based on molecular data of New World *Scrophularia* is available to date.

In the West Indies, *Scrophularia* has undergone an extensive diversification that corresponds to the generally high plant diversity in the Caribbean (Myers et al. 2000): while 11–13 species (depending on the synonymy employed) occur on the large North American mainland, the islands of the Greater Antilles host 7 or 8 species (7 of which are endemic to the Dominican Republic) characterized by notable morphological differences. As only a small number of species of *Scrophularia* have expanded into tropical regions, the understanding of their relationships to holarctic representatives is of special interest.

In this paper, we present a phylogenetic analysis of New World *Scrophularia* based on sequence data from nuclear internal transcribed spacer regions (ITS 1 and 2) and chloroplast *trnQ-rps16* and *psbA-trnH* intergenic spacers. The particular objectives were to (1) test the monophyly of the New World taxa and analyze phylogenetic relationships among them, especially regarding the origin of the West Indian taxa, (2) examine Stiefelhagen's taxonomic concept of reducing almost all North American species of *Scrophularia* into the synonymy of *S. nodosa*, (3) elucidate patterns of cytogenetic evolution based on the molecular phylogeny, (4) identify biogeographic diversity patterns within this alliance, and (5) gain insight into the colonization pathways leading from the assumed Eurasian origin of the genus to the New World.

This contribution is part of a more comprehensive study in preparation on the evolution of *Scrophularia*.

Materials and methods

Plant material

Leaf material for DNA extraction was removed from herbarium specimens in A, GH, M, MSB, UTEP, W, and WU. In three cases, material was taken from cultivated plants in the greenhouses of the Munich Botanical Garden. The sampling strategy was to include all species of *Scrophularia* occurring in North America and in the Caribbean as far as possible. *Scrophularia multiflora* and *S. serrata* Rydb. were sampled to gain more information about their status and taxonomic rank, which have been discussed for some time. No material could be obtained from *S. pluriflora* Urb. & Ekman, *S. tuerckheimii* Urb., and *S. bahorucana* Zanoni, three local endemics of Hispaniola. Several eastern Asian species were added to the sampling because preliminary molecular analyses revealed close relationships between nearctic and eastern Asian species of *Scrophularia* (results not shown). Furthermore, to assess the question of a close relationship of *S. nodosa* to the New World species of *Scrophularia* [or even conspecifity, as proposed by Stiefelhagen (1910)], we analyzed three accessions of *S. nodosa*, collected from Germany, Armenia, and the USA. One rather widespread Eurasian species (*S. umbrosa* Dumort.) completed the sampling. *Verbascum nigrum* (Scrophulariaceae) was chosen as an outgroup because *Verbascum* was found to be sister to the *Scrophularia* alliance by Olmstead and Reeves (1995) and Olmstead et al. (2001). Additionally, two more distant outgroup species, *Hemimeris centrodes* Hiern (Scrophulariaceae) and *Russelia verticillata* Kunth (Plantaginaceae), were sampled. Altogether, the analysis included 32 taxa: 12 species occurring in mainland North America, 5 from the Greater Antilles, 8 from eastern Asia, 1 Eurasian species, 3 accessions of *S. nodosa*, and 3 outgroup taxa. Table 1 lists all taxa and summarizes additional information such as voucher specimen data and GenBank accession numbers.

Table 1 Taxa included in the present study, with voucher information on country of origin, collector/collector's number, date of collection, herbarium, and GenBank accession numbers

Taxon	Source	Date	Collector	Coll. no.	Herbarium	GB (*T-R*)	GB (ITS)	GB (*P-T*)
Russelia verticillata Kunth	Costa Rica, Guanacaste Prov.	13.10.1990	P. Döbbeler	3795	M	–	HQ130062	–
Hemimeris centrodes Hiern	South Africa, Cape Prov.	04.09.1976	P. Goldblatt	4033	M	HQ130033	HQ130063	–
Verbascum nigrum L.	Germany, Bavaria	18.06.1998	H. Wunder	nn	M	HQ130034	HQ130064	HQ130094
Scrophularia atrata Pennell	USA, California, Santa Barbara Co.	30.04.1958	H. M. Pollard	nn	W 1960/21086	HQ130051	HQ130081	HQ130111
Scrophularia buergeriana Miq.	Greenhouse cultivated plants[a]	Cult. since 2008	Material from Kor. Nat. Arb.[b], cultivated by A. Scheunert	Cult. no 001/1-1	MSB	HQ130040	HQ130070	HQ130100
Scrophularia californica Cham. & Schltdl.	USA, California, Santa Cruz Co.	04.05.1976	A. L. and H. N. Moldenke	30898	M	HQ130049	HQ130079	HQ130109
Scrophularia desertorum (Munz) R. J. Shaw (*S. californica* Cham. & Schltdl. var. *desertorum* Munz)	USA, California, Mono Co.	28.05.1959	P. H. Raven	14265	GH	HQ130057	HQ130087	HQ130117
Scrophularia densifolia Urb. & Ekman	Dominican Republic	10.12.1969	A. H. Liogier	17213	GH	–	HQ130093	–
Scrophularia domingensis Urb.	Dominican Republic	28.02.1929	E. L. Ekman	H11711	GH	HQ130059	HQ130089	–
Scrophularia duplicato-serrata Makino	Japan, Shizuoka Pref.	09.11.2002	T. Sugawara	2110903	A	HQ130046	HQ130076	HQ130106
Scrophularia eggersii Urb.	Dominican Republic	30.05.1964	B. Augusto	1499	A	HQ130060	HQ130090	HQ130119
Scrophularia grayana Maxim. ex Kom.	Japan, Northern Honshu, Iwate Pref.	09.07.1986	H. Tohda, T. Nemoto, Y. Endo, H. Hoshi	1155	A	HQ130044	HQ130074	HQ130104
Scrophularia kakudensis Franch.	Greenhouse cultivated plants	Cult. since 28.07.08	Material from Bot. Gard. Tübingen, Germany, cultivated by A. Scheunert	2008/1036, cult. no 002/1-1	MSB	HQ130045	HQ130075	HQ130105
Scrophularia koraiensis Nakai	Greenhouse cultivated plants	Cult. since 28.07.08	Material from Kor. Nat. Arb., cultivated by A. Scheunert	Cult. no 003/1-1	MSB	HQ130039	HQ130069	HQ130099
Scrophularia laevis Wooton & Standl.	USA, New Mexico, Dona Ana Co.	06.07.1993	R. D. Worthington	22161	M	HQ130047	HQ130077	HQ130107
Scrophularia lanceolata Pursh	USA, Massachusetts, Hampshire Co.	28.06.1977	H. E. Ahles	83075	M	HQ130050	HQ130080	HQ130110
Scrophularia marilandica L.	nn	1990	V. Bates	10313	Harvard[c]	HQ130055	HQ130085	HQ130115
Scrophularia macrantha Greene ex Stiefelhagen	USA, New Mexico, Grant Co.	02.09.1996	C. E. Freeman	129	UTEP	HQ130122	HQ130092	HQ130121

Table 1 continued

Taxon	Source	Date	Collector	Coll. no.	Herbarium	GB (*T-R*)	GB (ITS)	GB (*P-T*)
Scrophularia micrantha Desv. ex Ham.	Dominican Republic	07/1910	H. v. Türckheim	3064	WU	HQ130058	HQ130088	HQ130118
Scrophularia minutiflora Pennell	Dominican Republic	14.08.1968	A. H. Liogier	12075	GH	HQ130061	HQ130091	HQ130120
Scrophularia montana Wooton	USA, New Mexico, San Miguel Co.	19.08.1984	S. R. Hill	15272	GH	HQ130052	HQ130082	HQ130112
Scrophularia multiflora Pennell	USA, California, Kern Co.	25.04.1951	W. J. Dress	3171	M	HQ130048	HQ130078	HQ130108
Scrophularia musashiensis Bonati	Japan, Toyama Pref.	26.06.1991	J. Jutila, H. Fujino, M. Yoshizoki	473	GH	HQ130043	HQ130073	HQ130103
Scrophularia ningpoensis Hemsl.	Japan, Toyama Pref.; garden culture	03.10.1991	J. Jutila	769	GH	HQ130041	HQ130071	HQ130101
Scrophularia nodosa L. (Germany)	Germany, Bavaria	29.06.1999	D. Podlech	nn	MSB 116671	HQ130037	HQ130067	HQ130097
Scrophularia nodosa L. (Armenia)	Armenia, Lori Prov.	01.07.2003	G. Fayvush, K. Tamanyan, H. Ter-Voskanian, E. Vitek	03-0549	MSB 123419	HQ130038	HQ130068	HQ130098
Scrophularia nodosa L. (USA)	USA, Massachusetts, Franklin Co.	23.08.1977	H. E. Ahles	84733	M	HQ130036	HQ130066	HQ130096
Scrophularia parviflora Wooton & Standl.	USA, Arizona, Santa Rita Mountains	25.08.1932	V. Douglas	1588?	GH	HQ130056	HQ130086	HQ130116
Scrophularia serrata Rydb.	USA, Idaho, Idaho Co.	13.07.1937	F. W. Pennell, L. Constance	20885	GH	HQ130053	HQ130083	HQ130113
Scrophularia umbrosa Dumort.	Iran, Chaharmahal va Bakhtiyari Prov.	17.07.2003	M. R. Parishani	14232	M	HQ130035	HQ130065	HQ130095
Scrophularia villosa Pennell	USA, California, L.A. Co.	10.05.1962	P. H. Raven	17711	GH	HQ130054	HQ130084	HQ130114
Scrophularia yoshimurae T. Yamaz.	Taiwan, Nantou Hsien	07.10.1992	C.-C. Liao	718	A	HQ130042	HQ130072	HQ130102

Pref. Prefecture, *Prov.* province, *Co.* county, *GB* Genbank accession number, *T-R trnQ-rps16* intergenic spacer, *P-T psbA-trnH* intergenic spacer

[a] Plants grown in the greenhouses of the Botanical Garden Munich, Germany

[b] Korean National Arboretum, Gwangwhanum Forest, Seoul, Rep. of Korea

[c] Harvard University, herbarium not known

DNA extraction, amplification, and sequencing

Total DNA was extracted from dried leaf material using the NucleoSpin Plant Kit (Macherey-Nagel). Protocols followed those provided by the manufacturer, except for an additional extraction step with phenol/chloroform to remove potentially interfering secondary compounds. The extracted DNA was resuspended in 50 µl elution buffer (10 mM Tris-HCl), and a standard amount of 1 µl of the solution was used for amplification (higher amounts up to 3 µl in cases where PCR yielded insufficient amounts of product).

Three noncoding regions from nuclear DNA (ITS) and chloroplast DNA (*trnQ-rps16* and *psbA-trnH* intergenic spacers) were chosen for phylogenetic analysis. The markers were amplified from total DNA using *Taq*-polymerase (AGS), or *Phusion*-polymerase (New England Biolabs) whenever *Taq*-polymerase did not provide satisfactory results. For ITS, we used the universal primers ITS1 and ITS4 (and in five difficult cases ITS2 and ITS3) described by White et al. (1990), alongside aITS1 and aITS4 designed for angiosperms by Meimberg (2002). In four cases, additional primers designed exclusively for problematic taxa were necessary, but these were used only in sequencing reactions, not for PCR (for exact position and additional information about all primers, see Fig. 1a–c). The *trnQ-rps16* intergenic spacer fragment was amplified using primers 1 (trnQ-F) and E (rps16-1R) described by Calviño and Downie (2007). These primers, originally designed for Apiaceae, were here shown to work for Scrophulariaceae as well. Two internal primer sets designed for the *trnQ-rps16* spacer fragment were used for sequencing (see Fig. 1a). The chloroplast *psbA-trnH* intergenic spacer was amplified with primers psbA (forward) and trnH2R (GUG) (reverse) as described by Shaw and Small (2004).

PCR reactions were performed in volumes of 50 µl (or rarely 100 µl) containing a dNTP solution of 2.5 mM, *Taq*-polymerase with 1 U/µl, primer solutions with a concentration of 100 pmol/µl, and differing amounts of unquantified genomic DNA. When necessary, an alternative preparation containing 0.05% bovine serum albumin (BSA) and 100% dimethyl sulfoxide (DMSO) was used for ITS. The 10× Thermo Pol (TP) reaction buffer used for "standard" PCR with *Taq* polymerase consisted of 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl, 20 mM $MgSO_4$, 10% Triton X-100 at a pH of 8.8 (25°C).

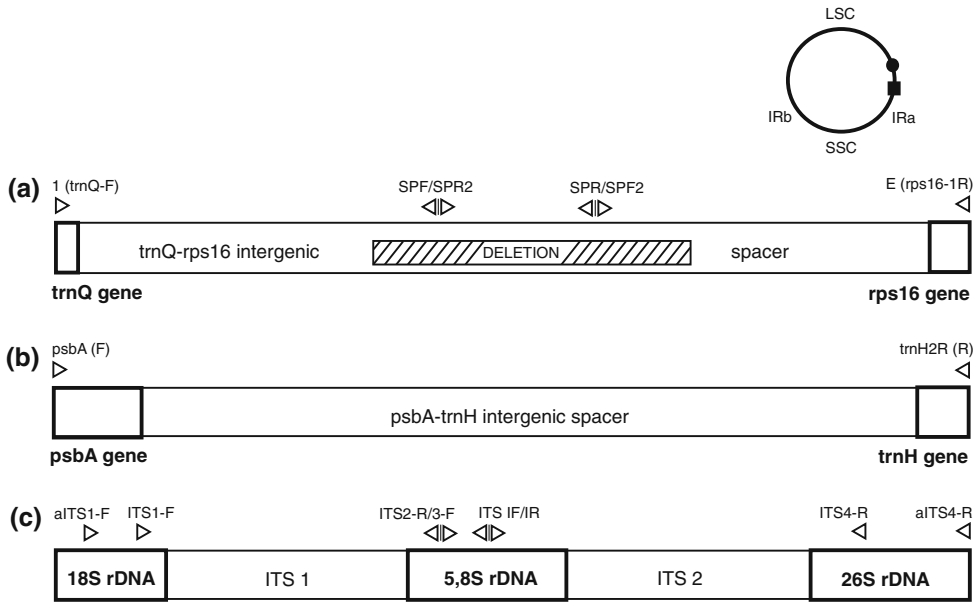

Fig. 1a–c Schematic illustration of marker regions in *Scrophularia* used for this study, with relative positions of primers. *F* indicates forward, *R* indicates reverse primers. The *trnQ-rps16* and *psbA-trnH* intergenic spacers are part of the large single copy (LSC) unit of the plastome (*overview above marker diagrams*). **a** Map of the *trnQ-rps16* intergenic spacer region of *Scrophularia* showing the deletion within the spacer; position within the LSC is indicated by a *black circle*. Primers 1 (trnQ-F) and E (rps16-1R) according to Calviño and Downie (2007). Primer sequences designed for this study, written 5′ to 3′: SPF: GAC AAC TGT TCA GTC TAT CTG, SPR: CAC GTT TGA TCT TCA TAG G, SPF2: CCT ATG AAG ATC AAA CGT G, SPR2: CAG ATA GAC TGA ACA GTT GTC. **b** Map of the *psbA-trnH* intergenic spacer region of *Scrophularia*; position within the LSC indicated by a *black square*. Primers psbA (*F*) and trnH2R (*R*) according to Shaw and Small (2004). **c** Map of the ITS region of *Scrophularia* with relative positions of the internal transcribed spacers 1 and 2. ITS1-F, ITS2-R, ITS3-F, and ITS4-R according to White et al. (1990); aITS1-F and aITS4-R according to Meimberg (2002). Primer sequences designed for this study, written 5′ to 3′: ITS-IF: AAT CCC GTG AAC CAT CGA GTT, ITS-IR: AAC TCG ATG GTT CAC GGG ATT

The modified TP buffer for BSA/DMSO ITS-PCR consisted of 50 mM KCl, 200 mM $(NH_4)_2SO_4$, 750 mM Tris-HCl, 15 mM $MgSO_4$. The 5× HF reaction buffer for Phusion PCR (Finnzymes) contained 7.5 mM $MgCl_2$. Detailed amounts of the components for each PCR reaction are given in Table 2.

A thermocycler type T-Personal 48 (Biometra), type Primus 96 plus (MWG-Biotech), or type 2720 (Applied Biosystems) was used for amplification. ITS/*trnQ-rps16* spacer amplification programs started with a 5 min initial denaturation step at 94°C; followed by 40 cycles of 30 s/1 min denaturation (94°C), 30 s annealing (54°C), and 1 min 15 s/2 min extension (72°C); ending with a final extension step of 10 min (72°C). The *psbA-trnH* spacer program was identical to that of ITS except for a lower annealing temperature (48°C). Successful PCR reactions were either purified with NucleoSpin® Extract II-Kit (Macherey-Nagel) following the manufacturer's instructions or were reduced to 25 µl and then purified in 4 µl units with 0.025 µl exonuclease I and 0.25 µl shrimp alkaline phosphatase (Sap) in a 5 µl preparation with 0.0725 µl 10× TP buffer (ExoSap purification). Purification using columns proved particularly useful with ITS as ExoSap seemed to work inefficiently on ITS fragments for unknown reasons.

Cycle Sequencing was carried out using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in a final volume of 20 µl. Runs were performed on an ABI 3730 48 capillary sequencer (Applied Biosystems). In all cases, markers were sequenced bidirectionally using the same primers as in PCR reactions, sometimes supplemented by sequences from the internal sequencing primers, to achieve maximum reliability of the results. Bidirectional sequencing proved essential for the *psbA-trnH* intergenic spacer because sequencing terminated regularly and from both ends at two nucleotide repeat regions in the central part of the sequence.

Alignment, indel coding, and phylogenetic reconstruction

All sequences generated in this study were assembled, edited, and aligned manually using BioEdit 7.0.5.1 (Hall 1999). The alignment is available from the corresponding author upon request. Ambiguously aligned characters and mononucleotide repeat units were excluded from further analyses. The beginning and end of the alignments where not all of the taxa provided complete data were also excluded. For Bayesian and parsimony analyses, informative indels (i.e., indels presumably containing phylogenetic information) resulting from the alignment were coded using the simple indel coding algorithm proposed by Simmons and Ochoterena (2000), which is implemented in SeqState (Müller 2005). A present/absent indel matrix (coded as 1/0) was added at the end of the alignment. The three markers were analyzed in a single combined dataset as well as in one ITS and one combined chloroplast dataset.

In accordance with Bull et al. (1993), the combined matrix was tested for incongruence between nuclear and chloroplast markers. To assess cases of "hard incongruence" (Mason-Gamer and Kellogg 1996) between the two datasets, two maximum parsimony bootstrapping analyses, one for each dataset, were conducted (using the same settings as for the bootstrap analysis of the combined dataset) and the resulting trees visually examined for well-supported contradicting placement of taxa, using a cutoff of 70% bootstrap support following Mason-Gamer and Kellogg (1996).

In addition, two statistical tests were used: the incongruence length difference (ILD) test as a suitable first step in detecting incongruences (Cunningham 1997; Hipp et al. 2004), and Templeton's significantly less parsimonious test (SLP_t; Johnson and Soltis 1998; Templeton 1983) to compensate for putative weaknesses of the ILD test pointed out by Barker and Lutzoni (2002). The ILD test, called the partition homogeneity test (PHT) in PAUP* 4.0b10 (Swofford 2003), computed 1,000 replicates with MAXTREES option set to 100 and was executed on the combined dataset, excluding coded indels, and after removing constant characters from the matrix. The PHT was conducted on the combined chloroplast dataset as well, to test the two chloroplast markers against each other. For the SLP test (implemented in PAUP* 4.0b10 as the nonparametric pairwise test), we performed separate runs for the ITS and

Table 2 Components and corresponding amounts of chemicals used for PCR reactions conducted for this study

	Standard PCR (all markers)	BSA/DMSO PCR (ITS only)	Phusion PCR (ITS and *trnQ-rps16* spacer)
Primer (each) (µl)	0.125	0.1	0.25
Reaction buffer (µl)	5.0 (10× Thermo Pol)	5.0 (modified)	10 (5× HF)
dNTPs (µl)	2.5	4.0	4.0
Polymerase (µl)	1.0 (*Taq*)	1.0 (*Taq*)	0.25 (Phusion)
BSA/DMSO (µl)	–	0.5/2.5	–
Unquantified DNA (µl)	1.0 (−3.0)	1.0 (−3.0)	1.0 (−3.0)
Total (µl)	50.0	50.0	50.0

the chloroplast dataset. A 70% consensus tree from an unconstrained heuristic search was tested against the trees obtained from a constrained heuristic search (level of significance $P = 0.05$; the P value reported represents the maximum found in the respective test). As the constraint, we used an artificial tree reflecting the topology suggested by the other marker/dataset, but with only the outgroup and contradicting nodes resolved (topologies correspond to the 70% consensus trees obtained when assessing cases of hard incongruence, see respective paragraph in the "Results" section). The heuristic searches were run with the same settings as all maximum parsimony (MP) calculations in this study (see below).

Phylogenetic reconstruction analyses were performed with a Bayesian inference (BI), an MP, and a maximum likelihood (ML) approach. MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) was used for BI calculations using the metropolis-coupled Markov Chain Monte Carlo (MCMC) algorithm. A mixed dataset was defined for MrBayes [DNA characters in part 1, binary characters (coded indels) in part 2]; for the DNA partition, the analyses assumed the general time reversible (GTR) model (Tavaré 1986) with rates differing across sites following a gamma distribution, and allowing a proportion of invariant sites; for the coded indel characters, settings as recommended in the MrBayes 3.1 manual were employed. MrBayes was then executed with two runs and four chains (hot chains with the default temperature $t = 0.2$) for 2 million generations, sampling every 100th generation. In all cases, average standard deviation of split frequencies fell below 0.01 long before 2 million generations were computed. A burn-in of 1/4 of the samples was discarded, and the remaining trees were summarized in an 80% majority rule consensus tree. MrBayes settings were identical for all analyses of combined and individual ITS and cpDNA datasets.

Maximum parsimony analyses were performed with the combined (ITS and cpDNA) dataset including coded indels using PAUP* 4.0b10 (Swofford 2003) with the following parameters: all characters unordered and equally weighted, coded indel characters not treated as separate data partition but added at the end of the alignment; heuristic search with random sequence addition, TBR branch swapping, 50 random-addition-sequence replicates, and MAXTREES option set to 1,000. Bootstrapping was done using the same settings and computing 5,000 bootstrap replicates (summarized in a 50% bootstrap majority rule consensus tree as a cladogram and a phylogram).

Maximum likelihood analysis was carried out with RAxML v. 7.0.4 (Stamatakis et al. 2005, 2008) as implemented in the CIPRES (Cyberinfrastructure for Phylogenetic Research) portal v. 1.14 (Miller et al. 2009), using only the combined dataset without coded indels. A bootstrap run with 5,000 replicates and subsequent maximum likelihood optimization was performed with *Russelia verticillata* as the outgroup and no constraint tree defined. Rapid bootstrap analysis used the CAT model; for the final ML search, rate heterogeneity was modeled using a gamma distribution and allowing a proportion of invariant sites, which is estimated in the course of the run (model as in Bayesian analysis).

Dating of divergence times

A BI approach was used to estimate divergence times of the *Scrophularia* lineages examined in this study. The algorithm implemented in BEAST v. 1.5.2 (Drummond and Rambaut 2007) employs the MCMC to co-estimate topology, substitution rates, and node ages (Drummond et al. 2002) and was executed on the combined dataset. A NEXUS file analogous to the one used for MP analyses (but without coded indels) was used for calculations in BEAST, but to avoid a basal polytomy, *Hemimeris centrodes* was excluded from the analysis; its unresolved position between *Russelia verticillata* and *Verbascum nigrum* would have made correct assignment of calibration points problematic. As in all analyses with the combined dataset, *S. lanceolata* and *S. serrata* were excluded as well so as to avoid incongruence problems during topology estimation. Rate variation among sites was modeled using the same substitution model as in MrBayes and PAUP (GTR model) to ensure comparability of the results. We employed a relaxed molecular clock model (Drummond et al. 2006) relying on uncorrelated rates drawn from a log-normal distribution, and a Yule tree prior for speciation. To further support the results of the phylogenetic analyses, tree topology was co-estimated during the BEAST runs for comparison purposes. The clock was calibrated using the assumed divergence time between Plantaginaceae and Scrophulariaceae as proposed by Bremer et al. (2004); see the "Discussion" section for further details. The value of 76 mya, referred to as "Plantaginaceae stem age" by the authors, was assigned to node 1 of Fig. 6 (value indicated by brackets) with a normal-distributed prior and a standard deviation of 0.5 my. Two independent MCMC runs were performed with 15,000,000 generations each, with every 1,000th generation sampled. A burn-in of 10% per run was discarded after assessing convergence with Tracer v. 1.4.1. Input data and results were edited and processed with the respective programs included in the BEAST program package. The resulting maximum clade credibility tree displaying mean heights was edited using FigTree v. 1.2.3.

Results

Amplification

The length of the *trnQ-rps16* intergenic spacer fragment differed considerably across the examined species. Within the alignment of 1,362 basepairs (bp), some species showed a 480 bp deletion that drastically shortened sequence length (see also Table 3). This deletion can easily be recognized in PCR products (Fig. 2) and is shared by all North American as well as Japanese taxa and *S. nodosa*. Likewise, *S. umbrosa* is characterized by a 262 bp deletion.

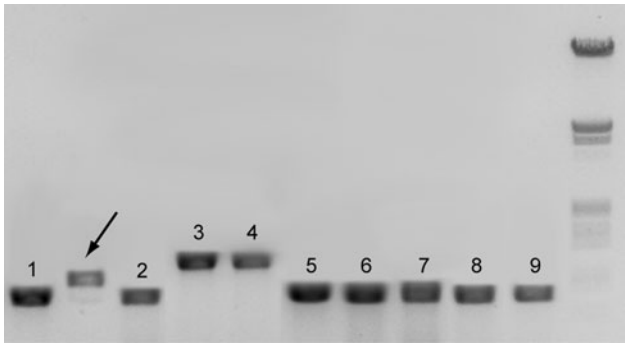

Fig. 2 Gel electrophoresis image *of trnQ-rps16* intergenic spacer PCR products from different species of *Scrophularia*. Mainland Asian species show the full sequence length (*3 S. ningpoensis*, *4 S. yoshimurae*). North American mainland species (*5 S. montana*, *6 S. serrata*, *7 S. villosa*, *9 S. marilandica*), West Indian species (*8 S. minutiflora*), and Japanese species (*1 S. musashiensis*, *2 S. duplicato-serrata*) show reduced length due to a 480 bp deletion. A taxon with a smaller deletion (such as in *S. umbrosa*) is indicated by an *arrow*

Sequence divergence and alignment

The combined data matrix of the internal transcribed spacer (ITS) and the *trnQ-rps16* and *psbA-trnH* intergenic spacers comprised a total of 2,940 aligned characters. Detailed information about alignment characteristics and statistics of MP analysis is given in Table 3. Sequence length of the *trnQ-rps16* intergenic spacer was 657 bp (with deletion) and 1,061 bp (complete) on average; ITS sequences had an average length of 583 bp, *psbA-trnH* sequences were 493 bp long.

Whenever possible, taxa were analyzed using sequence information from all three markers; in some cases, where sequencing of single markers failed, the taxa were included and scored as missing data for the respective markers: for *Russelia verticillata*, no chloroplast sequences could be obtained; *Hemimeris centrodes* provided *trnQ-rps16* spacer sequences only. No *psbA-trnH* sequences could be generated from two of the West Indian taxa (*S. densifolia* Urb. & Ekman, *S. domingensis* Urb.); *trnQ-rps16* and ITS sequence information of these taxa was included nevertheless so as to increase the sampling with respect to the West Indian species.

Phylogenetic reconstruction

The combined chloroplast dataset showed no significant heterogeneity in the PHT ($P = 0.132$). Consequently, both chloroplast markers were combined for all remaining analyses in favor of a more robust phylogenetic hypothesis.

Table 3 Alignment characteristics and statistics of maximum parsimony (MP) analysis for *trnQ-rps16* intergenic spacer, *psbA-trnH* intergenic spacer, ITS, and combined dataset

	Combined	*trnQ-rps16* spacer	ITS	*psbA-trnH* spacer
No. of taxa	32[a]	30	32	28
Sequence length (bp) (mean)	572–2,287 (1,714.59)	349–756 (656.87); 992–1,176 (1,061.33)	395–862 (583.34)	435–554 (493.29)
Aligned length (bp)	2,940	1,362	910	668
Excluded characters (bp)	688	136	346	206
Constant characters (bp)	1,834	1,048	388	391
Parsimony-uninformative characters (bp)	294	143	100	53
Parsimony-informative characters (bp)	164	49	88	32
Parsimony-informative characters (%)	7.16	3.95	15.28	6.72
No. of coded indels	40	14	12	14
Unknown characters within sequences (bp) (mean)	3–841 (43.5)	0–837 (35.13)	0–54 (7.69)	0–16 (3.29)
Average G-C content (%)	37.99	28.69	59.67	23.03

Number of constant characters, parsimony-(un)informative characters, and % parsimony-informative characters refer to nonexcluded characters. G-C content is without outgroup taxa. Sequence length is given separately for species with/without the 480 bp deletion (see text)

[a] Number of taxa includes *S. lanceolata* and *S. serrata*; see text for details

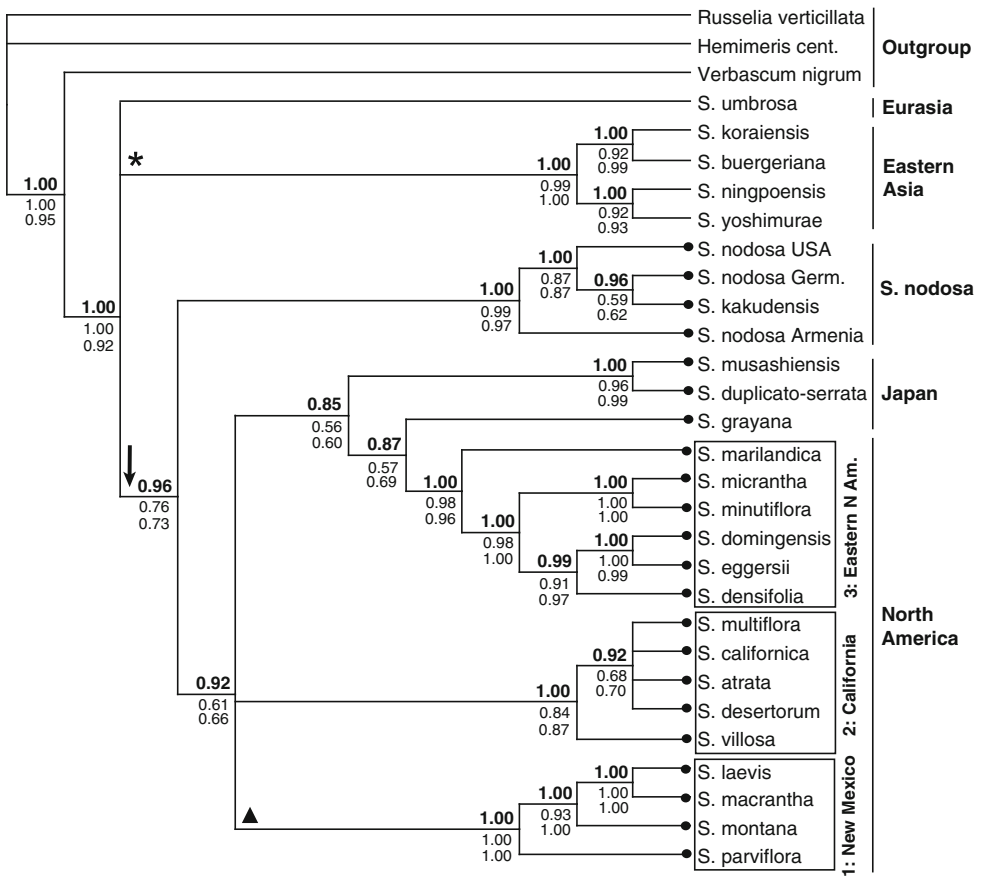

Fig. 3 Bayesian consensus tree (cladogram), from a combined dataset (ITS and *trnQ-rps16* and *psbA-trnH* intergenic spacers) excluding *S. lanceolata* and *S. serrata*. Geographic distribution and names are given for each clade. *Black dots* on terminal branches indicate presence of the 480 bp deletion in the *trnQ-rps16* sequence of the respective taxon; the position where the deletion was introduced is marked by an *arrow*. Posterior probabilities (PP) exceeding 80% are given *above each node*, corresponding bootstrap support (BS) values from a MP 50% majority rule consensus tree (not shown) are indicated *below*. Maximum likelihood (ML) bootstrap support values of the respective nodes are plotted *beneath the BS values*. PP values were obtained from 30,002 trees, BS values from 5,000 bootstrap replicates with 1,000 saved trees per replicate, and ML values from 5,000 bootstrap replicates with subsequent maximum likelihood optimization. *Asterisk* indicates the position of an additional node supported by MP analysis only (79% BS), and therefore not shown in this figure, assigning the Asia clade as sister to the Japan-New World (J-NW) clade and the *S. nodosa* clade (see also the "Discussion" section). *Triangle* indicates the position of an additional, yet insufficiently supported node (therefore not shown in this figure) in Bayesian (62% PP), BEAST (74% PP), and ML (54% ML bootstrap support) analyses, assigning the New Mexico clade as sister to the remaining New World clades (see the "Discussion" section). *Hemimeris cent. Hemimeris centrodes*, *Germ* Germany

For the nuclear (ITS) and combined chloroplast (*trnQ-rps16* and *psbA-trnH* spacer) dataset, the PHT displayed significant heterogeneity ($P = 0.004$). A series of PHT tests (results not shown), tentatively removing single species from the dataset, revealed *S. lanceolata* and *S. serrata* to be responsible for the conflict. When these taxa were excluded from the combined dataset, the PHT found the remaining data to be highly congruent ($P = 0.704$). Visual inspection of the majority rule consensus trees obtained from MP bootstrapping analyses of the nuclear and chloroplast datasets (not shown) supports the results of the PHT: the only instance of a hard incongruence sensu Mason-Gamer and Kellogg (1996), i.e., differences in topology supported by 70% BS or more, involves the placement of *S. lanceolata* and *S. serrata*. In the chloroplast gene tree, the two taxa are in one clade with *S. marilandica*; in the ITS tree, they are sister to *S. laevis* Wooton & Standl., *S. macrantha*, and *S. montana* Wooton.

We also examined possible incongruences between the datasets using Templeton's significantly less parsimonious (SLP) test. When performed on the ITS dataset, the test was significant ($p_{max} = 0.0045$), which means that the nuclear tree (70% consensus from the unconstrained search) is a significantly better fit to these data than the rival chloroplast topologies (trees obtained in the constrained search). The reciprocal test also rejected the null hypothesis ($p_{max} = 0.0018$), suggesting the rival ITS topology is significantly noncongruent as compared to the unconstrained dataset. The number of character sites available for the test was adequate in both tests

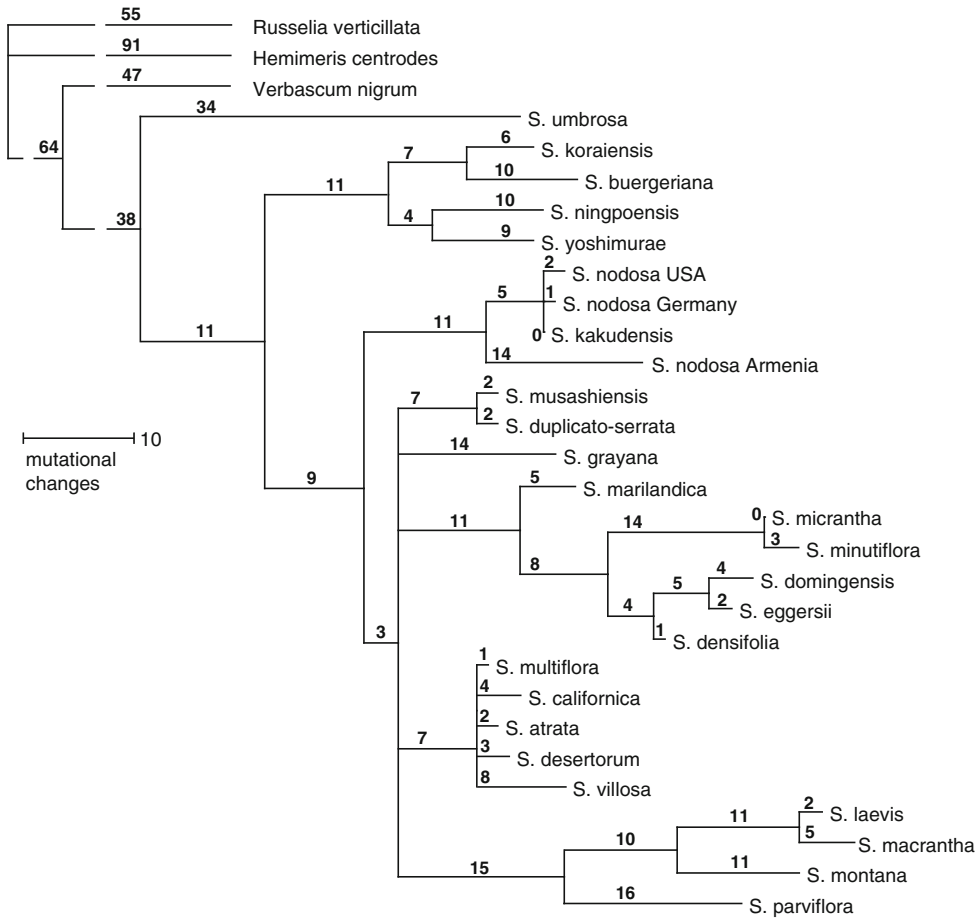

Fig. 4 The 50% majority rule consensus tree (phylogram) from the PAUP* maximum parsimony bootstrap analysis, from a combined dataset (ITS and *trnQ-rps16* and *psbA-trnH* intergenic spacers) excluding *S. lanceolata* and *S. serrata*. The consensus was generated from 5,000 bootstrap replicates with 1,000 saved trees per replicate. *Numbers above branches* indicate mutational changes of the respective branch, *dashed branches* are not shown with correct lengths, but were shortened

($N = 13$–15/18–20), assuring the reliability of the results. Trees obtained from the constrained search in the ITS test were 12 steps longer than the unconstrained 70% consensus; constrained trees in the chloroplast test had 17 steps in excess.

Taking into account the results of the statistical analyses, a combined nuclear/chloroplast dataset was used for phylogenetic analyses, but *S. lanceolata* and *S. serrata* were excluded and examined in two separate analyses (nuclear and chloroplast markers). The Bayesian consensus tree from the combined analysis is shown in Fig. 3, each node displaying its posterior probability (PP) values.

Maximum parsimony analysis of the combined dataset resulted in three most parsimonious trees. The bootstrap (BS) 50% majority rule consensus tree (length 620 steps, CI = 0.82, RI = 0.75) yielded the same topology as the Bayesian consensus tree, yet with much weaker support values in five cases, whereas one additional node was generated. The BS support values were attached to their corresponding nodes in the BI cladogram in Fig. 3. The MP 50% majority rule consensus including branch lengths is shown in Fig. 4.

Maximum likelihood optimization of the combined dataset resulted in a final optimization likelihood of −6,180.96, with the alpha parameter being 0.566429. The proportion of invariant sites was 0.16, the best-scoring ML tree had a length of 0.340060 and corresponded to the topology of the BI and MP analyses. ML bootstrap support values were attached to their respective nodes (below the maximum parsimony BS values) in the Bayesian cladogram in Fig. 3. Support values are highly similar to those of the MP analysis, with six values being weakly supported compared to the Bayesian consensus (five of them similarly weak in MP analysis).

All New World species of *Scrophularia* form a strongly (92% PP) to weakly (61% BS / 66% ML) supported clade. Apart from three New World subclades related to geographical distribution, the clade includes the three Japanese taxa and is referred to as the Japan-New World clade (J-NW clade). The Japanese taxa form a grade towards the New World Eastern North America (ENA) clade and indicate an intercontinental disjunction (though weakly supported): *S. grayana* Maxim. ex Kom. is linked somewhat more closely to the eastern North American species (87% PP / 57% BS / 69% ML), while support for the whole Japan–Eastern North America clade (J-ENA clade) including *S. musashiensis* Bonati and *S. duplicato-serrata* Makino remains very weak (85% PP / 56% BS / 60% ML).

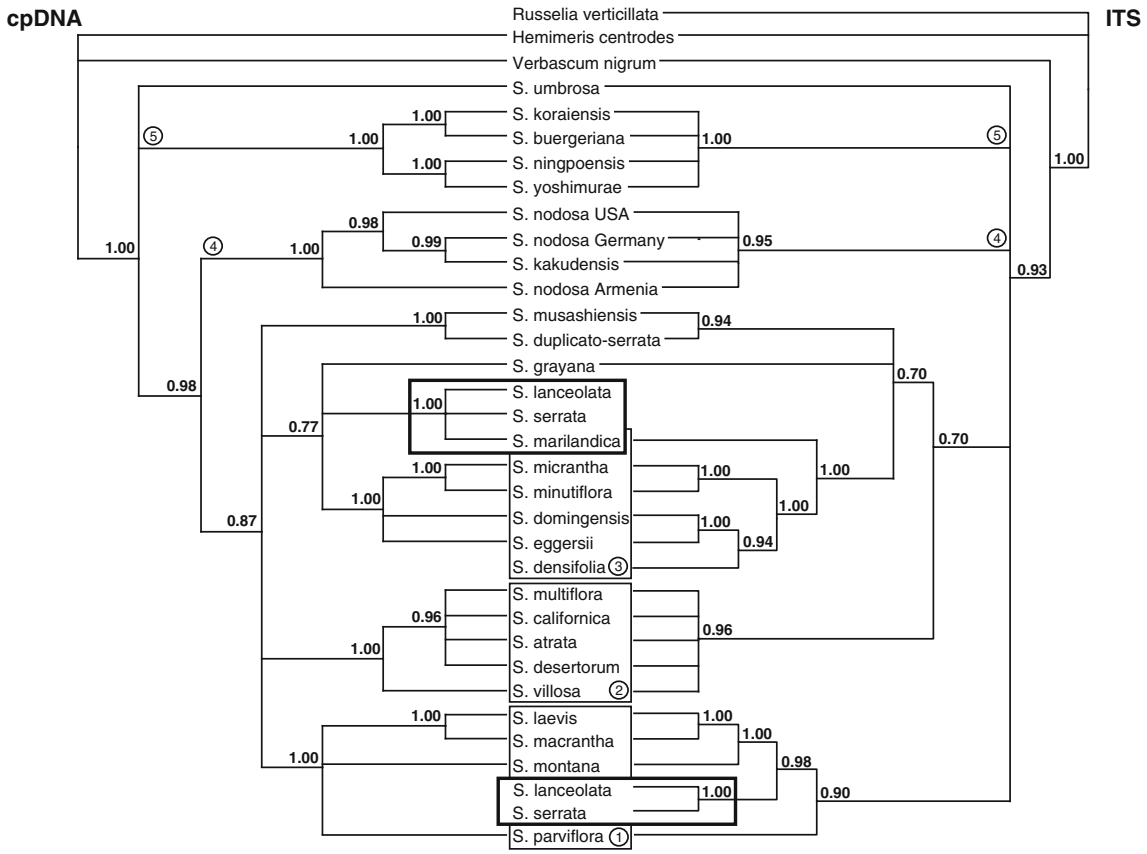

Fig. 5 Bayesian consensus trees (cladograms) from a combined chloroplast dataset (*trnQ-rps16* and *psbA-trnH* intergenic spacer) (*left side*) compared to the ITS dataset (*right side*). Posterior probabilities (obtained from 30,002 trees) exceeding or equaling 70% are given *above each node*. New World clades 1–3 are indicated by *boxes*. *Scrophularia lanceolata* and *S. serrata* are shown within *boldly lined boxes* at their differing positions within each tree. *1* New Mexico clade, *2* California clade, *3* ENA clade, *4 S. nodosa* clade, *5* Asia clade

Apart from that, three strongly supported North American subclades can be distinguished: a "New Mexico clade" (clade 1) with *S. laevis* and *S. macrantha* grouped together with high support (100% PP / BS / ML); *S. montana* is sister to this subclade (100% PP / 93% BS / 100% ML). *Scrophularia parviflora* Wooton & Standl., which is indicated as sister to all the rest of the New Mexico clade, is confined to New Mexico and Arizona, while the other members of the clade are exclusively distributed in New Mexico. The whole clade receives strong support in all analyses (100% PP / BS / ML). A well supported "California clade" (clade 2; 100% PP / 84% BS / 87% ML) shows *S. villosa* as sister to four Californian species that remain within an unresolved polytomy. Apart from California, *S. californica* occurs in British Columbia and Canada, and *S. desertorum* (Munz) R. J. Shaw is also found in Nevada. *Scrophularia atrata* and *S. villosa* are only very locally distributed. The ENA clade (clade 3) contains a highly supported subclade (100% PP / 98% BS / 100% ML) of five species endemic to several islands of the Greater Antilles ("West Indies clade"; distribution map in Fig. 7). These again are divided into two subclades, one with three species (*S. densifolia*, *S. domingensis*, *S. eggersii* Urb.) restricted to Hispaniola, the other with two more widespread taxa (*S. micrantha* Desv. ex Ham. and *S. minutiflora* Pennell). *Scrophularia marilandica* receives high support as sister to that group (100% PP / 98% BS / 96% ML).

Bayesian inference (96% PP), MP (76% BS), and ML (73% ML bootstrap support) analyses show high support for a sister relationship of all New World taxa (including the Japanese subgroup) to the widely distributed *S. nodosa*. All *S. nodosa* accessions included in the dataset form a strongly (100% PP / 99% BS / 97% ML) supported clade, with *S. kakudensis* Franch., a species distributed in eastern China, Korea, and Japan, nested within.

The remaining Asian species (*S. koraiensis* Nakai, *S. buergeriana* Miq., *S. ningpoensis* Hemsl., and *S. yoshimurae* T. Yamaz.) are placed in a single clade receiving high support (100% PP / 99% BS / 100% ML). This "Asia clade" is indicated within an unresolved polytomy at the base of the ingroup in BI and ML analysis.

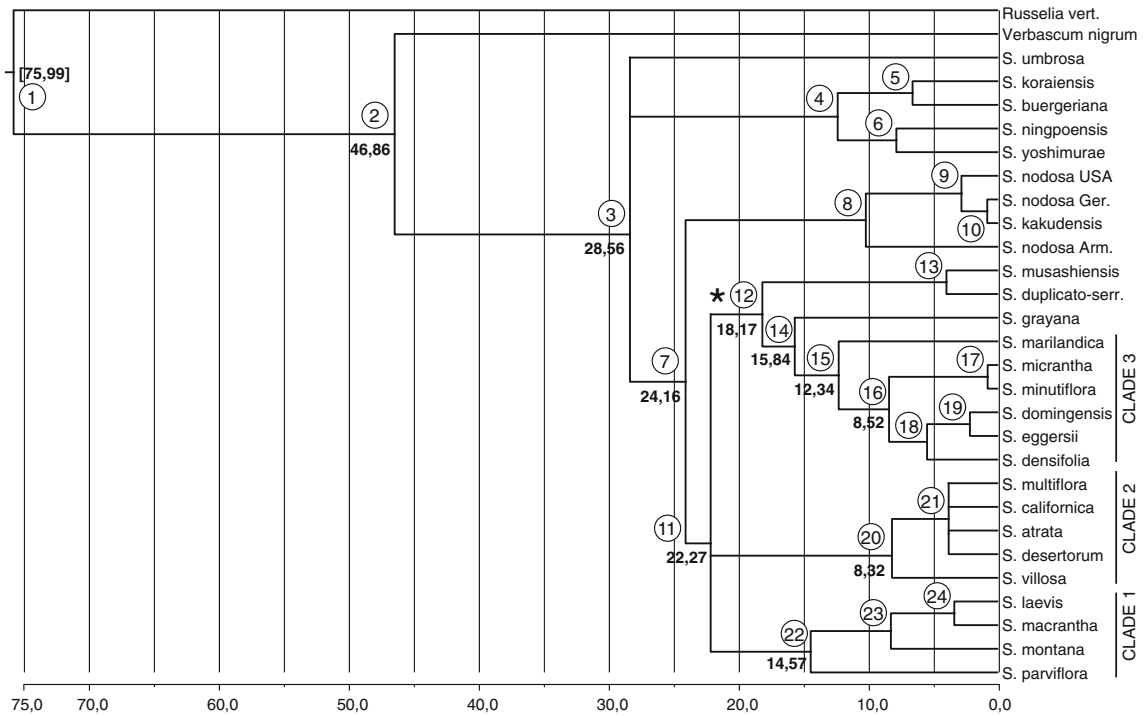

Fig. 6 Maximum clade credibility tree (chronogram with time scale) estimated by BEAST from a combined dataset (ITS and *trnQ-rps16* and *psbA-trnH* intergenic spacers) excluding *Hemimeris centrodes*, *S. lanceolata*, and *S. serrata*. Nodes receive posterior probability values exceeding 80% (PP values not shown); one exception with 0.68/0.69% PP is marked by an *asterisk* (see text). A time constraint of 76 mya (75–76.98 mya) was employed at node 1 for the Plantaginaceae stem age according to Bremer et al. (2004). Mean age and 95% higher posterior density values (HPD) for each node are given in Table 4. Divergence time estimates of important groups are indicated *below the node numbers*. Values are in millions of years before present (mya); value in *square brackets* represents the constrained age of the calibration node. *Russelia vert. Russelia verticillata*, *Ger.* Germany, *S. duplicato-serr. S. duplicato-serrata*

Table 4 Divergence time estimates calculated by BEAST (maximum clade credibility tree shown in Fig. 6)

Node	Mean age (mya)	95% HPD	Node	Mean age (mya)	95% HPD
1	75.99	75.00–76.98	13	4.20	0.4–10.2
2	46.86	25.83–72.59	14	15.84	7.57–26.11
3	28.56	13.97–46.5	15	12.34	5.64–20.71
4	12.41	4.68–22.5	16	8.52	3.63–14.75
5	6.82	1.88–13.25	17	1.03	0.01–2.8
6	7.99	2.22–15.69	18	5.66	1.86–10.32
7	24.16	11.51–39.15	19	2.39	0.37–5.12
8	10.44	3.11–19.89	20	8.32	2.09–17.24
9	3.03	0.39–7.08	21	4.00	0.94–8.44
10	1.08	0.02–2.9	22	14.57	6.22–25.2
11	22.27	10.9–36.35	23	8.34	2.88–15.34
12	18.17	8.68–29.95	24	3.57	0.65–7.61

Mean ages in million years before present (mya) with corresponding 95% higher posterior density values (HPD) are given for each node. Node numbers correspond to labels in Fig. 6

However, MP analysis places it as sister group to *S. nodosa* and the J-NW clade with moderately high support (79% BS). As the node collapses in Bayesian and ML analyses and is therefore not reliable, it was excluded from Fig. 3 (position marked by an asterisk). *Scrophularia umbrosa*, which is widely distributed but does not occur in the New World, is linked to none of the clades and appears as sister to the rest of the *Scrophularia* taxa sampled.

To further reveal the phylogenetic positions of the excluded *S. lanceolata* and *S. serrata*, two separate BI analyses were performed, one from ITS and one from the combined chloroplast dataset (*trnQ-rps16* and *psbA-trnH*). The resulting consensus trees are compared in Fig. 5.

The basal branches of the combined chloroplast cladogram strongly correspond to the *trnQ-rps16* gene tree (not shown), while the combination of both chloroplast markers increases support for the terminal clades. The topology is highly similar to that of the ITS tree, but with better resolution in the basal nodes. A paraphyletic grade leading from the Japanese taxa to the ENA clade is not supported in any case, but all Japanese taxa (ITS) or at least *S. grayana* (chloroplast tree) are positioned in an unresolved polytomy with the ENA clade. Like the Asia clade and *S. nodosa* clade, the three New World clades are highly supported by both markers (90–100% PP), however, the ENA clade does not include *S. marilandica* in the chloroplast tree. The placement of *S. lanceolata* and *S. serrata* in the two trees is

Fig. 7 Distribution of *Scrophularia* in the Greater Antilles. Symbols: *filled dot* *S. minutiflora*, *blank dot* *S. micrantha* (probably synonymous with *S. minutiflora*), *triangle* *S. domingensis*, *square* *S. eggersii*, *star S. densifolia*. The position of the symbols does not necessarily correspond to actual distribution areas. Present distribution areas of *S. marilandica* in Florida (USDA NRCS National Plant Data Center 2010) are marked by *crosses*

substantially different. In the ITS analysis, these taxa are part of the New Mexico clade. The combined chloroplast phylogeny places them in a clade with *S. marilandica* (causing the latter to split away from the ENA clade). Both placements are highly supported (98%/100% PP) and point out the ambivalent status of the most widespread species of *Scrophularia* in North America.

Divergence time estimation

The maximum clade credibility tree from the BEAST analysis is shown in Fig. 6 alongside a time scale and divergence times. It revealed the same topology as the cladogram inferred by MrBayes (some nodes with slightly lower/higher posterior probabilites; results not shown). One node supported with >80% PP in the Bayesian cladogram received only 68/69% support in the BEAST analysis (marked by an asterisk in Fig. 6), but was maintained nevertheless so as to allow interpretation of the tree on the basis of the MrBayes cladogram. The mean number of substitutions per site per million years across the tree was estimated to be 7.9296×10^{-4} (standard deviation: 8.8268×10^{-6}). The standard deviation σ of the uncorrelated log-normal relaxed clock was 0.598, which identifies moderate rate heterogeneity among branches; a strict clock is therefore rejected by the data.

In the absence of *Scrophularia* fossils and due to the limited fossil record of Scrophulariaceae and Lamiales, the clock was calibrated using time estimates from a study with better fossil backup (Bremer et al. 2004). Of course, results obtained by using approximations as a basis have to be treated with caution; see the "Discussion" section for a methodical review of that approach. The derived stem age of the ingroup (separation from its sister group) is 46.86 mya (Fig. 6, node 2). According to our results, the most recent common ancestor of New World and Japanese *Scrophularia* arose at the Oligocene-Miocene boundary (24.16 mya; Fig. 6, node 7). The split into the three North American clades occurred in the early Miocene (ca. 22.27 mya, node 11), with subsequent diversification within the New World clades themselves starting from 14.57 mya (late Miocene, New Mexico clade 1) to 8.32 mya (early Pliocene, California clade 2). The beginning of species diversification in the Antilles is dated to the early Pliocene as well (node 16). All estimated mean ages/divergence times and corresponding 95% higher posterior densities (HPD) are shown in Table 4.

Discussion

Diversification and character evolution within New World *Scrophularia*

Analyses of a combined dataset using nuclear ITS and plastid *trnQ-rps16* and *psbA-trnH* revealed three highly supported North American clades (Fig. 3). It is evident that tree topology is correlated with geographical distribution. The clades are confined to California, New Mexico, and eastern North America including the West Indies (i.e., ENA clade), respectively. Three Japanese taxa form a grade leading towards the ENA clade, representing a (weakly supported) Japan–Eastern North America species alliance (J-ENA clade).

Within the California clade, *S. villosa* is sister to all other taxa. This perennial, shrubby taxon, which can reach up to 3.5 m in height, apparently diverged early within the Californian lineage. Its restricted distribution on three Californian and Mexican islands (Santa Catalina, San Clemente, and Guadalupe) supports this assumption. Its densely villose inflorescence with slender white glandular hairs even covering the sepals is unique within the clade. Other features are shared with *S. atrata*, a very local species restricted to two Californian counties: a dark maroon to almost blackish urceolate corolla with a constricted orifice, and a lanceolate-oblong or (in the case of *S. villosa*) rudimental and awn-like staminode. Although *S. atrata* is placed in an unresolved polytomy with the other Californian taxa, a close relationship to *S. villosa* is apparent from a morphological viewpoint. The remaining species of the clade have a more or less bicolored corolla with a red or brownish dorsal upper lip and a pale to greenish lower lip, and a clavate to obovoid brownish staminode. In contrast to *S. atrata* and *S. villosa,* which are not found above 500 m, these species occur up to 1,000 m (*S. californica*) and 3,000 m (*S. desertorum*) above sea level.

Scrophularia californica, the most widespread species within the California clade, is native to coastal California. In the inland regions and in southern California, it is replaced by *S. multiflora*. Various names and taxonomic ranks have been assigned to this taxon. The name was introduced by Pennell (1947) as a nomen novum for *S. californica* var. *floribunda* Greene (described in Greene 1894) because of the earlier *S. floribunda* Boiss. & Bal. (described in Boissier 1856). *Scrophularia californica* var. *floribunda* had already been elevated to species rank as *S. floribunda* (Greene) A. Heller (1906), mainly because of its distinctive geographical distribution. Shaw (1962) modified its taxonomic status again by treating it as *S. californica* subsp. *floribunda* (Greene) Shaw. We agree with Pennell's (1947) taxonomic concept and accept it as a distinct species because of the smaller, shorter maroon corolla and the more paniculately branched inflorescences. *Scrophularia desertorum* (Munz) Shaw occurs on dry mountain slopes farther inland and extends into western Nevada. It was described as a variety of *S. californica* by Munz (1958), but elevated to species rank by Shaw (1962) because of its ecological characteristics.

The second distinct New World clade comprises four species largely confined to New Mexico. Shared morphological features within this group include a slender conical capsule and ovate to broadly lanceolate, decurrent leaves. *Scrophularia parviflora*, distributed in southern New Mexico and central and southeastern Arizona, is sister to the rest of the clade. While its stem is densely puberulent and the flowers possess a spatulate light brownish staminode, the other species have glabrous stems (*S. montana* in the lower parts only) and rounded staminodes. *Scrophularia laevis* and *S. montana* are restricted to the mountains of central New Mexico, with *S. laevis* distributed even more locally in the southern parts of that area. Both species are highly similar, but triangular-lanceolate, acute sepals set *S. laevis* apart from *S. montana* with its ovate, obtuse sepals. A conspecific status as proposed by Shaw (1962) is not supported by molecular analyses. Sister to *S. laevis* is *S. macrantha*, an outstanding species that has large, showy red corollas of 13–22 mm length, with erect lobes and a slightly constricted orifice; the flowers are almost entirely covered with glandular trichomes. Pollination by hummingbirds has been proven for this species by Lightfoot and Sivinski (1994), which is unique within North American *Scrophularia* and probably the whole genus. *Scrophularia macrantha* is extremely rare and known only from two counties of southwestern New Mexico. Shaw (1962) mentions some localities of *S. montana* in this region as well.

Biogeography of the Caribbean species of *Scrophularia*

Like the North American mainland clades, the Antillean species form a highly supported lineage [West Indies clade, see Eastern North America (ENA) clade in Fig. 3, but without *S. marilandica*]. A distribution map of all West Indian species is shown in Fig. 7. Putative morphological synapomorphies of the West Indies clade are as follows: lanceolate to linear, acute sepals, ovate to globular capsules, a filiform or rudimentary staminode, and a characteristic blackening of leaves and stems upon drying. The species of the first subclade are most likely synonymous: *S. micrantha* Desv. ex Ham. was described in Hamilton (1825) and is mentioned in Urban (1898, 1903). Later, it appears as synonym to *S. minutiflora* (see, e.g., Britton and Wilson 1925), a taxonomic decision accepted today (Liogier 1994). The long shared branch in the phylogram (Fig. 4) with low resolution of the terminal taxa corroborates this view and is additionally supported by the almost identical morphological features. *Scrophularia minutiflora* is rather widespread across the West Indies and occurs on the islands of Cuba, Jamaica, Puerto Rico, and Hispaniola. The other subclade comprising *S. densifolia*, *S. domingensis*, and *S. eggersii* is restricted to Hispaniola.

Scrophularia marilandica, which is widely distributed in the eastern parts of North America and eastern Canada, is sister to the species of the Greater Antilles with high support in all analyses. While there are no distinctive shared morphological characters, the phylogenetic results get support from biogeographic reconstructions of the Antillean region. According to current knowledge, the Greater Antilles originated as a submerged volcanic arc between North and South America (Pindell and Barrett

1990) during the lower Cretaceous (130–110 mya) and subsequently drifted eastwards on the Caribbean Plate (Donovan and Jackson 1994). The landmasses are thought to have been subaerial only since the mid-Eocene (45 mya; Iturralde-Vinent and MacPhee 1999), although this topic is still being discussed. With the continued drifting, Cuba (attaching to the North American plate) separated from N-Hispaniola and proto-Puerto Rico (on the Caribbean plate) during the Oligocene (Hedges 1996; Iturralde-Vinent and MacPhee 1999) or the early Miocene (Graham 2003a, 2003b); eventually, all three islands were fixed to the North American plate. The separation of Puerto Rico from Hispaniola probably dates to the early Miocene as well (Graham 2003a).

Various colonization patterns have been proposed for several Antillean species, including tertiary vicariance due to separation of islands (Negrón-Ortiz and Watson 2003), dispersal (also repeatedly, leading to reticulate patterns) from North or South America (Fritsch 2003), combinations of vicariance and dispersal (McDowell et al. 2003), taxa originating from the early Tertiary boreotropical flora (Lavin et al. 2001), or from in situ adaptive radiation (Graham 2003b). Hedges (2006) states that dispersal is likely to be the key factor in Antillean colonization by terrestrial vertebrates, often followed by adaptive radiation. This also applies to *Scrophularia* with seven species occurring in the West Indies, while only one species inhabits the eastern North American mainland. Furthermore, the divergence time between *S. marilandica* and the Antillean species was dated to ca. 12 mya (late Miocene) in the relaxed molecular clock analysis (Fig. 6), a time when all Greater Antillean islands were already separated from each other. This excludes two other main colonization patterns being discussed: proto-Antillean vicariance (Rosen 1975) and the Aves Land Bridge model (Iturralde-Vinent and MacPhee 1999). Colonization of the Greater Antilles should thus have occurred via long distance dispersal. The inferred divergence time for the Antillean species is somewhat later, but still in concordance with the results provided by Hedges et al. (1992), who studied albumin evolution in West Indian terrestrial vertebrates to date divergences between West Indian and Central/South American taxa and found that most splits date to the mid-Cenozoic (Eocene to Miocene).

Scrophularia marilandica occurs in large parts of eastern North America today, for example in the northern parts of Florida as well (see Fig. 7), whereas no species of *Scrophularia* have ever been reported from tropical Central or South America. For this reason, the evidence is strong that the common ancestor of the West Indian lineage occurred in Florida and colonized the Antilles from there, dispersing to Cuba. This was probably facilitated by the fact that the wet forests of western Cuba are remarkably similar to the vegetation of Florida, and that presumably, Cuba was fully emergent in its present position by the late Miocene, 19–12 mya (Iturralde-Vinent and MacPhee 1999; Lewis and Draper 1990). Migration pathways to Hispaniola, Puerto Rico, and Jamaica remain speculative; Hispaniola may have been colonized before the early Pliocene, when the Hispaniola endemic lineage diverged from the more widespread *S. minutiflora* (Fig. 6). Jamaica most likely was not colonized before that time as well because it remained inundated from the latest Eocene until ca. 10 mya (Lewis and Draper 1990) and was never connected to a land mass after that time (Buskirk 1985).

While dispersal in principle is the usual mechanism of Antillean colonization and was found in many other Antillean and New World taxa [e.g., *Poitea* (Fabaceae; Lavin 1993), *Styrax* sect. *Valvatae* (Styracaceae; Fritsch 2003), *Cuphea* (Lythraceae; Graham 2003b), *Erithalis* (Rubiaceae; Negrón-Ortiz and Watson 2003)], the modes of diaspore dispersal are diverse and in this case cannot be fully elucidated. However, as the Florida coastal region is influenced by the northeast trade winds that come across the Atlantic, incidental dispersal over the relatively short distance (Florida–Cuba: 160 km) is likely (Graham 2003a, b). Between the islands, the distances involved are even shorter (Cuba–Haiti: 72 km, Dominican Republic–Puerto Rico: 96 km) and there are various possibilities for overcoming these distances: hurricanes with unusual yet proven west to east tracks are mentioned in Hedges (2006), and Powell (1999) discussed strong easterly surface winds associated with westerly hurricanes. In addition, seeds could have been transported via local water currents (Hedges et al. 1992); the transport of plant seeds in soil stuck to drifting vegetation was described by Renner (2004), and this mode of dispersal has already been documented for West Indian vertebrates (Censky et al. 1998; Knapp 2000). Altogether, species diversification subsequent to colonization from the North American mainland and dispersal within the Antilles presumably accounts for the current distribution of *Scrophularia* in the Caribbean.

The colonization of the Greater Antilles is accompanied by morphological changes. The large reddish brown corollas of *S. marilandica* do not suggest a close relationship to *S. minutiflora* with its small, white corollas, but the latter are typical features for much of the flora of the Antilles and especially Cuba. According to Borhidi (1996), plants with very small flowers, so-called micranthia (cf. *S. micrantha*!), are adapted to pollination by minute, endemic insects. Again, the Hispaniola clade differs from *S. minutiflora* in possessing ovate-lanceolate leaves with crenate to serrate margins (in contrast to triangular-ovate, coarsely serrate leaves) and large yellow corollas (up to 12 mm). The species are perennial herbs or subshrubs and are adapted to coniferous forests of higher elevations (up to

3,100 m). This as well is in contrast to the annual life cycle of *S. minutiflora*, which inhabits shaded banks and humid forests between 800 and 2,000 m elevation. The appearance of woody lifeforms derived from herbaceous ancestors in the course of island colonization [so-called insular woodiness as coined by Carlquist (1974)] has been discussed for the pantropic giant lobelias (Campanulaceae) by Knox et al. (1993) and for *Echium* (Boraginaceae) and *Pericallis* (Asteraceae) on the Canary Islands (Böhle et al. 1996; Panero et al. 1999).

The origin of *Scrophularia lanceolata*

Scrophularia lanceolata is the most widespread species within New World *Scrophularia*. It is found throughout the United States except in a few southern states and extends to southern Canada as well, while all other American species (except *S. marilandica*) have a restricted distribution. *Scrophularia lanceolata* in all analyses forms a highly supported clade with *S. serrata*, a species which is known from only Idaho and Colorado and is generally regarded as synonymous with *S. lanceolata*. The only distinguishing feature is the color of the staminode, which is yellowish-green in *S. lanceolata* and purple in *S. serrata*. But as purple staminodes can also be found in individuals of *S. lanceolata* (D. E. Boufford, pers. comm.), different staminode color may not be sufficient for assigning species rank to *S. serrata*.

In all tree topologies, both species are closely linked. When the nuclear and chloroplast datasets are analyzed separately (Fig. 5), the *trnQ-rps16/psbA-trnH* subset groups the two taxa with *S. marilandica*, while the ITS phylogeny shows them within the New Mexico clade. Consequently, a different evolution of the markers concerned is apparent, which is best explained by a hybridization event involved in the origin of *S. lanceolata*. Under this assumption, crossing of *S. marilandica* with an ancestral species from the New Mexico clade (possibly resembling *S. parviflora*) is most likely. Successful crossings between North American *Scrophularia* species have already been conducted by Shaw (1962), who found that most North American species will cross fairly easily, also due to pollination by non-species-exclusive insects such as wasps and syrphid flies (Trelease 1881; Robertson 1891). In *S. marilandica* and *S. parviflora*, similar habitats and overlapping flowering times additionally facilitate hybridogenesis.

Numerous morphological characters shared between *S. lanceolata* and its putative parental species support a hybrid origin hypothesis. Morphologically, *S. lanceolata* is remarkably similar to *S. marilandica*; the two species can be distinguished only by leaf margin shape (more finely serrate in *S. marilandica*), sepal shape (somewhat acute in *S. lanceolata*, while broadly ovate in *S. marilandica*), and color of the staminode (yellow-greenish vs. purple in *S. marilandica*); however, none of these features is reliable given the great variability within the species. For example, *S. lanceolata* occasionally develops purple staminodes (see above) and Pennell (1935) reported an aberrant form (*S. marilandica* var. *viridis*) with green staminodes. The striking similarity has already led to confusion regarding species delimitation in the past. Pennell (1935), in his treatment of the eastern temperate Scrophulariaceae, transferred 10 species into synonymy with *S. lanceolata* and *S. marilandica*. On the other side, morphological features shared between *S. lanceolata* and *S. parviflora* include similar color patterns of the corolla (greenish with a shade of red-brown on the upper lip in *S. lanceolata*, green with reddish lobes in *S. parviflora*) and the spatulate staminode, which is unique within the New Mexico clade. The slender ovoid capsules in *S. lanceolata* are intermediate between the conical capsules in *S. parviflora* and the globular ones in *S. marilandica*, while capsules and flowers in *S. lanceolata* are larger than in *S. marilandica*. The combination of advantageous characters acquired through hybridization might have been the key factor enabling this unique species of *Scrophularia* to colonize almost the whole North American continent.

Phylogenetic position of New World *Scrophularia* within the genus

Scrophularia nodosa, which is widely distributed in Eurasia and the New World, is sister to the J-NW clade with high to moderate support in all analyses. Therefore, conspecificity of *S. nodosa* and the New World species as proposed by Stiefelhagen (1910) must be rejected based on our molecular analyses. However, close relationships between the taxa are apparent as *S. nodosa* (in addition to the three Japanese species) is the only known Old World species characterized by the large *trnQ-rps16* deletion typical for New World species of *Scrophularia*.

Preliminary studies conducted on *Scrophularia* placed *S. nodosa* and a set of New World taxa in one highly supported clade with several eastern Asian species (results not shown). An Asian origin of the North American taxa is suggested by several of our results. First, the affiliation of three Japanese taxa (possessing the deletion in *trnQ-rps16*) to the ENA clade (although weakly supported) indicates a link between eastern Asia and North America. Intercontinental biogeographic relationships of that kind have been observed in many plant genera and correspond to a general Eastern Asian–Eastern North American (EA–ENA) disjunct pattern (see Xiang et al. 2000), which has been extensively discussed (e.g., Boufford and Spongberg 1983; Li 1952; Boufford 1998; Wen 1999). Sometimes, eastern

Asian and North American taxa form sister clades, as in *Torreya* (Taxaceae; Li et al. 2001) and *Aesculus* (Sapindaceae; Xiang et al. 1998); several other examples are mentioned in Soltis et al. (2001). In this study, Japanese species are even linked to one of the New World clades and thus are part of the clade containing all North American taxa. Asian plant species that are more closely related to their New World relatives than to species of their own continent were discussed by Li et al. (2003). This pattern of relationships often involves Japanese taxa and was found, e.g., in *Hamamelis* (Hamamelidaceae) by Wen and Shi (1999) and Li et al. (2000); the latter state that the same relationships are found in several other groups (see also Kim and Jansen 1998).

Second, *Scrophularia nodosa*, which is sister to all North American taxa, has been shown in this study for the first time to have an eastern Asian species nested within. *Scrophularia kakudensis* was put into synonymy with *S. nodosa* by Stiefelhagen (1910) and has the same chromosome number as *S. nodosa* ($2n = 36$; Nishikawa 1985; Scherer 1939). Consequently, it can be assumed that *S. nodosa* itself, an ancestor, or a close relative occurs in eastern Asia, which also supports an Asian origin hypothesis. Third, further evidence comes from an additional node in the MP analysis, placing the eastern Asian species as sister to *S. nodosa* and the J-NW clade (Fig. 3, position of the node marked by an asterisk). Although this node collapses in ML and Bayesian analyses and was therefore not included in the figure, it receives a bootstrap support value of 79% in MP, which indicates a weak signal for the proposed relationship. Altogether, there is strong evidence that the J-NW clade arose from a *S. nodosa*-like Asian ancestor characterized by a large deletion in the *trnQ-rps16* marker.

Chromosome evolution

Regarding phylogenetic reconstructions, there is evidence that Asian *Scrophularia* species were involved in the formation of the Japan-New World clade. Regarding reported karyological data, all members of the three New World clades are high polyploids with $2n = 86$–96 (e.g., Shaw 1962). It has been suspected that all species endemic to the New World have $2n = 96$, with the lower counts being attributable to problems in identifying very small chromosomes. An exception occurs in *S. montana*, which possesses $2n = 70$–76 chromosomes (Shaw 1962). Polyploidy is also documented for the Japanese species of the J-NW clade: *S. grayana* has $2n = 94$ chromosomes (Kamada et al. 2007). No counts are available for *S. musashiensis* or *S. duplicato-serrata*, but it is likely that the whole J-NW clade is characterized by high polyploidy in contrast to *S. nodosa* with a chromosome number of $2n = 36$. Assuming a polyploid ancestor that gave rise to the J-NW clade, interspecific hybridization and autopolyploidization with *S. nodosa* or its ancestor involved is conceivable. Although no direct evidence is available at present, this hypothesis gains support from the existence of polyploid *Scrophularia* species and a remarkable variation of chromosome numbers in eastern Asia [from $2n = 24$ in *S. dentata* Royle ex Benth. (Mehra and Vasudevan 1972) and $2n = 30$ in *S. buergeriana* (Lee 1967), to $2n = 50$–56 in *S. incisa* Weinm. (Vaarama and Hiirsalmi 1967) and $2n = 90$ in *S. ningpoensis* (Ma et al. 1984)].

Phylogenetic dating

Estimating divergence times within families of Lamiales is extremely difficult due to the limited fossil record. There are two studies on a larger scale using reference fossils as calibration points to estimate clade ages: Bremer et al. (2004) dated families of asterids, Wikström et al. (2001) timed angiosperm cladogenesis. The stem and crown ages of Lamiales have been estimated by these authors at 106/97 mya (mid Cretaceous) and 77–81/71–74 mya (late Cretaceous), respectively.

To compensate for missing fossils, authors dealing with families belonging to Lamiales have used inferred ages from the studies mentioned above for calibration of their phylogenies, e.g., Phrymaceae (Nie et al. 2006) and also Scrophulariaceae (Datson et al. 2008). Nie et al. (2006) additionally dated divergence times by calibration with three reference fossils from Bignoniaceae and Oleaceae. This approach is likely to produce more accurate results because calibration points are closer to the examined family. However, calibration based on these fossils was not possible in our study due to problems in aligning distant outgroups when using markers variable enough to resolve structures on an infrageneric level. From families other than Scrophulariaceae, only *Russelia verticillata* (Plantaginaceae) could be included successfully into the sampling. Calculations of divergence times were also complicated by the incomplete sampling within the genus. As outlined by Linder and Hardy (2004), the potential absence of basal lineages can lead to younger dates obtained for the start of radiation. Thus, all divergence times provided in this study must be regarded as an initial attempt to establish a chronological framework for the radiation of lineages within *Scrophularia*.

To calibrate the present phylogeny, we decided to follow Bremer et al. (2004). Their results are probably more reliable than those provided by Wikström et al. (2001), as six chloroplast regions (compared to three) and six reference fossils (compared to one) were used. Furthermore, the obtained divergence times for Lamiales (106 mya) was recently corroborated by Janssens et al. (2009), assessing the age at 104 ± 8.2 mya. As for the group concerned in

this study, Bremer et al. (2004) date the separation of Scrophulariaceae from the Plantaginaceae at ca. 76 mya. Many asterids appear in the fossil record of the late Cretaceous (Magallon et al. 1999), and the values fit the minimum age of Scrophulariaceae based on their appearance in the fossil record (late Eocene; Magallon et al. 1999). This "Plantaginaceae stem age" of 76 mya was used as calibration point. Although Plantaginaceae and Scrophulariaceae are not sister groups in the phylogeny provided by Bremer et al. (2004) but form a grade leading towards the Lamiales crown group families (such as Verbenaceae, Acanthaceae, Bignoniaceae), a study on Scrophulariaceae has already been conducted successfully including only Plantaginaceae and a few related Lamiales stem group taxa (Datson et al. 2008). With calibration applied, the resulting divergence time of the ingroup (46.86 mya) is optimally congruent with Bremer et al. (2004) who estimate *Selago* (which is less closely related to *Scrophularia*) to have split away 48 mya. Datson et al. (2008), using calibration points according to Wikström et al. (2001), specify this divergence at 39.5 mya. The split between *Verbascum* and *Scrophularia* as inferred by Datson et al. (2008) dates to ca. 24.3 mya, compared to 46.86 mya in the present study.

Intercontinental disjunction

According to Stiefelhagen (1910), the origin and native range of *Scrophularia* is assumed to be in Asia, more precisely, in the Himalayan region. Southwestern China is one of the biologically richest temperate regions in the world today (Wu 1988; Sun 2002), and it is hypothesized that the uplift of the Himalaya-Tibetan plateau (which started 40–50 mya) and subsequent increase in geological complexity in that region (resulting in a diverse mixture of habitats) has contributed to the process of diversification (Wu 1988; Nie et al. 2005). As the sampling of the present study does not allow any implications regarding the center of origin, we follow Stiefelhagen's (1910) assumption in that point. Relaxed molecular clock estimates suggest the divergence of *Scrophularia* from its sister taxon at ca. 47 mya. From its center of origin, it would then have spread to eastern Asia, to Europe, and to North America (Stiefelhagen 1910).

Based on an Asian origin of New World *Scrophularia*, the observed relationships point towards ancient trans-Beringian migrations. As outlined by Tiffney (1985), the Beringian Land Bridge (BLB) allowed migrations from the early Tertiary until the late Eocene, and also in the second half of the Tertiary (until 5.5–4.8 mya) for cool-temperate taxa (Marinovich and Gladenov 1999; Tiffney and Manchester 2001). In contrast, migration via the North Atlantic Land Bridge (NALB) seems unlikely because of the closure of this pathway at the Eocene-Oligocene boundary (Tiffney 1985), which is considerably earlier than the earliest possible migration of *Scrophularia* to the New World (early Miocene, between nodes 7 and 11 in Fig. 6) as determined by relaxed molecular clock analyses. Thus, migration to the New World from Asia through Beringia has to be assumed for *Scrophularia*, a hypothesis which was doubted but not ruled out by Hong (1983). Other Northern Hemisphere genera are also known to have dispersed using that route: Scheen et al. (2004) found that North American species of *Cerastium* (Caryophyllaceae) originated as a result of Asian taxa migrating across the BLB prior to its breakup. An ancestral species of *Aralia* (Araliaceae) was hypothesized by Wen et al. (1998) to have migrated into North America via Beringia and diversified into the present-day species. Asian *Aralia* species occur in China, Japan, Korea, and the Himalayas, while the North American species are found in California, New Mexico, Arizona, and eastern North America, a distribution that is identical to that of *Scrophularia*.

Although relationships among the three New World clades are not resolved in the tree topologies, a rough scenario for the colonization of the North American continent can be outlined. Assuming a common ancestor in Asia, the most parsimonious explanation for the current distribution pattern involves one or two migration events across the BLB in the Miocene (between 22.27 and 8.32 mya), with subsequent diversification into the Californian and New Mexican lineages (clades 1 and 2). Independently, another lineage underwent a radiation in Japan before likewise colonizing the New World in the late Miocene (between 15.84 and 12.34 mya) and giving rise to the eastern North American/Caribbean lineage (clade 3).

An additional node in BI, BEAST, and ML analyses placed the New Mexico clade sister to the other New World clades, but support values were too low for the node to be reliable (62 / 74% PP and 54% ML bootstrap support), so it was not included in Fig. 3 (position marked by a triangle). However, if this weak signal reflects the true relationships, another, less likely scenario has to be considered, where one single migration event across the BLB led to the colonization of western North America. From there, a recolonization of Japan via the BLB with subsequent return to the New World would have created the EA-ENA disjunction present in the phylogeny, and eventually the West Indies clade.

However, as long as no reliable evidence supports any of the possibilities, we tend to favor the most parsimonious explanation. Apart from that, a molecular phylogenetic study focusing on *Scrophularia* on a worldwide scale is in preparation and should provide answers to the remaining questions.

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4.3. Article III

Diversification of *Scrophularia* (Scrophulariaceae) in the Western Mediterranean and Macaronesia - Phylogenetic relationships, reticulate evolution and biogeographic patterns.

by Agnes Scheunert & Günther Heubl

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Diversification of *Scrophularia* (Scrophulariaceae) in the Western Mediterranean and Macaronesia – Phylogenetic relationships, reticulate evolution and biogeographic patterns

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ABSTRACT

The flora of the Mediterranean region and Macaronesia is characterized by high levels of species diversity and endemism. We examined phylogenetic relationships of *Scrophularia* within one of its secondary centers of diversity located in the Iberian Peninsula and adjacent Macaronesia. In total, 65 ingroup accessions from 45 species, representing an almost complete sampling of the region, were analyzed using sequences from the internal transcribed spacer region (ITS) and the plastid *trnQ-rps16* intergenic spacer. Phylogenetic relationships were inferred using Bayesian inference, maximum likelihood and statistical parsimony networking. Incongruence between datasets was assessed with statistical tests and displayed by split networks. Biogeographic inferences incorporating information from both markers (despite low resolution in some parts of the trees) and all incongruent taxa were accomplished with a novel combination of methods, using trees generated with the taxon duplication approach as input for Bayesian binary MCMC (BBM) analysis as implemented in RASP.

Nuclear and chloroplast markers support a clade which comprises the majority of Iberian and Macaronesian species and consists of three subclades. Analyses of the substantial incongruence observed among markers indicate reticulate evolution and suggest that *Scrophularia* species diversity in this region is largely attributable to hybridization; a combination of both polyploidy and dysploidy in the karyotypic evolution of Western Mediterranean *Scrophularia* taxa is proposed. Our results provide support for an ancient hybridization event between two widespread lineages, which resulted in an allopolyploid ancestor of the Iberian – Macaronesian group with $2n = 58$ chromosomes. The ancestor then diverged into the three main lineages present in the Iberian Peninsula, Northern Africa and Macaronesia today. Subsequent interspecific hybridizations at different ploidy levels additionally generated new species. Presumably, hybridization and diversification within the genus in the Western Mediterranean have not been restricted to one particular event, but occurred repeatedly. It can be assumed that the topographical complexity found in the Iberian Peninsula has promoted diversification and hybrid speciation processes in *Scrophularia*, and that isolation in glacial refugia has preserved recent and ancient lineages. For the Macaronesian taxa, biogeographic analyses support several origins, by colonizations from at least four distinct lineages.

1. Introduction

The Mediterranean basin is recognized as a global biodiversity hotspot and harbors app. 25,000 vascular plant species (Comes, 2004; Médail and Myers, 2004; Myers et al., 2000). As defined by Médail and Quézel (1997), important areas of plant endemism and floristic richness in the Western Mediterranean and Macaronesia are the High and Middle Atlas mountains, the Baetic – Rifan complex, the Maritime and Ligurian Alps, the Tyrrhenian Islands, and the Canary Islands and Madeira. One of two main centers of biodiversity in the Mediterranean basin is found in its western part and includes the Iberian Peninsula and Morocco (Médail and Quézel, 1997). It is assumed that patterns of plant speciation in the Mediterranean Basin have been shaped by climatic shifts and geological events, like the Betic Crisis and Messinian Salinity Crisis (Garcia-Castellanos et al., 2009; Krijgsman et al., 1999; Lonergan and White, 1997), the onset of the Mediterranean climate rhythm (Suc, 1984; Thompson, 2005), and the Quaternary glaciations (Suc, 1984). Additionally, its topographical complexity was suggested to promote diversification and speciation processes. Fragmentation and contraction of distribution ranges have triggered the isolation of populations during glacial periods, thereby causing allopatric speciation. Following inter- and postglacial range expansion,

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hybrid zones developed where different genetic lineages came into contact (Blanco-Pastor et al., 2012).

The genus *Scrophularia* L. (Scrophulariaceae), belonging to the order Lamiales and closely related to *Verbascum* L. (Datson et al., 2008; Olmstead et al., 2001), includes app. 270 extant species (Ortega Olivencia, 2009). Representatives of the mainly holarctic genus occur in both the Old and New World; the primary center of diversity is located in the Irano – Turanian region including the Caucasus and Central Asia (Gorschkova, 1997; Grau, 1981; Lall and Mill, 1978). One of two secondary diversity centers is found in the Iberian Peninsula and adjacent Macaronesia with 28 species, more than half of which are endemic (Dalgaard, 1979; Ortega Olivencia, 2009). The last taxonomic treatment of the genus was done by Stiefelhagen (1910), who classified the genus according to two sections: section *Anastomosantes* Stiefelhagen (= section *Scrophularia*), with two subsections *Vernales* Stiefelhagen and *Scorodoniae* (Benth.) Stiefelhagen (= subsection *Scrophularia*), and section *Tomiophyllum* Bentham, comprising subsections *Farinosae* Stiefelhagen, *Orientales* Stiefelhagen, and *Lucidae* Stiefelhagen (which largely corresponds to sect. *Canina* G.Don). Leaf venation, petal length, shape of the corolla tube, and life form were used as distinguishing characters. In a revision of the genus for the Iberian Peninsula, Ortega Olivencia and Devesa Alcaraz (1993a) confirmed the assignment of the respective species to sections *Anastomosantes* / *Scrophularia* (20 species) and *Canina* G.Don (three species); *S. tanacetifolia* was moved to section *Scrophularia* by the authors.

Species of *Scrophularia* are annual, biennial or perennial herbs, subshrubs or small shrubs with pinnate to undivided leaves of various forms. The inflorescence is a thyrse with cymose, often dichasial or monochasial partial inflorescences. The flowers are characterized by a more or less equally 5-lobed calyx, a mostly zygomorphic, distinctly 2-lipped corolla and typically a rudimentary fifth stamen of various shapes at the base of the upper lip. The fruit is a capsule with septicidal – septifragous dehiscence (Fischer, 2004). Representatives of the genus inhabit regions from coasts and lowlands to high plateaus and alpine regions, where the majority of species is found. Preferred habitats include mountain slopes and rock crevices, but also forests, scrubs and grassland as well as roadsides or disturbed areas. Species occurring in moist habitats (e.g. on river banks) are mainly restricted to section *Anastomosantes*, while section *Tomiophyllum* also contains more xerophytic elements; however, real desert plants are rare.

Karyological studies on taxa of the genus were confined to particular geographic areas (Carlbom, 1969, 1964; Dalgaard, 1979; Grau, 1976; Ortega Olivencia and Devesa Alcaraz, 1990; Shaw, 1962) or merely reported chromosome numbers (Vaarama and Hiirsalmi, 1967), which range from $2n = 22$ (e.g. in *S. divaricata* Ledeb.; Vaarama and Leikas, 1970) to $2n = 96$ (e.g. in *S. desertorum* (Munz) R.J. Shaw; Shaw, 1962). Many taxa from the Iberian Peninsula and Macaronesia are characterized by $2n = 58$ (Grau, 1976); and most of the species occurring in the New World are high polyploids with $2n = 70–96$ (Shaw, 1962). Shaw (1962) and Carlbom (1969) postulated that allopolyploid evolution increased species diversity and variability within *Scrophularia*. Indeed, hybridization is frequent in the genus (natural hybrids have been erroneously described as distinct species by Menezes, 1908, 1903), and is additionally supported by flower morphology and pollinator preferences. While some of the few large-flowered *Scrophularia* (*S. sambucifolia* L., *S. grandiflora* DC. and *S. trifoliata* L. from the Western Mediterranean, and *S. calliantha* Webb. & Berthel. from Gran Canaria) were shown to possess a mixed pollination syndrome between insects and passerine birds (and even juvenile lizards in *S. calliantha*; Ortega-Olivencia et al., 2012), wasp pollination was recently revealed to be the ancestral condition within the genus (Navarro-Pérez et al., 2013). Wasps (Vespidae) are considered the main pollinators (Faegri and van der Pijl, 1979; Ortega Olivencia and Devesa Alcaraz, 1993b; Wilson, 1878); other insects like bees (Valtueña et al., 2013) and syrphid flies (Knuth, 1909; Ortega Olivencia and Devesa Alcaraz, 1993b; Robertson, 1891) complete the pollinator spectrum, which thus mostly consists of generalist pollinators unable to distinguish between different species of *Scrophularia*. In addition, the mostly protogynous flowers are often self-compatible (although rarely self-pollinating; Ortega Olivencia and Devesa Alcaraz, 1993b, 1993c; Shaw, 1962; Trelease, 1881; Valtueña et al., 2013), and reproductive barriers among related species tend to be weak as well. Artificial cross-pollination experiments have shown the potential for hybridization: successful crossings were performed by Shaw (1962) and Carlbom (1964) on North American, Dalgaard (1979) on Macaronesian, and Goddijn and Goethart (1913) and Grau (1976) on some European species. Altogether, the above-mentioned ecological and morphological factors make interspecific hybridization, in combination with karyotype evolution including polyploidy and dysploidy, very likely to have played an important role in the diversification and speciation history of *Scrophularia*.

In the current work, which is part of an extensive molecular phylogenetic study of the genus, we specifically address the following questions: (1) What are the phylogenetic relationships among *Scrophularia* taxa in the Iberian Peninsula and in Macaronesia? (2) Is the high species diversity observed in the Iberian Peninsula the result of hybridization events or due to other factors? (3) Which parental taxa were involved in the origin of the polyploid Iberian species? Which event gave rise to the unusual chromosome number $2n = 58$ found in many taxa? (4) Which historical biogeographic processes have affected present distribution patterns of *Scrophularia* in the Iberian Peninsula? Are the species occurring on the Canary Islands and Madeira monophyletic (implying a single colonization event), or were there multiple colonizations?

To achieve this goal, we analyzed the plastid *trnQ-rps16* intergenic spacer, which has been successfully employed in a recent study on New World *Scrophularia* (Scheunert and Heubl, 2011), and the nuclear ribosomal internal transcribed spacer (ITS) region, which continues to be widely used for phylogenetic analyses on the interspecific level (Nieto Feliner and Rosselló, 2007).

2. Material and methods

2.1. Plant material, DNA extraction, sequencing

The taxon sampling strategy was designed to span the distribution range of *Scrophularia* in the Western Mediterranean (especially the Iberian Peninsula), Macaronesia and Northern Africa. The present study comprises all 22 species of the Iberian Peninsula according to Ortega Olivencia (2009), 27 out of 28 species occurring in the Western Mediterranean as a whole (e.g. Cartier, 1975; Ortega Olivencia, 2009; Zángheri, 1976; *S. heterophylla* was not sampled but in the region is confined to Istria only), 17 out of 21 species occurring in Northern Africa (Ibn Tattou, 2007; Qaiser, 1982; Quézel and Santa, 1963; Täckholm, 1974; the four absent species are either narrow endemics with close association to sampled species, or putative synonyms), and all of the nine species from Macaronesia (Dalgaard, 1979). Additionally, four species from other diversity centers and the putative center of origin (“Asian species”) were sampled; these include three Southern Asian/Eastern Asian taxa (*S. urticifolia* Wall., *S. ningpoensis* Hemsl., *S. yoshimurae* T.Yamaz.) and one species distributed in Southwestern Asia, Turkey and the Caucasus (*S. amplexicaulis* Benth.). Altogether, the sampling thus consists of 45 ingroup species. For a rough estimate of the degree of intraspecific genetic variation, 13 species were sampled with two or more accessions (see Table 1), laying

Table 1
Analyzed taxa with voucher information on collectors, localities and collection years, herbaria, and GenBank accession numbers. Abbreviated identifiers for individual accessions as used in the text are given after the respective locality (in quotes). Reference citations of previously published sequences: (1), Vargas et al. (2009); voucher information for the respective sequence as given there; (2), Kornhall and Bremer (2004); (3), Scheunert and Heubl (2011). CULT., plants grown in the greenhouses of the Botanical Garden Munich, Germany – the original locality and the supplier (in brackets) are given where known. Herb., Herbarium; acc. no., accession number; Bot. Gard., Botanical Garden, n/a, information not available.

Taxon	Year	Locality (country, province/district)	Collector	Collector no.	Herb.	Acc. no. trnQ-rps16	Acc. no. ITS
Antirrhinum majus L.	1999	Spain, Cordoba	M. Nydegger	36531	MSB	KF447311	FJ487615[1]
Russelia verticillata Kunth	1990	Costa Rica, Guanacaste	P. Döbbeler	3795	M	–	HQ130062[3]
Hemimeris centrodes Hiern	1976	South Africa, Cape	P. Goldblatt	4033	M	HQ130033[3]	HQ130063[3]
Nemesia cheiranthus E.Mey. ex Benth.	1974	South Africa, Cape	P. Goldblatt	2534	M	KF447312	KF447249
Selago corymbosa L.		South Africa, Beaufort West	Vlok	2514	S	–	AJ550603[2]
Verbascum arcturus L.	1962	Crete, Chanía	D. Phitos	603	M	KF447313	KF447250
Verbascum nigrum L.	1998	Germany, Bavaria	H. Wunder		M	HQ130034[3]	HQ130064[3]
S. amplexicaulis Benth.	1977	Iran, Tehran	K.H. Rechinger	57228	M	KF447315	KF447252
S. auriculata L.	1993	Morocco, Tétouan	H. Förther	7104	M	KF447247	KF447287
S. auriculata "balbisii Hornem."	2008	CULT., orig.: Spain, Cantabria (J. Grau)	A. Scheunert	005/1-1	MSB	KF447248	KF447291
S. alpestris J.Gay ex Benth.	1998	France, Pyrénées-Atlantiques	D. Podlech	55135	MSB	KF447332	KF447269
S. arguta Sol.	1905	Fuerteventura, Puerto del Rosario	C.J. Pitard		M	KF447368	KF447308
S. arguta Sol.	2010	CULT., orig.: Lanzarote, Teguise (M. Erben)	A. Scheunert	015/1-1	MSB	KF447367	KF447307
S. arguta Sol.	1995	Morocco, Tiznit	D. Podlech	52494	MSB	KF447366	KF447306
S. bourgaeana Lange	1994	Spain, Salamanca	M. Martinez Ortega	(MA 631819)	MA	KF447333	KF447270
S. calliantha Webb. & Berthel.	2011	CULT., orig.: Gran Canaria, n/a	A. Scheunert	010/1-1	MSB	KF447362	KF447302
S. canina ssp. canina L.	1995	Morocco, Tiznit	D. Podlech	52525	MSB	KF447320	KF447257
S. canina ssp. ramosissima (Loisel.) P.Fourn.	1976	Sardinia, Oristano	U. Hecker	I 774 (Hec. 1560)	MJG	KF447323	KF447260
S. crithmifolia Boiss.	1983	Spain, Malaga	E. Bayer, J. Grau & G. López González		M	KF447321	KF447258
S. deserti Delile	1991	Egypt, Sinai Peninsula	D. Podlech	49719a	MSB	KF447325	KF447262
S. eriocalyx Emb. & Maire	1933	Morocco, Rif-Atlas	Sennen (& Mauricio)	8461	W	KF447351	KF447289
S. frutescens L.	1973	Spain, Cadiz	H. Merxmüller & W. Gleißner	29073	M	KF447322	KF447259
S. glabrata Aiton	1988	La Palma, Fuencaliente; "pal"	M. Nydegger	25415	M	KF447360	KF447300
S. glabrata Aiton	2010	CULT., orig.: Tenerife, Monte del Cuchillo; "ten"	A. Scheunert	011/1-1	MSB	KF447361	KF447301
S. grandiflora DC.	2010	CULT., orig.: n/a (Bot. Gard. Erlangen 218/2007)	A. Scheunert	004/1-1	MSB	KF447348	KF447285
S. herminii Hoffmanns. & Link	2009	CULT., orig.: n/a (Bot. Gard. Madrid 262-80); "na"	A. Scheunert	016/1-1	MSB	KF447370	KF447278
S. herminii Hoffmanns. & Link	1987	Spain, Zamora; "za"		(MA 510365)	MA	KF447342	KF447279
S. hirta Lowe	2010	CULT., orig.: Madeira, n/a; "mad. (1)"	A. Scheunert	009/1-1	MSB	KF447364	KF447304
S. hirta Lowe	2010	CULT., orig.: Madeira, n/a; "mad. (2)"	A. Scheunert	013/1-1	MSB	KF447365	KF447305
S. hirta Lowe	2010	CULT., orig.: Madeira, Pico de Ruivo (M. Erben); "mad. (r)"	A. Scheunert	014/1-1	MSB	KF447363	KF447303
S. hispida Desf.	1989	Morocco, Tadla-Azilal	W. Lippert	25095	M	KF447353	KF447292
S. laxiflora Lange	1995	Spain, Cadiz, Algeciras; "alg"	M. Nydegger	33671	MSB	KF447337	KF447274
S. laxiflora Lange	1996	Spain, Cadiz, Los Barrios; "bar"	M.A. Carrasco, S. Castroviejo & M. Velayos	13801SC	MA	KF447336	KF447273
S. libanotica Boiss.	1957	Iraq, Sulaymaniyyah (Kurdistan)	K.H. Rechinger	10358	M	KF447329	KF447266
S. lowei Dalgaard	2010	CULT., orig.: Madeira, n/a (Bot. Gard. Madeira 61/2009)	A. Scheunert	007/1-1	MSB	KF447369	KF447309
S. lucida L.	1967	Greece, Attica	J. Grau		M	KF447319	KF447256
S. lyrata Willd.	1971	Crete, Chanía	G. & W. Sauer	12546	M	KF447350	KF447288
S. macrorrhyncha (Humbert, Litard. & Maire) Ibn Tattou	2006	Morocco, n/a	M. Staudinger	7444	W	KF447334	KF447271
S. ningpoensis Hemsl.	1991	CULT., orig.: n/a	J. Jutila	769	GH	HQ130041[3]	HQ130071[3]
S. nodosa L.	2003	Armenia, Lori	G. Fayvush, K. Tamanyan, H. Ter-Voskanian & E. Vitek	03-0549	MSB	HQ130038[3]	HQ130068[3]
S. nodosa L.	1999	Germany, Bavaria	D. Podlech	(MSB 116671)	MSB	HQ130037[3]	HQ130067[3]
S. oxyrhyncha Coincy	1995	Spain, Badajoz	J.L. Perez Chiscano	(MA 560760)	MA	KF447343	KF447280
S. peregrina L.	1987	Croatia, Dalmatia	E. & M. Mayer	12049	M	KF447317	KF447254
S. pyrenaica Benth.	1971	France, Pyrénées-Atlantiques	H. Merxmüller & B. Zollitsch	27178	M	KF447335	KF447272
S. racemosa Lowe	2011	CULT., orig.: Madeira, n/a (Bot. Gard. Madeira 47/2009)	A. Scheunert	006/1-1	MSB	KF447354	KF447293
S. reuteri Daveau	1974	CULT., orig.: Spain, Avila (J. Grau)	J. Grau	Sc-143	M	KF447344	KF447281
S. sambucifolia L.	1996	Spain, Cadiz	D. Podlech	54066	M	KF447352	KF447290
S. scopolii var. scopolii Hoppe ex Pers.	1967	CULT., orig.: Poland, Małopolskie (Bot. Gard. Wroclaw)	J. Grau	Sc-63	M	KF447330	KF447267
S. scopolii var. grandidentata	1965	Italy, L'Aquila	H. Merxmüller & J. Grau	20788	M	KF447331	KF447268

Table 1 (*continued*)

Taxon	Year	Locality (country, province/district)	Collector	Collector no.	Herb.	Acc. no. trnQ-rps16	Acc. no. ITS
(Ten.) Boiss.							
S. scorodonia L.	1986	Madeira, Funchal; "mad"	H. Hertel	33369	M	KF447338	KF447275
S. scorodonia L.	2011	CULT., orig.: Tenerife, Anaga mountains (M. Erben); "ten"	A. Scheunert	017/1-1	MSB	KF447339	KF447276
S. smithii ssp. smithii Hornem.	2010	CULT., orig.: Tenerife, Chamorga (M. Erben); "cha"	A. Scheunert	018/1-1	MSB	KF447355	KF447295
S. smithii ssp. smithii Hornem.	2010	CULT., orig.: Tenerife, n/a; "ten. (1)"	A. Scheunert	019/1-1	MSB	KF447310	KF447294
S. smithii ssp. smithii Hornem.	1971	Tenerife, Taganana; "tag"			M	KF447356	KF447296
S. smithii ssp. langeana (Bolle) Dalgaard	2010	CULT., orig.: Tenerife, Aguamansa; "ag"	A. Scheunert	008/1-1	MSB	KF447357	KF447297
S. smithii ssp. langeana (Bolle) Dalgaard	2007	Tenerife, Los Erjos; "erj"	W. Nezadal		M	KF447358	KF447298
S. smithii ssp. langeana (Bolle) Dalgaard	2010	CULT., orig.: Tenerife, Los Silos; "sil"	A. Scheunert	012/1-1	MSB	KF447359	KF447299
S. sublyrata Brot.	1986	Portugal, Estremadura	E. Bayón & R. Vogt	4564	M	KF447345	KF447282
S. syriaca Benth.	1992	Israel, Negev; "isr"	K. Tielbörger		M	KF447326	KF447263
S. syriaca Benth.	1980	Tunisia, Gafsa; "tun"	D. Podlech	34195	MSB	KF447324	KF447261
S. syriaca "hypericifolia Wydler"	1957	Iraq, Al Ramadi	K.H. Rechinger	9514	M	KF447327	KF447264
S. tanacetifolia Willd.	1983	Spain, Valencia	A. Aguilella & I. Mateu	15531	M	KF447340	KF447371
S. tenuipes Coss. & Durieu	1986	Algeria, Skikda	A. Dubuis, H. Maurel & R. Rhamoun	18440	M	KF447318	KF447255
S. trifoliata L.	1977	Corsica, Cap Corse	H. Merxmüller & W. Lippert	31405	M	KF447349	KF447286
S. umbrosa Dumort.	2003	Iran, Chaharmahal & Bakhtiyari	M.R. Parishani	14232	M	HQ130035[3]	HQ130065[3]
S. urticifolia Wall.		n/a; voucher specimen: LP0908740	JL	17	HU/HZU	KF447314	KF447251
S. valdesii Ortega Oliv. & Devesa	1982	Spain, Salamanca	J.L. Fernández Alonso	(MA 519560)	MA	KF447341	KF447277
S. vernalis ssp. clausii (Boiss. & Buhse) Grau	1974	Iran, Azerbaijan	W. Rechinger & J. Renz	49744	M	KF447316	KF447253
S. viciosoi Ortega Oliv. & Devesa	2002	Spain, Malaga, Antequera; "ant"	B. Cabezudo	(MA 789425)	MA	KF447346	KF447283
S. viciosoi Ortega Oliv. & Devesa	1973	Spain, Malaga, El Torcal; "tor"	H. Merxmüller & W. Gleißner	29144	M	KF447347	KF447284
S. xanthoglossa Boiss.	1992	Israel, Negev	K. Tielbörger		M	KF447328	KF447265
S. yoshimurae T.Yamaz.	1992	Taiwan, Nantou Hsien	C.-C. Liao	718	A	HQ130042[3]	HQ130072[3]

particular emphasis on Macaronesian taxa. Samples do not represent the whole distribution range of the respective species as this would have gone beyond the scope of this study; therefore, as no accessions from the Azores (*S. auriculata*) and the Cape Verdes (*S. arguta*) were available, biogeographic conclusions regarding Macaronesia were restricted to the Canary Islands and Madeira. Seven outgroup species were chosen from the Scrophulariaceae (*Verbascum nigrum* L., *Verbascum arcturus* L., *Selago corymbosa* L., *Hemimeris centrodes* Hiern, *Nemesia cheiranthus* E.Mey. ex Benth.) and Plantaginaceae (*Russelia verticillata* Kunth, *Antirrhinum majus* L.) based on results by Olmstead et al. (2001), Datson et al. (2008) and Scheunert and Heubl (2011). Information on voucher specimens as well as accession numbers is provided in Table 1. Chromosome numbers for sampled ingroup taxa were obtained from the database of Index to Plant Chromosome Numbers (IPCN; http://www.tropicos.org/Project/IPCN; last accessed on 10.05.2013) and from the literature, especially Grau (1976) and Ortega Olivencia and Devesa Alcaraz (1990).

Leaf material for DNA sequencing was obtained from herbarium specimens (55 accessions from collections in A, GH, HU/HZU, M, MA, MJG, MSB, nd W), and from plants cultivated in the greenhouses of the Botanical Garden in Munich (16 accessions, vouchers deposited in MSB; See Table 1). Seeds for cultivation were acquired from seed banks, economic providers or collected during field trips; to avoid confounding effects of uncontrolled hybridization in the greenhouses, plants were grown in isolation; furthermore, generally no F1 plants were sampled for this study. Specimens were checked for correct species identification whenever possible.

One non-coding chloroplast (cp) region (the *trnQ-rps16* intergenic spacer) and one nuclear ribosomal (nr) region (the internal transcribed spacer region, ITS) were chosen for phylogenetic analyses. Total genomic DNA was extracted from dried leaf material using the NucleoSpin Plant Kit (Macherey–Nagel, Düren, Germany) following the manufacturer's standard protocol, while applying an additional phenol/chloroform extraction step to remove proteins and potentially interfering secondary compounds. PCR reactions as well as subsequent purifications and sequencing reactions were performed according to the procedure described in Scheunert and Heubl (2011; no ExoSap purification), using the following primers: for ITS, primers ITS1 and ITS4 (White et al., 1990), supported by ITS2 and ITS3 (White et al., 1990), aITS1 and aITS4 (Bräuchler et al., 2004) and ITSIR (Scheunert and Heubl, 2011) in problematic cases; for *trnQ-rps16*, primers 1 (trnQ-F) and E (rps16-1R) (Calviño and Downie, 2007), and SPF, SPR, SPF2 and SPR2 (Scheunert and Heubl, 2011). Primers were used for amplification and sequencing, except for aITS1 and aITS4 (PCR only), and SPF2 and SPR2 (sequencing only). Markers were sequenced bidirectionally in cases where the quality of single sequences proved insufficient.

2.2. Phylogenetic analyses

Alignments were generated with MAFFT v.6 (Katoh and Toh, 2008; Katoh et al., 2002) using the slow iterative refinement FFT-nS-I algorithm, 1PAM/κ = 2 as scoring matrix, a gap opening penalty of 1.5 and an offset value of 0.0. All alignments were refined manually using BioEdit v.7.1.11 (Hall, 1999); mononucleotide repeats and ambiguously aligned regions were excluded from further analyses. ITS sequences were checked for potential pseudogenes (Bailey et al., 2003; Hershkovitz and Zimmer, 1996; Jobes and Thien, 1997; Liu and Schardl, 1994). In order to assess their phylogenetic information content before incorporating them into the

final dataset, nuclear and chloroplast indels were tentatively added or removed from the single marker phylogenetic analyses and the results compared. Ingroup indels were coded as binary states (discarding excluded alignment regions) using the simple indel coding method by Simmons and Ochoterena (2000) as implemented in SeqState v.1.4.1 (Müller, 2005).

The two markers were first analyzed separately using both a Bayesian inference (BI) and maximum likelihood (ML) approach. MrModelTest v.2.3 (Nylander, 2004) suggested the GTR + Γ substitution model as best fit to the data, with a proportion of invariant characters for the nuclear matrix only (Akaike information criterion). For Bayesian analyses including indel data, a mixed dataset was defined, using the model settings recommended in Ronquist et al. (2009) for the binary partition. Bayes runs were performed with MrBayes v.3.2 for 64 bit systems (Ronquist et al., 2012), using one cold and three heated Markov Chain Monte Carlo (MCMC) chains with temperature $t = 0.10$ for ITS and $t = 0.05$ for *trnQ-rps16*. For each of two independent runs per marker, 10×10^6 generations were completed, sampling every 2000th generation. The first 10% trees of each run were discarded as burn-in and the remaining 9002 trees used for computation of the majority-rule consensus tree.

ML analyses were performed with RAxML v.7.2.8 (Stamatakis et al., 2008) using raxmlGUI v.0.95 (Silvestro and Michalak, 2012). Pairs of accessions with completely identical sequences were represented by only one sequence in the analysis; the removed accession was then manually added to the final tree. A rapid bootstrap run with 10,000 replicates was followed by an ML optimization, defining *Antirrhinum* as outgroup and using the same models as in Bayesian analyses for DNA data partitions, and BINGAMMA for the binary indel partition. Each analysis yielded one fully resolved best-scoring ML tree.

2.3. Sequence divergence and statistical parsimony network analysis

To obtain more detailed information about clades lacking internal resolution in Bayesian and ML analyses, levels of nucleotide divergence among sequences (uncorrected ("*p*") distances and numbers of total character differences) were determined separately for each partition using the "pairwise distance" option in PAUP v.4.0b10 (Swofford, 2003). Calculations were performed based on the sequence data taken into account for Bayesian and ML analyses. In addition, the number of plastid haplotypes and their relationships were inferred for a subset of 36 accessions (corresponding to the 23 species of the "IPM" clade, definition see Section 3.2.) by generating a statistical parsimony network (Templeton et al., 1992), using the median joining algorithm with subsequent MP calculation as implemented in TCS v.1.21 (Clement et al., 2000). Generally, gaps were regarded as missing data, while coded indels used in Bayesian and ML analyses were added to the sequence matrix. The number of mutations among haplotypes was calculated with a maximum parsimony connection limit of 95% (=14 steps), using equal weights and setting epsilon to zero.

2.4. Identification and testing of incongruence

Patterns of phylogenetic incongruence were explored using several methods as suggested by Hipp et al. (2004). First, the phylogenies yielded from single marker analyses were visually examined and compared for incongruent placements of individual accessions or whole clades; congruence was rejected if support values for the contradictory placements exceeded or equalled 70% bootstrap support (BS) ("hard incongruence", Mason-Gamer and Kellogg, 1996), a cutoff which has successfully been used in several studies (e.g., Maureira-Butler et al., 2008; Moline et al., 2007; Scheunert et al., 2012). A Bayesian posterior probability (PP) of $\geqslant 0.95$ was additionally defined as sign of hard incongruence. The respective accessions or clades were then subjected to the Incongruence Length Difference (ILD) test (Farris et al., 1995) implemented in PAUP as Partition Homogeneity Test; accessions within incongruent clades were additionally tested alone. Applying an approach also used by van der Niet and Linder (2008), all sequences were first pruned from the dataset and then re-added and tested separately. Significant accessions were excluded; from those yielding insignificant results ($p > 0.05$), a combination of as many as possible was re-included into the dataset. All excluded accessions were then duplicated for the ancestral area reconstruction (see Section 2.5); these are referred to as "conflicting taxa/accessions" in the text. Tests were run with 1000 replicates, maxtrees set to 100 and heuristic searches with 50 random addition sequence replicates. Constant characters were removed from the data matrix prior to the test (following Cunningham, 1997; Lee, 2001).

As the ILD test has been shown to have certain weaknesses, e.g. a high false positive rate (Barker and Lutzoni, 2002; Darlu and Lecointre, 2002), all hard incongruence accessions were additionally subjected to Templeton's significantly less parsimonious test (SLP test; Templeton, 1983; "nonparametric pairwise test" in PAUP); ILD-significant accessions or clades were only allowed for exclusion/duplication if the SLP test was significant for at least one of the two datasets. Furthermore, in order to assess whether partly (i.e., in one of the markers) unresolved or weakly supported positions represented insufficient information or a distinct phylogenetic hypothesis, several of such groups/species (especially present in the chloroplast tree) were also tested. These included the Macaronesia clade (sister to the Auriculata clade in ITS only), the Asian species (well supported as sister to *S. scopolii* and the IPM clade in ITS only), and *S. grandiflora*, *S. trifoliata* and *S. tanacetifolia* within the IPM clade (part of the Auriculata/Scorodonia clade in ITS only). Constraint topologies were simplified from clades in the Bayesian consensus tree of the other marker; the clades are indicated by the respective constraint numbers in Fig. 1 (exact constraint topologies are provided in Supplementary Fig. S1). Searches were done with maxtrees set to 5000, 50 replicates with five trees held at each cycle of the stepwise addition procedure, and the number of trees retained in each random-addition sequence replicate limited to 100. Significance was set at <0.05 following Templeton (1983).

Finally, to illustrate incongruities between the individual gene trees (Holland et al., 2004), a tree-based filtered super network (FSN; algorithm by Huson et al., 2006) was constructed based on 1501 trees from the posterior distribution of the first run of each single marker Bayesian analysis. Calculations were performed with SplitsTree v.4.12.3 (Huson and Bryant, 2006), applying the equal angle splits transformation and the Convex Hull algorithm, with no filtering of selected splits. Construction of the FSN was done using default settings, with edge weights displayed as "tree size weighted mean", and with the minimum number of trees set to 751 (corresponding to a 25% trees threshold any split must be present in to be displayed in the FSN).

2.5. Ancestral area reconstruction

Recently developed models for ancestral state inference are able to account for the uncertainty present in phylogenetic reconstructions. This is particularly important in topologies with polytomies and weakly supported nodes (Ronquist, 2004). For biogeographic analyses in this study, we used the Bayesian Binary Markov Chain Monte Carlo (MCMC) algorithm as implemented in RASP v.2.0b (Yu et al., 2011), which takes trees from the posterior distribution of a Bayesian analysis as input and infers ancestral distributions using a full hierarchical Bayesian approach. Another essential requirement for obtaining reliable results in biogeographic reconstructions is that all available data are included; to enable simultaneous analy-

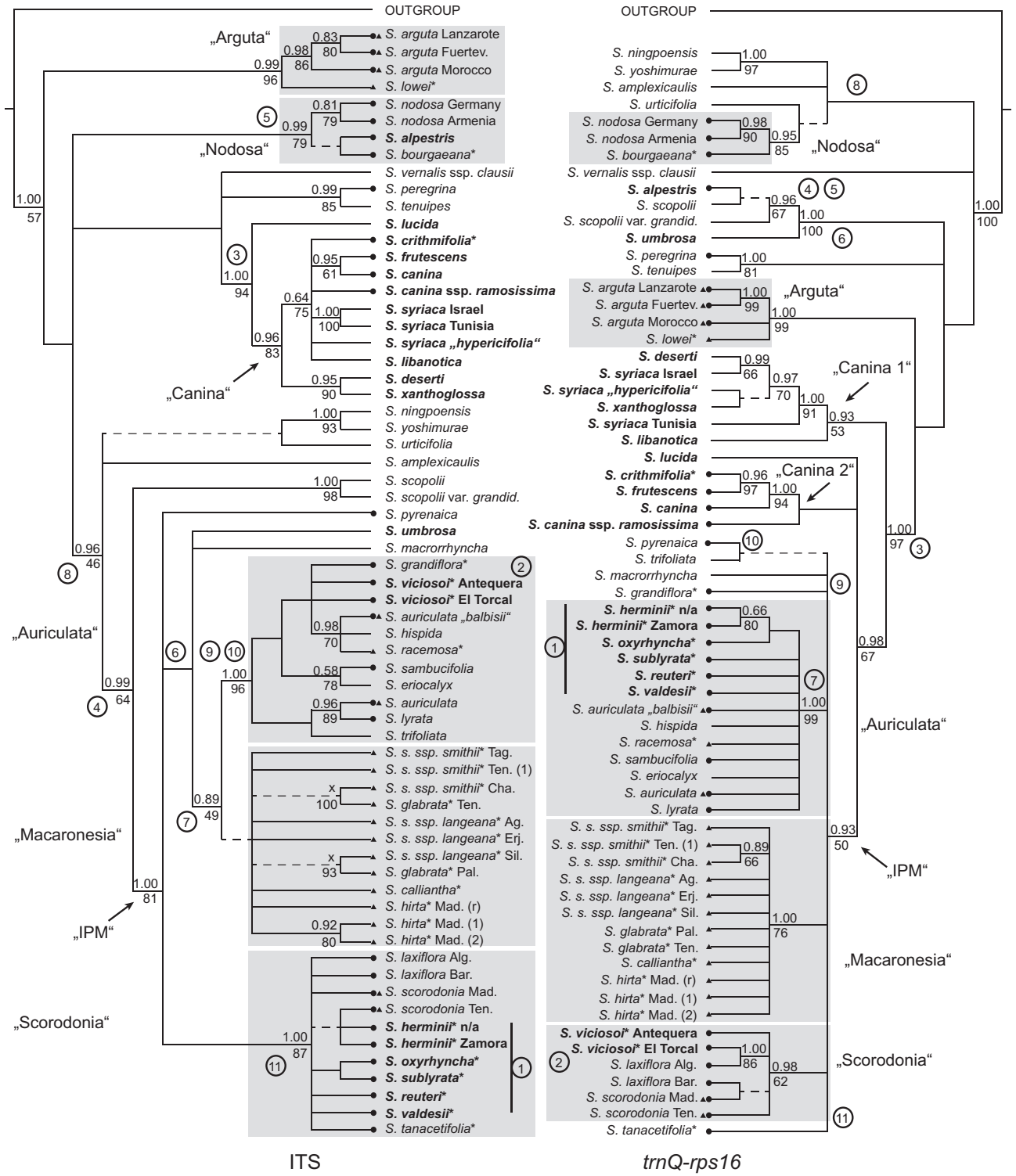

Fig. 1. Bayesian majority-rule consensus trees (cladograms) from the nuclear internal transcribed spacer (ITS) and the plastid *trnQ-rps16* intergenic spacer, with outgroup taxa reduced to a single branch. Posterior probabilities (PP) are given above each node; results from Maximum Likelihood (ML) analyses were plotted onto the cladograms, with bootstrap support values (BS) given below each node. Branches supported by at least 50% BS in the best-scoring ML trees but lacking in the Bayes consensus trees are represented by dashed lines. Both Bayesian and ML support values are given only if support at the node equals or exceeds either 0.85 PP or 75% BS ("*x*" denotes cases where the node was not present in the fully resolved tree of the respective analysis). Major clades are indicated by boxes, divergence of the IPM clade and the Canina clade is marked by arrows. Conflicting taxa (definition see Section 2.4.) are highlighted in bold. Solid dots (Iberian Peninsula) and triangles (Macaronesia) on terminal branches indicate the distribution of the respective (sub)species; endemic taxa are additionally marked by asterisks. Circled numbers refer to constraint topologies used in the SLP test. Fuertev., Fuerteventura; grandid., *grandidentata*; n/a, information not available; other abbreviations according to Table 1.

sis of all relevant data in a combined dataset containing several cases of hard incongruence and potential hybrid taxa, we constructed a combined *trnQ-rps16*/ITS data matrix following the "taxon duplication approach" by Pirie et al. (2009; 2008; also applied in e.g. Linder et al., 2013). All conflicting accessions (definition see Section 2.4.) were included twice, once as *trnQ-rps16*-only-sequence (with nuclear characters coded as missing), and once as ITS-only-sequence. The modified matrix which thus

comprised 93 sequences was used for a "duplicated analysis" using MrBayes with the same settings as for single marker analyses, except for the temperature being set to $t = 0.0001$ to permit conversion of the chains without having to enforce topological constraints. A ML analysis (using settings from the chloroplast marker calculations) was done for comparison purposes.

Distributions of species were assigned to 15 areas, subspecies were given their own respective distribution; to avoid erroneous inferences due to human influence, only native occurrences were taken into account. Single accessions from the same species were coded with the distribution of the respective species rather than their individual origin, to avoid mistakes caused by taxa not sampled across their whole distribution range. An exception is *S. auriculata "balbisii"* (see Table 1); as the distribution of *S. balbisii* Hornem. is difficult to infer (due to naming confusions and conspecificity with *S. auriculata* L. according to Ortega Olivencia, 2009), but cannot necessarily be assumed to be identical to that of *S. auriculata* L. given different phylogenetic positions in the ITS tree (Fig. 1), this accession was coded with its geographical origin. The chosen areas are mainly areas of endemism (here defined as a geographic region inhabited by two or more species displaying congruent distributions; Harold and Moor, 1994) based on present-day natural distributions of *Scrophularia* taxa; some of those were further subdivided according to palaeogeographic or climatic characteristics. In detail, areas were defined as follows: A, Azores; B, Canary Islands (including Cape Verde Islands); C, Madeira; D, Western Mediterranean (from Portugal to Italy including Sicily); E, Eastern Mediterranean (from Slovenia and Croatia to Crete and Cyprus including the Balkan Peninsula); F, Western North Africa (Morocco to Tunisia); G, Eastern Africa (Libya to Somalia); H, W-/N-/C-Europe (from the British Isles to Norway and Austria, excluding France); I, E-Europe and Western ("European") Russia (from Czech Republic eastwards, Baltic States, Russia as far as Ural and Pechora rivers); J, Lebanon/Syria/Israel s.l.; K, Southwestern Asia (Arabian Peninsula, Iran and Iraq); L, Turkey and the Caucasus (including the Talysh Mountains); M, Southern/Southeastern Asia (India to Myanmar, including Afghanistan and Pakistan); N, Central Asia and Siberia (Kazakhstan and southwards, Southern Siberia (Russia), northwards as far as species of the genus occur); O, Eastern Asia, Mongolia and "Russian Far East" (China to Japan and Taiwan, southeasternmost Russia from Sakhalin to the Zeya River).

The outgroup taxa, as well as the virtual outgroup, were assigned a wide distribution (i.e., occurring in all defined areas), which matches the real distribution of *Verbascum*, the closest relative of *Scrophularia*. Ancestral area distributions were estimated for ingroup nodes only. The maximum number of ancestral areas inferred at each node was constrained as recommended by Ronquist (1997): assuming that ancestral ranges were similar to those of present-day descendants (Sanmartín, 2003), "maxareas" was set to five as the majority of species (39 out of 45) now occur in no more than five areas. Five independent runs of the Bayesian Binary MCMC were conducted with one million generations each, sampling every 100th tree and discarding 1/5th of the trees as burn-in. State frequencies were estimated (F81 model) with a Dirichlet distribution of 1.0, and among-site rate variation was modeled across a gamma distribution as suggested by MrModeltest. An additional run with identical settings but maxareas set to two yielded congruent results.

3. Results

3.1. Sequence variation

Between *Scrophularia* and outgroup, *p* distances from chloroplast DNA sequences were smallest between *Verbascum nigrum* and *S. nodosa* L. from Germany (0.04074) and largest between *Hemimeris* and *S. hispida* Desf. (0.16982). In the nuclear dataset, values ranged from 0.05060 between *Verbascum nigrum* and *S. arguta* Sol. from Morocco, to 0.19403 between *Antirrhinum* and *S. nodosa* from Armenia. Among ingroup taxa, chloroplast and nuclear DNA distances varied from 0.00000 to 0.03515 in *trnQ-rps16* (between *S. vernalis* L. and *S. hispida*), and from 0.00000 to 0.05602 in ITS (between *S. syriaca* Benth. from Tunisia and *S. tanacetifolia* Willd.). Completely identical sequences in *trnQ-rps16* as well as ITS were found in several cases, especially within the Auriculata, Scorodonia and Macaronesia clades (definitions see Section 3.2). All uncorrected distances and total pairwise character differences are provided in Supplementary Table S1.

3.2. Phylogenetic analyses

ITS sequences showed no length changes in conserved parts indicative of pseudogenes, and G + C contents (see Table 2) were similar to those previously published for the genus (Scheunert and Heubl, 2011), so all sequences were regarded as derived from functional copies. Altogether, the sampling covers 72 accessions, of which new sequences were generated for 71 accessions; the ITS sequences of two outgroup taxa (*Antirrhinum*, *Selago*) were obtained from GenBank (NCBI). As sequencing of the *trnQ-rps16* intergenic spacer failed for *Russelia* and *Selago*, and no sequence was available in GenBank, the species were coded as missing for the respective marker. The aligned *trnQ-rps16* matrix thus consisted of 70 accessions and 1345 characters and the ITS matrix of 72 accessions and 617 characters. Detailed information on average lengths of sequences and further alignment characteristics including parsimony – informative characters is given in Table 2.

Thirty-four ingroup indels were coded for the trnQ-rps16 dataset and 17 for the ITS dataset. Analyzing the trnQ-rps16 dataset in MrBayes with and without indels showed that support values increased in 12 cases (decrease in six cases) when using indels, and that four new nodes were supported (results not shown). Using indels with the ITS dataset (results not shown) generally did not alter support values severely; however, disregarding them resulted in one additional ingroup node and three considerably increased support values. Consequently, indels were only coded for the trnQ-rps16 dataset in the final calculations.

MrBayes runs on the single marker datasets had reached convergence after 10,000,000 generations (standard deviation of split

Table 2
Sequence and alignment characteristics, and statistics from maximum likelihood (ML) analysis for *trnQ-rps16* intergenic spacer and ITS. Percentage of parsimony – informative characters referable to non-excluded characters; lengths and G + C content calculated based on the sequences as present in the alignment without any exclusions (aligned length). Sequence divergence values based on calculation of *p* distances (dissimilarity distances). alpha, the alpha value of the gamma shape parameter as inferred by ML calculations; SD, sequence divergence; avg., average; bp, basepairs; No., number.

	trnQ-rps16	ITS
No. of taxa (including outgroups)	70	72
Sequence length (avg.)	622–1126 bp (1022 bp)	506–587 bp (556 bp)
Aligned length	1345 bp	617 bp
Non-excluded characters	1306 bp	591 bp
Parsimony-informative characters	133 bp (10.18%)	129 bp (21.83%)
Average G + C content	26.65%	61.40%
Min – max SD Outgroup–Ingroup	4.07–16.98%	5.06–19.40%
Min – max SD Ingroup	0.00–3.52%	0.00–5.60%
ML tree score	−4512.522	−3554.120
ML tree length	0.626	2.145
Alpha	1.173	0.371

frequencies 0.004 and 0.003, respectively). Log-likelihood curves, acceptance rates, chain swap frequencies and potential scale reduction factors suggested effective mixing and stationarity of the chains. The majority-rule consensus trees for the single marker datasets are shown in Fig. 1. Information about likelihoods and tree lengths in ML analyses is given in Table 2; the alpha parameter was estimated at 1.173 for the chloroplast DNA partition and 17.947 for the chloroplast binary indel data partition, and at 0.371 for the nuclear dataset, while the proportion of invariant characters was 0.135. Results obtained by single marker ML analyses were consistent with those from Bayesian phylogenetic inference, so the Bayesian majority consensus trees are provided with both Bayesian posterior probabilities (above) as well as ML bootstrap supports (below) where either PP $\geqslant$ 0.85 or BS $\geqslant$ 75. Nodes supported in ML but absent in the majority-rule BI tree were added, applying a 50% BS threshold for displaying branches.

Large parts of the chloroplast and nuclear tree topologies are incongruent; however, there is support for a clade (cp PP: 0.93, BS: 50/nr PP: 1.00, BS: 81) containing the majority of the Iberian as well as the Macaronesian species (“Iberian Peninsula – Macaronesia” = “IPM” clade, Fig. 1). Within the IPM clade, three major subclades can be distinguished: one includes *S. scorodonia* L. (“Scorodonia” clade) and is highly to weakly supported by both analyses (cp PP: 0.98, BS: 62/nr PP: 1.00, BS: 87). Another comprises several species alongside *S. auriculata* (“Auriculata” clade) and receives high support (cp PP: 1.00, BS: 99/nr PP: 1.00, BS: 96). However, while these two clades as a whole are supported by cp and nr analyses, their composition is slightly different among the datasets: three taxa remain unresolved in *trnQ-rps16* (*S. tanacetifolia*, *S. grandiflora*, *S. trifoliata*) which are part of the Auriculata or Scorodonia clades in ITS. A third subclade forming a largely unresolved polytomy contains all but one of the Macaronesian perennial endemics (“Macaronesia” clade; does not contain the Madeiran *S. racemosa* Lowe) and is sufficiently supported by the chloroplast tree only (PP: 1.00, BS: 76). Relationships within as well as among the Scorodonia, Auriculata and Macaronesia clades are only poorly resolved; the Auriculata clade is subtended by an exceptionally long branch in both analyses, while the Scorodonia clade features a long branch in ITS only (see Supplementary Fig. S2). Altogether, the IPM clade comprises 68% of all Iberian/Macaronesian species and 13 of the 16 species endemic to the two regions. Apart from the IPM clade, two smaller clades were identified by both analyses: a “Nodosa” clade containing the two accessions of the holarctic *S. nodosa* as well as the Iberian endemic *S. bourgaeana* Lange (cp PP: 0.95, BS: 85/nr PP: 0.99, BS: 79), and an “Arguta” clade consisting of the three accessions of the mainly Northern African and Southwestern Asian *S. arguta* and the Madeiran endemic *S. lowei* Dalgaard (cp PP: 1.00, BS: 99/nr PP: 0.99, BS: 96). From the species included with more than one accession, only *S. nodosa* is revealed as monophyletic in both analyses (cp PP: 0.98, BS: 90/nr PP: 0.81, BS: 79).

3.3. Phylogenetic Incongruence

While several major clades identified in the nuclear phylogeny are also present in the cp tree topology, large degrees of incongruence are indicated between the two markers, on the level of single accessions as well as whole clades regarding their composition and relationships. Focusing only on cases of well-supported, hard incongruence ($\geqslant$70% BS/$\geqslant$0.95 PP as defined above), this is true for instance for the species of the “Canina group” (*S. canina* L. and other species from subsection *Lucidae* Stiefelhagen) and *S. lucida* L.: while in the chloroplast phylogeny they are part of two separate clades with moderate support (Fig. 1, cp tree, “Canina 1”, PP: 0.93, BS: 53; “Canina 2”, PP: 1.00, BS: 94) and *S. lucida* remains unresolved, they are merged into one well supported clade in the nuclear tree (Fig. 1, nr tree, “Canina”, PP: 0.96, BS: 83), with *S. lucida* highly supported as sister, and with no trace of subclades corresponding to the clades of the cp phylogeny. Another example is the IPM clade, which is sister to *S. scopolii* Hoppe ex Pers. in the ITS phylogeny (Fig. 1, nr tree; PP: 0.99, BS: 64), but to the Canina 2 clade and *S. lucida* in *trnQ-rps16* (Fig. 1, cp tree; PP: 0.98, BS: 67).

Within the clades, considerable amounts of hard incongruence can be found as well (see Table 3 for detailed information on support values): regarding the Canina group, *S. frutescens* L. is sister to *S. crithmifolia* Boiss. in the cp tree, but sister to *S. canina* in the nr tree; *S. deserti* Delile and *S. syriaca* from Tunisia occupy incongruent positions as well. The Scorodonia and Auriculata clades display incongruence “vice versa” regarding six of their species: *Scrophularia valdesii* Ortega Oliv. & Devesa, *S. herminii* Hoffmanns. & Link, *S. oxyrhyncha* Coincy, *S. reuteri* Daveau and *S. sublyrata* Brot. (referred to as “S/A taxa”) are part of the Scorodonia clade in ITS, but belong to the Auriculata clade in *trnQ-rps16*. The same is true for the two accessions of *S. viciosoi* Ortega Oliv. & Devesa (“A/S taxon”), but in the opposite way, as they are part of the Auriculata clade in ITS and the Scorodonia clade in *trnQ-rps16*. Single taxa with hard incongruent placements also include *S. umbrosa* Dumort. (part of the IPM clade in ITS, sister to *S. scopolii* and *S. alpestris* J.Gay ex Benth. in *trnQ-rps16*), *S. alpestris* (part of the Nodosa clade in ITS, sister to *S. scopolii* in *trnQ-rps16*), and *S. scopolii* and *S. scopolii* var. *grandidentata* (Ten.) Boiss. (sister to the IPM clade in ITS, sister to *S. alpestris* in *trnQ-rps16*).

As expected, the ILD test revealed severe incongruence within the complete dataset ($p = 0.001$); the test with all incongruent clades and single accessions mentioned above removed (51 accessions altogether) resulted in $p = 0.445$. However, when those accessions of the IPM clade which are congruent within the clade were re-included (28 accessions), the result remained insignificant

Table 3
Bayesian posterior probabilities (PP), ML bootstrap support values (BS) and ILD test results for four groups and four single species displaying hard incongruence among the chloroplast (cp) and nuclear (nr) markers. Accessions/clades were added to a pruned, congruent matrix ($p = 0.144$; see Sections 2.4. and 3.3.); accessions of *S. scopolii* were only tested together. Asterisks indicate significance at the $p = 0.05$ level, accessions with insignificant results are shown in bold. No separate support values are given for accessions unresolved or weakly resolved within their respective clade/group. Explanation of “S/A taxa” and “A/S taxon” see Section 3.3.; hyp., *hypericifolia*; ramos., ssp. *ramosissima*, other abbreviations according to Table 1.

Group/accession	cp PP/BS	nr PP/BS	*P* value
S/A taxa	1.00/99	1.00/87	0.001*
S. sublyrata	–	–	0.003*
S. reuteri	–	–	0.001*
S. oxyrhyncha	–	–	0.001*
S. valdesii	–	–	0.004*
S. herminii (za)	0.66/80	–	0.001*
S. herminii (na)	0.66/80	–	0.002*
A/S taxon	0.98/62	1.00/96	0.027*
S. viciosoi (tor)	1.00/86	–	0.028*
S. viciosoi (ant)	–	–	0.027*
Canina 1	1.00/97	0.96/83	0.008*
S. libanotica	–	0.64/75	0.040*
S. deserti	0.99/66	0.95/90	**0.073**
S. xanthoglossa	0.97/70	0.95/90	0.025*
S. syriaca “hyp.”	0.97/70	0.64/75	0.019*
S. syriaca (isr)	0.99/66	1.00/100	**0.070**
S. syriaca (tun)	1.00/91	1.00/100	0.008*
Canina 2	0.98/67	0.96/83	0.018*
S. canina ramos.	–	0.64/75	0.037*
S. canina	1.00/94	0.95/61	0.033*
S. frutescens	0.96/97	0.95/61	0.009*
S. crithmifolia	0.96/97	0.64/75	0.025*
S. scopolii (x2)	0.96/67	0.99/64	**0.135**
S. alpestris	0.96/67	0.99/79	**0.051**
S. umbrosa	1.00/100	1.00/81	**0.063**
S. lucida	0.98/67	1.00/94	**0.134**

($p = 0.144$), suggesting that the incongruence observed regarding the IPM clade did concern its sister groups (*S. scopolii* and the Canina group with *S. lucida*, respectively) rather than the clade itself. Therefore, further tests were conducted using this "congruent dataset" ($p = 0.144$) with only the remaining 23 hard incongruence accessions removed. Results for all clades and accessions tentatively re-included into the dataset are shown in Table 3. Seven accessions did not render the dataset incongruent when re-added; of these, all possible pairs were tested to find suitable combinations (results not shown). The final "reduced dataset" was chosen to comprise all taxa from the congruent dataset plus both accessions of *S. scopolii*, thereby including as much data as possible while keeping congruence of the dataset as large as possible ($p = 0.135$). The remaining 21 excluded accessions (=conflicting taxa; see Table 3) represent app. 1/3 of the ingroup. The results of the SLP test are shown in Table 4. Unlike ITS, the *trnQ-rps16* dataset rejected most of the foreign topologies, in particular regarding all hard incongruence taxa but also several partly unresolved/weakly supported positions, which suggests that the latter contain explicit information and are not the mere outcome of insufficient data.

Within the FSN constructed to visualize conflicting signals between and within markers (Fig. 2A), clades are represented in a tree-like way where single marker trees are fully congruent (e.g., Arguta and Nodosa clades). However, the structure of most groups is highly networked, and their relationships among each other are entangled. The Scorodonia and Auriculata clades within the IPM clade are connected by bundles of parallel edges (which indicate different signals within the data) representing the incongruent positions of the S/A and A/S taxa switching between clades in *trnQ-rps16* and ITS. The Macaronesia clade is situated in between both clades, while the IPM clade itself is closely connected to *S. umbrosa* (Fig. 2A, "5"). *Scrophularia scopolii* ("3") represents a connection between the IPM clade and the remainder of the sampling. When the taxa within the FSN are diminished to those of the reduced dataset as defined above (Fig. 2B), the number of parallel edges decreases substantially, leaving only few reticulations. Thus, a combined analysis with conflicting taxa duplicated can be assumed to produce largely reliable results not hampered by major incongruence issues.

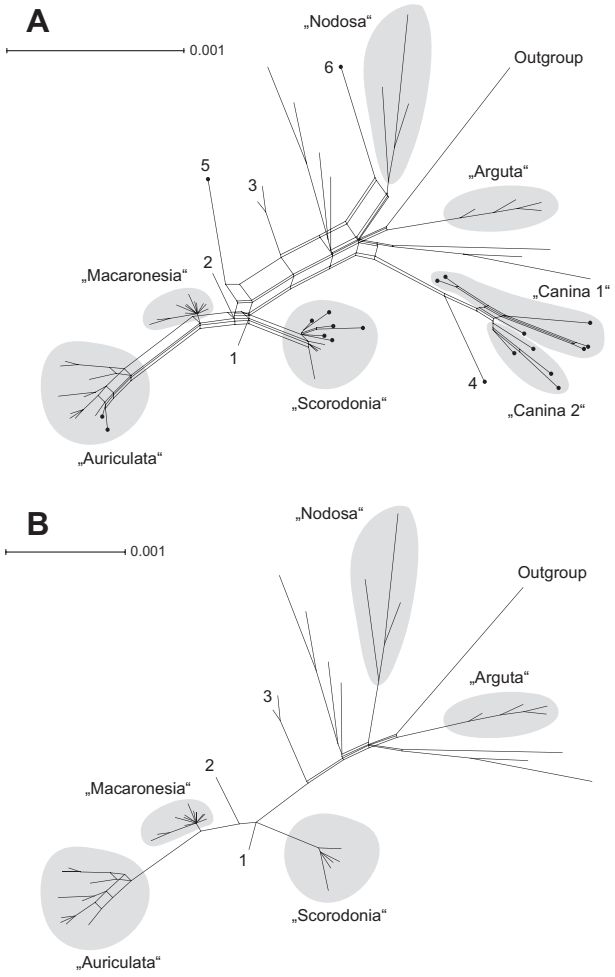

Fig. 2. Filtered super networks (split networks), based on each 1501 trees from the posterior distribution from two single marker Bayesian analyses (chloroplast *trnQ-rps16*, nuclear ITS) which yielded the consensus trees shown in Fig. 1. Scale bars represent treesize weighted mean edge weights. Major clades according to Fig. 1 are indicated in grey. Composition of the network based on (A) all 72 accessions included in the study (with seven outgroup taxa reduced to a single edge in the graphics), and (B) 21 conflicting accessions (explanation see Section 2.4.), marked by black dots on the edge tips in (A), removed using the "exclude selected taxa" option. 1, *S. pyrenaica*; 2, *S. macrorrhyncha*; 3, *S. scopolii* and *S. scopolii* var. *grandidentata*; 4, *S. lucida*; 5, *S. umbrosa*; 6, *S. alpestris*.

3.4. Plastid haplotype inference

The haplotype network analysis revealed 18 distinct haplotypes, connected by 20 missing intermediate (unsampled) haplotypes, and with no more than seven inferred mutational changes to connect sampled haplotypes. Six haplotypes are shared among up to eight accessions, and among one to five species, respectively.

Table 4
Results of SLP tests for hard incongruence taxa and partly unresolved groups/species (see Section 2.4.). Tests on chloroplast (cp, left side) and nuclear (nr, right side) dataset using 11 constrained topologies as provided in Supplementary Fig. S1. Length of most parsimonious tree(s), number of trees saved during heuristic search and minimum/maximum SLP test *P* values are given for each marker and constraint. Asterisks indicate significance at the $p = 0.05$ level, ° marks cases where only one value was significant. Constraints regarding the phylogenetic position of: 1, five "S/A taxa", explanation see Section 3.3.; 2, one "A/S taxon"; 3, the Canina/Canina 1/Canina 2 clades and *S. lucida*; 4, *S. scopolii*; 5, *S. alpestris*; 6, *S. umbrosa*; 7, the Auriculata and Macaronesia clades; 8, *S. ningpoensis*, *S. yoshimurae*, *S. urticifolia* and *S. amplexicaulis*; 9, *S. grandiflora*; 10, *S. trifoliata*; 11, *S. tanacetifolia*. No., number.

Constraint	Length (cp)	No. trees	*P* value (nr)	Length (nr)	No. trees	*P* value (cp)
None	499	5000	–	544	3800	–
1	509	5000	0.0016/0.0184*	557	3400	0.0003/0.0046*
2	509	5000	0.0016/0.0184*	557	3800	0.0003/0.0073*
3	500	5000	0.3173/0.7389	559	2800	0.0001/0.0011*
4	519	5000	<0.0001/0.0002*	549	4400	0.0956/0.2818
5	511	5000	0.0005/0.0047*	548	3800	0.1573/0.3785
6	518	5000	<0.0001/0.0003*	551	3900	0.0348/0.1488
7	507	5000	0.0047/0.0455°	546	3500	0.1573/0.5637
8	509	5000	0.0075/0.0124*	550	5000	0.1533/0.3692
9	507	5000	0.0047/0.0455°	547	3600	0.0833/0.4054
10	508	5000	0.0027/0.0290*	547	3600	0.0833/0.4054
11	501	4800	0.1573/0.5271	545	4100	0.3173/0.7630

In one case, haplotypes are shared among present-day allopatric species. Of the eight species represented by more than one accession, five feature two different haplotypes. The three groups in the network correspond to the Scorodonia, Auriculata and Macaronesia clades in the chloroplast tree (Fig. 1). Groups are separated by at least four mutational changes, while haplotypes within groups differ by two mutations at most. The "biggest outgroup probability" according to TCS, i.e., the most likely ancestral haplotype was found in eight accessions/four species of the Macaronesia clade.

3.5. Biogeographic reconstruction

The two runs from the Bayesian analysis of the duplicated dataset had converged after 10,000,000 generations (standard deviation of split frequencies 0.006). The best-scoring ML tree (results not shown) largely corroborated the Bayesian majority-rule consensus. The latter mostly reproduced the relationships of the single marker analyses, however, with reduced resolution and lower support values. Results from the RASP analyses as plotted onto the Bayesian consensus are shown in Fig. 4. Exact marginal probabilities for each ancestral range and Bayesian posterior probabilities for tree nodes are available from Supplementary Table S2.

The different RASP runs mostly yielded congruent results as well; one node (node 17) with almost identical marginal probabilities for two ancestral distributions, shifted in the different runs between including or excluding Western North Africa from the most frequent distribution. Inferred ancestral ranges had low marginal probabilities in some, especially more basal nodes; these results should therefore be treated with caution.

4. Discussion

4.1. Reticulate evolution within Scrophularia

The Nodosa, Arguta and IPM clades exclusively consist of species from section *Anastomosantes* subsection *Scorodoniae* sensu Stiefelhagen (= section *Scrophularia* subsection *Scrophularia*). Species of section *Tomiophyllum* subsection *Lucidae* sensu Stiefelhagen (or section *Canina* sensu G.Don) are restricted to the Canina clade. This is in accordance with Navarro-Pérez et al. (2013) who found a clade of semi-shrubby, sparsely foliate plants of section *Canina* embedded within section *Scrophularia* comprising mostly herbaceous representatives with numerous and often large leaves featuring clearly anastomosing nerves.

The species of the IPM clade (Fig. 1) as presented here can be characterized as subshrubs or perennial (biennial) herbs, with undivided to 3-pinnatisect, lanceolate to suborbicular leaves. A scarious margin, more or less distinct, is always present on the calyx lobes. The corolla is usually indistinctly bicolored, with the posterior part showing predominantly purple or brownish tones, while the anterior is greenish, brown, yellowish or reddish in color. The staminode is suborbicular, obovate, reniform, or transversely elliptical in shape and of considerable variability. The globose, ovoid, or subconical capsule is often apiculate, or the base of the style is persisting as mucro on mature capsules. However, none of these morphological characters can be regarded as synapomorphic for the clade. Similarly, the monophyly of the IPM clade (with or without *S. umbrosa* depending on the marker) is supported by only two nucleotide synapomorphies in the ITS sequence alignment and one in *trnQ-rps16*. In constrast, the Auriculata and Scorodonia clades mostly receive high supports in phylogenetic analyses, but also lack clear morphological synapomorphies. This is best explained by the comparatively young age of the IPM clade (inferred at around 3.7my by Navarro-Pérez et al., 2013) and is also reflected in the weakly resolved relationships among and within the IPM clade, as well as low levels of sequence divergence with several cases of identical sequences (see Supplementary Table S1).

As a consequence, especially regarding the high frequency of hybridization and polyploidy within the group, reticulate events among the closely related species studied here are to be expected, and the large amount of incongruence found between trees from nuclear and plastid markers corroborates this assumption (see Fig. 2). Similar examples within the Lamiales are known from e.g. Plantaginaceae (Albach and Chase, 2004; Blanco-Pastor et al., 2012) or Lamiaceae (Bräuchler et al., 2010), among others. Reticulation can be due to e.g. hybridization (Arnold, 1997; Rieseberg et al., 1996), introgression (Mason-Gamer, 2004; Rieseberg and Wendel, 1993) or incomplete lineage sorting (persistence of ancient polymorphism/deep coalescence; Degnan and Rosenberg, 2009; Maddison, 1997), all of which have frequently been considered as explanation for cases of well-supported topological incongruence among phylogenetic trees (hybridization: e.g. Albaladejo et al., 2005; Doyle et al., 2003; Nieto Feliner et al., 2002; see further references in Vriesendorp and Bakker, 2005; lineage sorting: e.g. Jakob and Blattner, 2006; Vilatersana et al., 2010). However, artificial factors can cause topological incongruence as well (Wendel and Doyle, 1998); such factors were excluded as far as possible in the present study. Sampling error was avoided by re-determination of nearly all accessions; species coverage is close to 100% for the main study regions, avoiding insufficient taxon sampling (Stockley et al., 2005). Methodical mistakes caused by model misspecification were ruled out by performing all analyses with two different methods. Furthermore, only robustly supported cases of incongruence were taken into account. Undetected ITS pseudogenes can also introduce topological incongruence into datasets (Álvarez and Wendel, 2003); however, no signs of sampled non-functional nrDNA copies were found in the sequences. Some sister groups with long branches in the basal (and sparsely sampled) part of the trees (see Supplementary Fig. S2) could possibly be the result of long branch attraction (Felsenstein, 1978), so no implications are made regarding these taxa.

In cases where incongruence reflects some kind of reticulate evolutionary history, forcing conflicting signals into one phylogenetic tree might blur real relationships (Bull et al., 1993; Lecointre and Deleporte, 2005); on the other hand, pruning hard incongruent taxa from a combined analysis (Huelsenbeck et al., 1996; Johnson and Soltis, 1998) will disregard much of the available data, and valuable information about possible parent species will be lost in cases where hybridization is frequent (Rieseberg and Brunsfield, 1992; see examples in Albaladejo et al., 2005; Fehrer et al., 2007; Okuyama et al., 2005; Soltis and Kuzoff, 1995). The duplication approach (Pirie et al., 2009, 2008) as used in this study provides a suitable solution to this problem.

In the case of *Scrophularia* as sampled here, the ITS phylogeny generally seems to better reflect the relationships known from morphological studies (e.g. Bentham, 1846; Ortega Olivencia, 2009; Stiefelhagen, 1910). Single accessions of the same species are more often retrieved as monophyletic (*S. scopolii*, *S. arguta*, *S. nodosa*). It has already been recognized that in many cases, the ITS topology is congruent with phylogenetic hypotheses established from morphological or biogeographical data (Baldwin et al., 1995; Fehrer et al., 2007; Kellogg et al., 1996). However, species – independent geographical structuring as described by Wolf et al. (1997) is hardly if at all present in the *trnQ-rps16* tree. Therefore, we chose to refer to the ITS topology (or the duplicated topology, Fig. 4) when inferring species relationships, and to draw additional information from the chloroplast tree where useful.

Although detailed differentiation between evolutionary mechanisms lies beyond the scope of this study, parts of the reticulation observed here are very likely to be the result of hybridization. As an example, six species within the IPM clade display hard

incongruence, changing positions between the Auriculata and the Scorodonia clades in ITS/*trnQ-rps16* (S/A and A/S taxa). Results from Navarro-Pérez et al. (2013) match this observation in at least two cases; three of the other species remain unresolved in the plastid tree, which is probably due to the choice of a different marker (*trnL-trnF*) providing weaker resolution. In contrast, the incongruence regarding the sixth of the species, *S. herminii*, as found here clearly contradicts results by Navarro-Pérez et al. (2013); unless more specimens are analyzed, we refrain from making any conclusions regarding this species here. The evolutionary split between the Auriculata and Scorodonia clades has been shown to be rather young (mid-Pliocene; Navarro-Pérez et al., 2013); consequently, lineage sorting is a possible explanation for the observed pattern (Maddison, 1997). On the other hand, if incongruent placements in the ITS tree resulted from sorting of ancestral polymorphisms in the IPM clade ancestor, one would expect, from the stochastic nature of the process, that accessions appeared at any position in the IPM clade (Buckley et al., 2006). Here, the species change positions between two clades exclusively. Furthermore, both clades are highly supported and well separated (Figs. 1 and 3, also see Navarro-Pérez et al., 2013) which makes lineage sorting a less likely cause for the incongruence (Morgan et al., 2009). Introgression by geographically close populations of the other clade cannot be excluded due to the lack of populational sampling; however, the "Sambucifolia" haplotype (Fig. 3) encloses species from very different geographic regions, which precludes introgression as the only cause for haplotype sharing and suggests common ancestry of the respective species (Gutiérrez Larena et al., 2002). On the other hand, viable hybrids between *S. scorodonia* and *S. auriculata* have already been created by Grau (1976) and Dalgaard (1979), and the natural hybrid *S.* × *moniziana* Menezes was shown to be derived from *S. scorodonia* and *S. racemosa* (belonging to the Auriculata clade; Fig. 1). Therefore, it seems more likely that *S. sublyrata*, *S. reuteri*, *S. oxyrhyncha*, *S. valdesii* and *S. viciosoi* have originated through homoploid hybrid speciation (Mallet, 2007), with ancestors or members of the Scorodonia clade being the female parent in *S. viciosoi*, and ancestors/members of the Auriculata clade acting as female donor in the remaining cases, contributing the maternally inherited plastid.

Morphologically, *S. sublyrata* shares characters with *S. sambucifolia* (Richardson, 1972), and *S. reuteri* is similar to *S. sambucifolia* (Daveau, 1892) and to some extent also *S. sublyrata* (Grau, 1976). This corresponds to the identical haplotype found in the three species (Fig. 3). Morphological similarities are also present between *S. oxyrhyncha* and *S. sublyrata* (Coincy, 1898; Stiefelhagen, 1910). Furthermore, *S. oxyrhyncha* is connected to *S. reuteri* by a distinctive long - subconical capsule (Grau, 1976); both are local endemics of Western Spain. *Scrophularia valdesii* is a threatened narrow endemic known from only 14 populations occurring in the Duero Basin in Spain and Portugal (Bernardos et al., 2006). It shares the haplotype found in *S. sambucifolia*, *S. sublyrata* and *S. reuteri* (Fig. 3) and is closely related to the latter morphologically (Ortega Olivencia and Devesa Alcaraz, 1991). *Scrophularia viciosoi* is the only hybrid with its paternal source found in (ancestors of) the Auriculata clade. Ortega Olivencia and Devesa Alcaraz (1991) relate the species to *S. grandiflora*, a local endemic of the Coimbra region in Portugal; indeed, the ITS sequence of *S. viciosoi* from Antequera is identical to that of *S. grandiflora* (Supplementary Table S1). Morphological similarities include the densely pubescent – glandular, pinnatisect leaves possessing many small intercalars, and the subsessile peduncles (Ortega Olivencia and Devesa Alcaraz, 1991).

4.2. Chromosome number evolution, origins of polyploidy and ancestral hybridization within Iberian Scrophularia

Hybridization and polyploidization are considered as driving forces in the diversification history of the genus *Scrophularia*, e.g. in the high level polyploids occurring in North America (Carlbom, 1969; Scheunert and Heubl, 2011; Shaw, 1962); they also play an important role in plant evolution and speciation in general (Hegarty and Hiscock, 2005; Leitch and Leitch, 2008; Otto and Whitton, 2000; Schubert, 2007). Besides the evidence for homoploid hybrid speciation as discussed above, our phylogenetic reconstructions support allopolyploid hybridization in several cases.

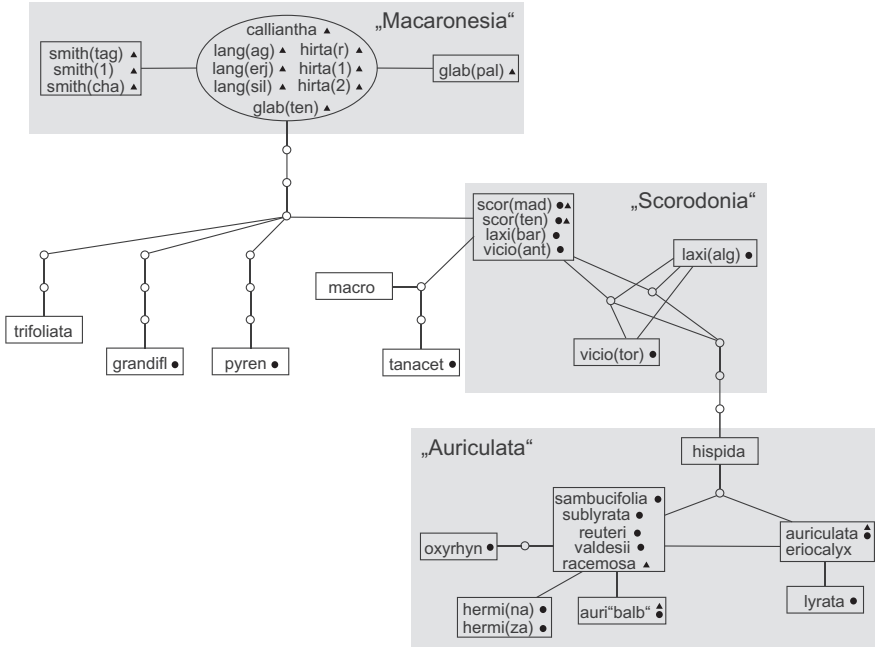

Fig. 3. Statistical parsimony network obtained from analysis of the *trnQ-rps16* intergenic spacer, limited to taxa from the IPM clade. Lines represent single mutational steps, small circles represent inferred haplotypes. The size of boxes is relative to the number of accessions possessing the respective haplotype; the oval represents the most likely ancestral haplotype as inferred by TCS. Major plastid lineages indicated by grey boxes correspond to clades in Fig. 1. Solid dots (Iberian Peninsula) and triangles (Macaronesia) indicate the distribution of the respective (sub)species. For complete taxon names and other abbreviations see Table 1.

Scrophularia alpestris, distributed in montaneous regions of Southern France and Northern Spain, with a chromosome number of $2n = 68$ (Grau, 1976), is sister to *S. scopolii* ($2n = 26$; Grau, 1976) in the *trnQ-rps16* phylogeny and, according to ML estimations, sister to *S. bourgaeana* ($2n = 42$; Ortega Olivencia and Devesa Alcaraz, 1990) in the ITS topology. Regarding the long branches of *S. alpestris* and *S. bourgaeana* in ITS, their sister relationship could theoretically be a result of long branch attraction (Supplementary Fig. S2). However, *S. alpestris* was already proposed to be an allopolyploid (with *S. scopolii* and *S. bourgaeana* as progenitors) by Grau (1976) and Ortega Olivencia and Devesa Alcaraz (1990). This hypothesis is corroborated by morphology and by molecular phylogenetic reconstructions as presented here.

A similar case seems to be apparent in *S. auriculata* which typically possesses $2n = 84$ (Grau, 1976) chromosomes. Grau (1979) suggested the species to result from allopolyploid hybridization between (ancestors of) *S. lyrata* Willd. ($2n = 58$; Grau, 1976) and *S. umbrosa* ($2n = 26, 52$; Vaarama and Hiirsalmi, 1967). This is supported by intermediate morphological traits connecting *S. auriculata* to its putative parents; e.g., bracts and bracteoles in *S. lyrata* are scariously margined across their whole length as opposed to the non-margined *S. umbrosa*, while the bracts of *S. auriculata* have no or narrow margins generally confined to the tip of the leaf. Furthermore, the staminode is reniform to bilobed in *S. umbrosa*, obovate to suborbicular in *S. lyrata*, and subreniform in *S. auriculata*.

A close relationship of *S. auriculata* to *S. lyrata* is evident from the ITS phylogeny in one of the two accessions only (Fig. 1); connections to *S. umbrosa* are present in neither plastid nor nuclear trees. Possibly, this unexpected result is due to fixation of the maternal ITS copy in the hybrid species through concerted evolution (Álvarez and Wendel, 2003), an event that would leave no trace of a hybrid origin as long as no additional markers are included (see e.g. Blöch et al., 2009; Joly et al., 2006; Sang et al., 1997).

The second accession, *S. auriculata "balbisii"* (originally determined as *S. balbisii* Hornem, a name synonymized with *S. auriculata* L. ssp. *auriculata* by Ortega Olivencia, 2009), does not cluster with the first specimen, but instead is sister to the Algerian - Moroccan endemic *S. hispida* (Fig. 1). This species is morphologically similar to *S. lyrata* and has the same chromosome number ($2n = 58$; Grau, 1976). With respect to the considerable variability found within *S. auriculata* (visible in e.g. *S. auriculata* ssp. *valentina* with lyrate-pinnatisect leaves, as well as several synonyms listed in Ortega Olivencia, 2009), and the great potential for hybridization, a definite conclusion about the phylogenetic position of *S. auriculata* does not seem advisable based on two inconsistently placed specimens. However, an involvement of *S. hispida* in the origin of *S. auriculata* should be considered, especially with regard to the third taxon in the respective ITS clade, the Madeiran endemic *S. racemosa*. This species has been related to *S. auriculata* and also possesses $2n = 84$ chromosomes (Dalgaard, 1979); its position is clearly separated from the remaining Macaronesian perennial endemics which are part of the Macaronesia clade.

Disregarding hybrid species with higher chromosome numbers (*S. auriculata*, *S. racemosa*) as described above, the IPM clade is characterized by the derived chromosome number $2n = 58$ (with aneuploidy in some taxa, e.g. *S. sublyrata*, *S. glabrata* Aiton) as already pointed out by Grau (1976). The number seems to be exclusive for the species of the IPM clade; this points toward a single evolutionary event, which generated a presumably allopolyploid ancestor with that particular chromosome number. Some suggestions have been made regarding its origin, but no concrete evidence was provided: Ortega Olivencia and Devesa Alcaraz (1990) hypothesized an allopolyploid taxon with $2n = 60$ derived from progenitors with $2n = 36$ (as present in *S. nodosa* and *S. peregrina*; Grau, 1976; Vaarama and Hiirsalmi, 1967) and $2n = 24$ (as present in *S. crithmifolia* and occasionally found in *S. canina*; Ortega Olivencia and Devesa Alcaraz, 1990), with subsequent chromosome number reduction to $2n = 58$. Based on the sister relationships as present in the nuclear and plastid phylogeny, our molecular data support an allopolyploid origin, by ancient hybridization involving an ancestor from the Canina group (possibly with $2n = 30$ chromosomes as occasionally found in *S. canina*; Ortega Olivencia and Devesa Alcaraz, 1990) as maternal parent. Interestingly, almost all species of the Canina group themselves yield highly significant ILD test results (Table 3), and their cp and nr sequences occupy different positions in the duplicated tree (Fig. 4; Canina clade vs. Canina 1 and 2 clades). This could be explained by assuming reticulation present in their origin as well, possibly involving groups not sampled for this study. Regarding the paternal source, a contribution by a taxon with $2n = 36$ chromosomes as proposed by Ortega Olivencia and Devesa Alcaraz (1990) is not supported; instead, an *S. umbrosa* – like species ($2n = 26$) is suggested by the duplicated tree (Fig. 4), as *S. umbrosa* is placed in a polytomy with the Canina clade and *S. lucida* with all being sister to the IPM clade. *Scrophularia umbrosa* was already mentioned as potentially involved in the origin of the group; it does not occur in the Iberian Peninsula today (westernmost populations reach Norway, the British Isles, and France), but could easily have done so in the past according to Grau (1976). A natural hybrid between *S. auriculata* and *S. umbrosa* (sub *S. alata* Gilib.) was already noted by Stiefelhagen (1910); this confirms the close association of the species. Surprisingly, when consulting the ITS tree, *S. scopolii* is sister to the IPM clade and *S. umbrosa* is nested within the latter. However, the position of *S. umbrosa* is characterized by a long branch in the phylogram (Supplementary Fig. S2); the substitutions shared with the rest of the IPM clade species could thus be homoplasious. The low alpha value of the gamma shape parameter in the ITS dataset (Table 2) supports this view. If we finally consider the fact that the chromosome number of *S. umbrosa* with $2n = 26$ would be unique within the IPM clade, we can conclude that this position as a member of, and not sister to, the IPM clade is likely artificial.

If we assume an *S. canina* – like ($2n = 30$) and an *S. umbrosa* – like species ($2n = 26$) as progenitors for the IPM clade ancestor (Fig. 4), and that subsequent chromosome doubling was necessary to enable fertility of the new hybrid, the resulting allopolyploid should have had $2n = 56$ chromosomes. Ascending aneuploidy could then have resulted in the present-day $2n = 58$ for the group; the latter process was also proposed to account for the deviant chromosome number of *S. viciosoi*, counted with $2n = 58$ as well as $2n = 64$ (Grau, 1976, sub *S. sublyrata*; Ortega Olivencia and Devesa Alcaraz, 1990).

4.3. Biogeographic implications

The primary diversity center of the genus *Scrophularia* is assumed in the Irano - Turanian region (Grau, 1981; Lall and Mill, 1978). Most of the species studied here are part of a secondary center of diversity located in the Iberian Peninsula (Ortega Olivencia, 2009; Ortega Olivencia and Devesa Alcaraz, 1990). According to ancestral area reconstructions as performed by RASP, the most recent common ancestor (MRCA) of the IPM clade was distributed in the Western Mediterranean, however with low Bayesian support for the underlying node (Fig. 4, node 31; for node supports and exact frequencies of occurrence see Supplementary Table S2). Regarding the progenitors of the IPM clade as discussed above, RASP inferred that the ancestor of the Canina group was distributed in a region ranging from Eastern Africa, Israel, Lebanon and Syria to Southwestern Asia, the Caucasus and Turkey (node 18, 27). However, the marginal probabilities for the inferred range are low (42.19, 43.23); a reconstruction with possible areas at each node restricted to two, narrows the most frequent ancestral range

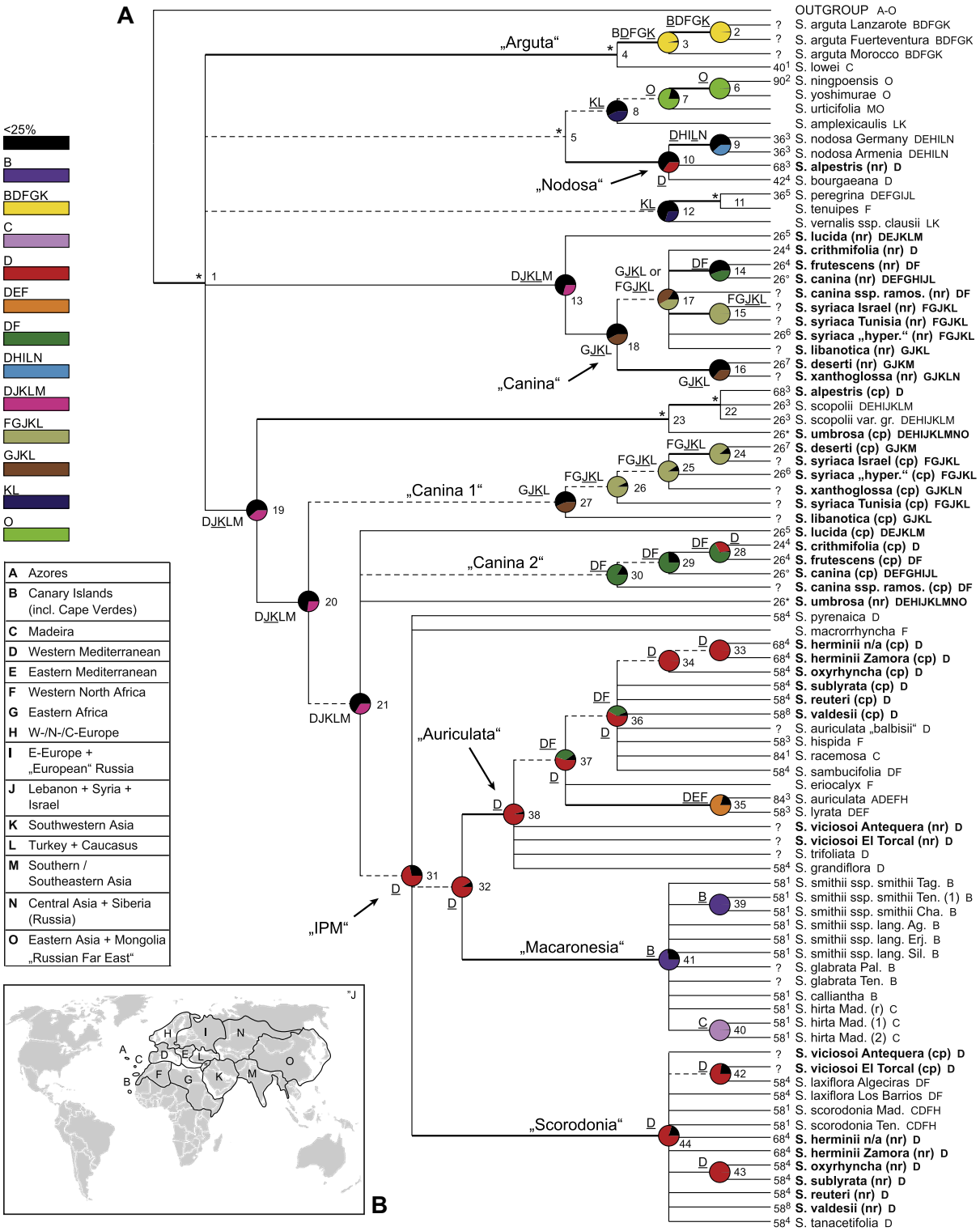

Fig. 4. (A) Biogeographical optimization as performed by RASP (maxareas = 5), using the majority-rule consensus of 9002 trees from a duplicated Bayesian analysis of the ITS region and the plastid *trnQ-rps16* intergenic spacer. Conflicting taxa duplicated for the analysis are highlighted in bold and suffixed with "nr" and "cp", for the nuclear and chloroplast sequences, respectively (see Section 2.5.). Branches with PP < 0.85 in the consensus tree are shown by dashed lines, branches with PP ⩾ 0.95 in bold. Outgroups were reduced to one branch in the diagram, clade names correspond to those in Fig. 1. Pie charts illustrate inferred distributions of MRCAs from one of five RASP runs (only ranges supported by all five runs are shown). Color – coded fractions represent the frequency of occurrence/marginal probability (⩾25) of the respective ancestral distribution over the Bayesian sample of trees. Asterisks mark nodes where no ancestral distribution reached a marginal probability of ⩾25 in all five runs. Areas additionally supported by an optimization with maxareas set to two are underlined. Contemporary distribution and known chromosome numbers are denoted next to each taxon, "?" indicate cases where counts yielded inconsistent results; *: known autopolyploidy (2n = 26, 52; Vaarama and Hiirsalmi, 1967) within *S. umbrosa*. °: apart from 2n = 26, occasionally counted numbers in *S. canina* also include 2*n* = 24 and 2*n* = 30 (Ortega Olivencia and Devesa Alcaraz, 1990; Vaarama and Leikas, 1970). [1]: Dalgaard, 1979; [2]: Ge and Li, 1989; [3]: Grau, 1976; [4]: Ortega Olivencia and Devesa Alcaraz, 1990; [5]: Vaarama and Hiirsalmi, 1967; [6]: Murin and Sheikh, 1971; [7]: Mohamed, 1997; [8]: Ortega Olivencia and Devesa Alcaraz, 1991. ramos., *ramosissima*; hyper., *hypericifolia*; gr., *grandidentata*; lang., *langeana*; other abbreviations according to Table 1. (B) Table and map showing 15 areas defined for ancestral area reconstructions, and color codes for inferred ancestral ranges.

to Israel, Lebanon, Syria and Southwestern Asia with a higher frequency of occurrence (85.96, 58.58). According to the reconstructions, east–west migrations would then have expanded the distribution range of the group to Western North Africa (nodes 14, 15, 17, and 24-26). The ancestral range inferred for the Canina 2 clade (chloroplast; node 30, marginal probability: 83.59) suggests that the ancestor of this part of the Canina group (here represented by *S. canina*, *S. frutescens* and *S. crithmifolia*) at some point reached the Iberian Peninsula via the Strait of Gibraltar and diversified in situ. This is supported by the ancestral areas inferred for nodes 14, 28 and 29 with mostly sufficient marginal probabilities and Bayesian node supports. Similar biogeographical patterns involving expansion from east to west have been recorded in several Mediterranean groups, e.g. in elements of the Spanish steppe flora (Polunin and Smithies, 1973), in Asteraceae (Font et al., 2009), Araceae (Mansion et al., 2008), Rutaceae (Salvo et al., 2011), and in insects (Sanmartín, 2003).

For *S. umbrosa*, the second assumed progenitor of the IPM clade, biogeographic reconstructions were ambiguous and marginal probabilities insufficient; hypotheses about the biogeography of this widespread species must remain speculative at this point. In every case, the contact of an *S. umbrosa* ancestor with a taxon from the Canina group in the Iberian Peninsula should have resulted in the hybridization event generating the allopolyploid ancestor of the IPM clade.

In the course of the diversification of the IPM clade, an early dispersal of *S. macrorrhyncha* (Humbert, Litard. & Maire) Ibn Tattou into Northern Africa is suggested by its position within the clade. This subshrub species is adapted to semi-arid conditions and is endemic to Morocco today. From the three main lineages derived from the MRCA, one dispersed to Macaronesia (Macaronesia clade, see Section 4.4. and Fig. 4, node 41); the Scorodonia clade underwent local radiation and mostly remained restricted to the Iberian Peninsula (Fig. 4, nodes 42–44). In contrast to that, RASP reconstructions show that Northern Africa might have played a larger role in the diversification of the Auriculata lineage (nodes 35, 36, and 37). Both Scorodonia and Auriculata clades also contain more widespread elements which have dispersed into Macaronesia, the Eastern Mediterranean, and Europe (*S. scorodonia*, *S. auriculata*).

Diversification of *Scrophularia* in the Western Mediterranean and especially the Iberian Peninsula as mentioned above is likely to be, to a great extent, the result of repeated hybridization as discussed in Sections 4.1 and 4.2. In addition, there is evidence that glacial refugia also played a role in promoting and preserving species diversity. Five taxa of the IPM clade remain unresolved in the chloroplast tree (Fig. 1; *S. pyrenaica* Benth., *S. macrorrhyncha*, *S. grandiflora*, *S. trifoliata*, *S. tanacetifolia*); their positions were shown to contain distinct phylogenetic signal in two of three tested cases (Table 4) and correspond to haplotypes which are isolated from the remainder of the IPM clade by up to seven steps (Fig. 3). In the duplicated tree (Fig. 4), these taxa are unresolved as well (*S. pyrenaica*, *S. macrorrhyncha*), are sister to the remainder of the Auriculata clade (*S. grandiflora*, *S. trifoliata*) or part of the Scorodonia clade (*S. tanacetifolia*). All except the latter species are restricted to only small areas, and exclusively or predominantly inhabit regions classified as refugia within the Mediterranean bioclimatic region (Médail and Diadema, 2009): the central Pre-Pyrenees and Pyrenees (*S. pyrenaica*; Ortega Olivencia, 2009), Sardinia and Corsica (*S. trifoliata*; Gamisans and Marzocchi, 1996), Beira Litoral of Western Portugal (*S. grandiflora*; Ortega Olivencia, 2009), and the High, Middle and Anti Atlas mountains of Morocco (*S. macrorrhyncha*; Ibn Tattou, 2007). Given their distinctive genetic features and their occurrence in refugia, these four species are likely to represent more ancient lineages within the IPM clade which persisted in the favorable conditions of the climatically stable refugial areas. Long isolation in restricted regions likely accumulated the genotypic changes and accounts for the long branches in the phylograms (Supplementary Fig. S2). *Scrophularia tanacetifolia* does not have a restricted distribution, but is more widespread in the eastern and southeastern parts of the Iberian Peninsula. Unlike in *S. grandiflora* and *S. trifoliata*, the SLP test was insignificant for this species (Table 4), suggesting that its position in the chloroplast tree might also be due to a lack of informative characters. However, it is sister to a clade containing *S. laxiflora* and *S. scorodonia* in the combined analysis by Navarro-Pérez et al. (2013), which corroborates its isolated position. It is also subtended by a long branch in the *trnQ-rps16* phylogram (Supplementary Fig. S2), and, like *S. macrorrhyncha*, is characterized by 2–3 pinnatisect leaves resembling those of the Canina group (confusions with *S. crithmifolia* were reported by Ortega Olivencia, 2009), a rather plesiomorphic character within the IPM clade. Possibly, this species dispersed to its present distribution area in Southeastern Spain from refugia located in the area.

Scrophularia species are distributed throughout nearly all regions of the Iberian Peninsula today, occurring from sea level up to 2500 m. Hybrid species from the Scorodonia and Auriculata clades (*S. sublyrata*, *S. reuteri*, *S. oxyrhyncha*, *S. valdesii* and *S. viciosoi*) are confined to granite or siliceous substrates; their distribution corresponds to a biogeographical pattern as shown by Moreno Saiz et al. (2013), which divides the Peninsula into two distinct distributional areas characterized by different soil conditions. Whether substrate characteristics influenced hybridization in this area remains unclear; but furthermore, the present-day distributions of these species likely reflect the influence of the varied topography in the region, also in the context of the climatic conditions during their formation. Divergence time estimations by Navarro-Pérez et al. (2013) suggest their very recent divergence in the Pleistocene; this is corroborated by identical haplotypes in three of five cases (Fig. 3) and very low levels of nuclear sequence divergence among each other and to closely related non-hybrid species (Supplementary Table S1) as shown here. Possible parental taxa and hybrid offspring are allopatrically distributed within the Peninsula in several cases; thus, it is conceivable that range shifts in the parental lineages, promoted by climate fluctuations during the Pleistocene (Hewitt, 2000), enabled hybridization in contact zones. The narrow distributions of three of the hybrids, (including one threatened species; Bernardos et al., 2006) indicate that geographic features might also have had special influence, by isolating new species e.g. on the sierras of the Cordillera Central (*S. reuteri*) and the Sierra Morena (*S. oxyrhyncha*). Isolation is regarded essential for survival of homoploid hybrids (Rieseberg and Willis, 2007) as it prevents backcrossing with the already established lineages. Likewise, the role of the sierras of Central Spain as refugia for plant and animal species has been highlighted (Crochet et al., 2004; Médail and Diadema, 2009). Isolation and range shifting in glacial refugia have promoted speciation in several genera, amongst others *Erodium* (Geraniaceae; Fiz-Palacios et al., 2010) and *Armeria* (Plumbaginaceae; Gutiérrez Larena et al., 2002).

4.4. The origin of the Macaronesian taxa

Although our sampling of the Macaronesian taxa does not allow detailed inferences on colonization pathways among the islands, some general patterns are clearly supported by the present data (Fig. 5). According to phylogenetic reconstructions, at least four distinct lineages of *Scrophularia* have colonized the Macaronesian archipelago; these roughly correspond to the three groups defined by Dalgaard (1979). Multiple independent introductions into Macaronesia have also been shown in e.g. *Asteriscus* (Asteraceae; Goertzen et al., 2002), *Ilex* (Aquifoliaceae; Cuénoud et al., 2000), *Lavatera* (Malvaceae; Fuertes-Aguilar et al., 2002), or *Plantago* (Plantaginaceae; Rønsted et al., 2002), and have been reviewed in Carine et al. (2004). Regarding *Scrophularia*, Madeira was colonized

at least four times (by members of the Scorodonia, Auriculata, Macaronesia and Arguta clades, see Figs. 4 and 5). At least two dispersals to the Canary Islands can be assumed (by members of the Arguta clade and the ancestor of the Macaronesia clade). In particular, the perennial endemics of the Canary Islands (*S. smithii* Hornem., *S. glabrata*, *S. calliantha*) are shown to be the result of a dispersal event from the Western Mediterranean mainland to the Atlantic islands (Fig. 4, node 32, marginal probability: 91.50). The dated phylogeny by Navarro-Pérez et al. (2013) places the split between mainland taxa of the Scorodonia clade and Macaronesian perennial endemics in the late Pliocene. Indeed, many species and lineages of the Macaronesian islands have been shown to be recently derived from continental ancestors, rather than being relictual elements of the flora (Barber et al., 2002; Carine et al., 2004, see also studies reviewed therein; Francisco-Ortega et al., 1997; Helfgott et al., 2000); this also seems to be the case for *Scrophularia*. The ancestors of the Madeiran perennial endemic *S. hirta* were inferred to have originated on the Canary Islands (Fig. 4, node 41, marginal probability: 71.76). Colonization routes from the Canary Islands to Madeira were also found in e.g. *Sonchus* (Asteraceae; Lee et al., 2005), *Bystropogon* (Lamiaceae; Trusty et al., 2005) or *Micromeria* (Lamiaceae; Meimberg et al., 2006). A second colonization event, from the Western Mediterranean or possibly also Western North Africa (Fig. 5), was inferred for the other perennial endemic on Madeira, *S. racemosa* (Fig. 4, node 36, marginal probabilities: 58.20 and 35.06, respectively). Two of the species occurring in Macaronesia are more widespread (*S. scorodonia, S. auriculata*); therefore, their biogeography should be examined using more specimens from different parts of their distribution range, and no conclusions are made here. The annual taxa occurring in Macaronesia today are part of a clade whose closest relatives remain unclear; accordingly, no informative ancestral distributions could be inferred.

4.5. Conclusions and perspectives

This study provides an initial framework in understanding the complex evolutionary history of *Scrophularia* lineages from the Western Mediterranean, Northern Africa and Macaronesia. Interspecific hybridization and polyploidization have significantly influenced the diversification of the genus in this area and explain the major incongruences found between nuclear and chloroplast datasets. Hybrid speciation is favored by the pollination biology of the genus and the absence of reproductive barriers among closely related taxa. The comparatively young age of the lineages might explain the lack of resolution among Macaronesian and Iberian groups as well as single species (on the other hand, haplotypes are not necessarily identical within species). This also indicates that apart from hybridization, other reticulate processes like introgression and lineage sorting may have occurred among and within species. The sampling and methods employed in the present study are not intended for assessing these topics or for disclosing inter-island colonization patterns across the Atlantic archipelagoes in detail. A different approach involving an extensive geographic and intraspecific population-level sampling and appropriate markers (e.g. SSR, ISSR, AFLP etc.), together with additional chromosome counts, would help to further unravel the evolutionary history of the genus.

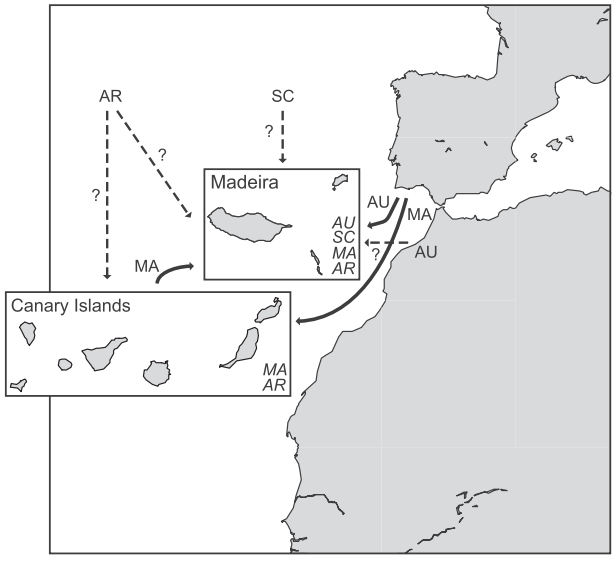

Fig. 5. Colonization of the Canary Islands and Madeira by different lineages of *Scrophularia*. Solid arrows represent dispersal events as inferred by the ancestral area reconstruction in Fig. 4 further possible or unknown migration routes are indicated by dashed arrows (see Section 4.4.). Abbreviations of *Scrophularia* lineages, corresponding to clade names in Fig. 1: AU, Auriculata; SC, Scorodonia; MA, Macaronesia; AR, Arguta. Present distributions for each archipelago are indicated in italics.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.09.023.

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4.4. Article IV

Against all odds: reconstructing the evolutionary history of *Scrophularia* (Scrophulariaceae) despite high levels of incongruence and reticulate evolution.

by Agnes Scheunert & Günther Heubl

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Against all odds: reconstructing the evolutionary history of *Scrophularia* (Scrophulariaceae) despite high levels of incongruence and reticulate evolution

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Abstract The figwort genus *Scrophularia* (Scrophulariaceae), widespread across the temperate zone of the Northern Hemisphere, comprises about 250 species and is a taxonomically challenging lineage displaying large morphological and chromosomal diversity. *Scrophularia* has never been examined in a large-scale phylogenetic and biogeographic context and represents a useful model for studying evolutionary history in the context of reticulation. A comprehensively sampled phylogeny of *Scrophularia* was constructed, based on nuclear ribosomal (ITS) and plastid DNA sequences (*trnQ-rps16* intergenic spacer, *trnL-trnF* region) of 147 species, using Bayesian inference and maximum likelihood approaches. Selected individuals were cloned. A combination of coding plastid indels and ITS intra-individual site polymorphisms, and applying Neighbor-Net and consensus network methods for adequate examination of within-dataset uncertainty as well as among-dataset incongruence, was used to disentangle phylogenetic relationships. Furthermore, divergence time estimation and ancestral area reconstruction were performed to infer the biogeographic history of the genus. The analyses reveal significant plastid-nuclear marker incongruence and considerable amounts of intra-individual nucleotide polymorphism in the ITS dataset. This is due to a combination of processes including reticulation and incomplete lineage sorting, possibly complicated by inter-array heterogeneity and pseudogenization in ITS in the presence of incomplete concerted evolution. Divergence time estimates indicate that *Scrophularia* originated during the Miocene in Southwestern Asia, its primary center of diversity. From there, the genus spread to Eastern Asia, the New World, Europe, Northern Africa, and other regions. Hybridization and polyploidy played a key role in the diversification history of *Scrophularia*, which was shaped by allopatric speciation in mountainous habitats during different climatic periods.

Keywords *Scrophularia* · Incongruence · Reticulate evolution · Intra-individual polymorphism · 2ISP · Allopatric speciation

Introduction

In recent years, an increasing number of phylogenetic studies in plants, based on molecular sequence information from numerous independent loci, have revealed discordance among chloroplast and nuclear gene trees as well as gene trees in general. Although methodological issues in data collection or analysis might be responsible for some of these observations, incongruence may also be due to conflicting genealogical histories (e.g., Rokas et al. 2003; van der Niet and Linder 2008). These can be caused by gene duplications or losses, or by incomplete lineage sorting (ILS; Maddison 1997; Degnan and Rosenberg 2009). Furthermore, processes involving reticulation, i.e., gene flow among species, have been identified, e.g., horizontal gene transfer, introgression, and homo- or polyploid hybridization. These phenomena are common in plants (Rieseberg and Wendel 1993; Rieseberg et al. 1996; Wendel and Doyle 1998; Mason-Gamer 2004; Richardson and Palmer 2007); reticulation is now even regarded as a

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major driving force in the evolution and diversification of plant lineages (Ellstrand and Schierenbeck 2000; Seehausen 2004; Soltis and Soltis 2009), as is polyploidization (Leitch and Bennett 1997; Abbott et al. 2013).

Researchers have aimed to trace reticulations within the evolutionary history of plants by examining incongruences among gene trees, often a combination of uniparentally (usually plastid) and biparentally (nuclear) inherited molecular markers (e.g., Rieseberg 1991; Baumel et al. 2002; Okuyama et al. 2005; Marhold and Lihová 2006; Fehrer et al. 2007; Scheunert and Heubl 2011, 2014). However, it has to be noted that the information content of phylogenetic reconstructions can be severely impeded in the presence of hybridization. Additionally, ILS/deep coalescences lead to incongruent patterns identical to those from hybridization and introgression. The use of nuclear ribosomal internal transcribed spacer (ITS) sequence information, although most popular due to its considerable advantages (Baldwin et al. 1995), can possibly mislead phylogenetic inference (reviewed by Álvarez and Wendel 2003; Nieto Feliner and Rosselló 2007). The arrangement of the 35S rDNA genes in tandem arrays at (sometimes several) separate ribosomal loci results in thousands of repeats per genome. Consequently, any ITS sequence will contain the summarized signal from many, often non-orthologous, not necessarily identical units. This leads to intra-individual site polymorphism, which can have a variety of origins (see "Discussion" section). Although these variabilities in rDNA units are often homogenized by the process of concerted evolution (Brown et al. 1972; Arnheim et al. 1980; Nei and Rooney 2005), several cases are known where it is slowed down, incomplete or non-operational (Grimm et al. 2007; Rosselló et al. 2007; Grimm and Denk 2008; Xiao et al. 2010; Hodač et al. 2014). All of the phenomena described above can have substantial impact on phylogenetic reconstruction (Álvarez and Wendel 2003; Linder and Rieseberg 2004; Beiko et al. 2008; Degnan and Rosenberg 2009), challenging traditional approaches which aim to depict evolutionary history and its underlying events using dichotomously branching phylogenetic trees. In such cases, the incorporation of network construction methods (e.g., Huson and Bryant 2006) into the analyses is a useful alternative.

The genus *Scrophularia* L., 1753 (Lamiales: Scrophulariaceae) represents a useful model for studying the influence of reticulation on evolutionary history and how it affects the inference of the latter. The genus consists of about 250 species, with estimates ranging from about 200 species in Mabberley (1997) and Fischer (2004), to more than 300 in Willis (1973). Plants are mostly herbaceous perennials, less often suffrutescent perennials or subshrubs and, more rarely, biennial or annual herbs. They are characterized by quadrangular, sometimes winged stems and considerably variable, undivided to 3-pinnatisect, generally opposite leaves. The inflorescence typically is a thyrse or a raceme with chasmogamous flowers. The five lobes of the calyx frequently possess a scarious margin, the often brownish, purplish or greenish corolla is sympetalous, bilabiate, and generally tubular or ventricose in shape. Apart from the four fertile stamens, the fifth (adaxial) stamen, if not completely absent, is generally sterile and forms a scale-like staminode of various shapes. The fruit is a septicidal, globose to subconical capsule containing numerous small seeds.

The most recent taxonomic treatment by Stiefelhagen (1910) divided the genus into two sections, *Scrophularia* sect. *Anastomosantes* Stiefelh. (= *S.* sect. *Scrophularia*) and *Scrophularia* sect. *Tomiophyllum* Benth. (= *S.* sect. *Canina* G.Don). *Scrophularia* extends throughout the temperate zone of the Northern Hemisphere (Asia, Europe and Northern America), with very few species expanding into tropical regions (e.g., the Greater Antilles). Based on floristic studies, species are concentrated in the Irano-Turanian floristic region sensu Takhtajan (1986), with particularly high species diversity found in Iran and Turkey (42 and 59 species according to Lall and Mill 1978; Grau 1981; Davis et al. 1988), but a large number also accounted for in the Flora of the USSR (Gorschkova 1997). Secondary centers of species richness are located in the Himalayan region and China (more than 36 species; Hong et al. 1998), as well as the Iberian Peninsula and adjacent areas including Macaronesia (28 species; Dalgaard 1979; Ortega Olivencia 2009). Representatives of the genus mainly inhabit highland plateaus and mountainous regions (all centers of species diversity comprise mountainous regions) but also coastal and lowland areas. Many species prefer shady and/or moist habitats, while others are xerophytic (especially within *S.* sect. *Tomiophyllum*) and can tolerate drier conditions; true desert plants are however rare. Most importantly, the genus is characterized by frequent natural hybridization, expressed in a variety of polyploid species and also linked to great morphological plasticity (Stiefelhagen 1910; Grau 1981). Natural hybrids in *Scrophularia* have been reported or even described as species by Menezes (1903, 1908), Stiefelhagen (1910), Pennell (1943) or Grau (1981). Artificial crossings were successful according to Goddijn and Goethart (1913), Shaw (1962), Carlbom (1964), Grau (1976), and Dalgaard (1979), and several cases of homoploid and allopolyploid speciation have been reported by Scheunert and Heubl (2011, 2014), who also found evidence for substantial tree incongruence.

Until now, phylogenetic relationships were mainly addressed on a restricted geographical scale, regarding e.g., species of the New World (Scheunert and Heubl 2011), Iran (Attar et al. 2011), and the Mediterranean and Macaronesia (Scheunert and Heubl 2014). Based on a time-calibrated phylogeny, Navarro Pérez et al. (2013) recently found support for monophyly of the genus and its divergence in the Miocene. However, a robust phylogenetic framework comprising all important lineages has been missing to date.

Here, we use sequences from the nuclear ribosomal ITS region and two plastid DNA regions (the *trnQ-rps16* intergenic spacer and the *trnL-trnF* region) to infer phylogenetic relationships. We aim to test the extent to which it is possible to reconstruct evolutionary history in the face of reticulation and incomplete lineage sorting without resorting to cloning or whole genome analyses. The main objectives of our study are (1) to establish a comprehensive evolutionary framework for *Scrophularia* based on a broad taxon sampling; (2) to assess the amount of intra-individual polymorphisms in ITS sequence data and to explore their possible causes; (3) to identify inconsistencies between nuclear and plastid DNA phylogenies and to examine their relation to reticulate evolution; (4) to reconstruct the biogeographic history of *Scrophularia* and to reveal which processes account for its current distribution patterns and species diversity.

Materials and methods

A broad range of methods has been applied in the present work; these are generally outlined below. Additionally, as the intent of this study is also to provide a workflow for researchers dealing with similarly complex groups, the information provided here is complemented by detailed descriptions including settings and procedures, available from Online Resource 1.

Taxon sampling

The taxon sampling is the most comprehensive presented so far in a molecular study on the genus and comprises 147 of the approximately 250 extant *Scrophularia* species. Sampled taxa include representatives from throughout the distribution area and cover all proposed sections and subsections. Known hybrid taxa were only exceptionally included to avoid unnecessary introduction of further conflicts into the dataset. Five widespread or morphologically diverse species (*S. vernalis* L., 1753; *S. scopolii* Hoppe ex Pers., 1806; *S. heterophylla* Willd., 1800; *S. canina* L., 1753; *S. variegata* M.Bieb., 1798) were sampled with additional subspecies and/or varieties. To investigate intraspecific variability, five species were included with up to four representatives (*S. auriculata* L., 1753; *S. lyrata* Willd., 1805; *S. arguta* Sol., 1789; *S. nodosa* L., 1753; *S. olympica* Boiss., 1844). Altogether, the *Scrophularia* ingroup consisted of 162 accessions. Based on previously established relationships, 18 taxa, from the Scrophulariaceae (represented by five species) and other families within Lamiales (Calceolariaceae, Gesneriaceae, Plantaginaceae, Stilbaceae, Bignoniaceae, Verbenaceae), were selected as outgroups (Kornhall et al. 2001; Albach et al. 2005; Oxelman et al. 2005; Nie et al. 2006; Datson et al. 2008; Schäferhoff et al. 2010). In addition, the sampling included one species from *Oreosolen* Hook.f., 1884 (Scrophulariaceae), a genus which comprises one to four species endemic to the Himalayas and the Tibetan Plateau and was found to be most closely related to *Scrophularia* (Albach et al. 2005; Oxelman et al. 2005). Complete information on voucher specimens is provided in Online Resource 2 alongside accession numbers for all analyzed sequences.

DNA extraction, PCR, sequencing, and cloning

Leaf material for DNA extraction was obtained from herbarium specimens (169 accessions from collections in A, B, E, GH, HAL, HU/HZU, HSNU, KUN, KSC, LE, M, MA, MSB, W, WU, and WUK) and in nine cases from plants cultivated by the authors in the greenhouse of the Botanical Garden Munich (vouchers deposited in MSB). DNA extraction, PCR, purification and sequencing reactions were performed according to methods described in Scheunert and Heubl (2011, 2014). Two well-established loci from these studies were used, the non-coding chloroplast ("cp") *trnQ-rps16* intergenic spacer and the nuclear ("nr") ribosomal ITS region (internal transcribed spacer 1, 5.8S rRNA gene, internal transcribed spacer 2). Additionally, the plastid *trnL-trnF* region (consisting of the *trnL* intron, the *trnL* 3′ exon, and the *trnL-trnF* intergenic spacer; Taberlet et al. 1991) was used (see also Navarro Pérez et al. 2013). All primer sequences alongside references are provided in Online Resource 3. DNA sequences generated by Scheunert and Heubl (2011, 2014), Navarro Pérez et al. (2013) and others, as well as sequences from selected outgroup taxa were downloaded from NCBI's GenBank (http://www.ncbi.nlm.nih.gov, accessed 10 January 2014; see Online Resource 2). For further investigation of the considerable amount of intra-individual nr DNA variability and to support identification of putative hybrid species, six selected individuals (from *S. auriculata*; *S. incisa* Weinm., 1810; *S. lyrata*; *S. musashiensis* Bonati, 1911; *S. ruprechtii* Boiss., 1879; and *S. villosa* Pennell, 1923) were additionally cloned (for detailed information and PCR protocols see Online Resource 1). All clones were included into a separate phylogenetic analysis together with uncloned sequences (see *Phylogenetic inference*).

Data matrix composition and coding of chloroplast indels and ITS intra-individual site polymorphisms

Raw DNA sequence reads were edited and, where necessary, assembled into contigs with the CLC Main Workbench v. 6 (CLC Bio, Aarhus, Denmark). Ambiguously specified basepairs (due to poor sequence read quality or site polymorphism) were recorded using IUPAC ambiguity codes. Contigs were aligned using MAFFT v.7.110 (Katoh and Standley 2013; online version available at http://mafft.cbrc.jp/alignment/server/, accessed 13 October 2013); used

settings are reported in Online Resource 1. Manual refinements were done in BioEdit v. 7.1.11 (Hall 1999). Mononucleotide repeats and ambiguously aligned regions were excluded from further analysis. ITS sequences were checked for potential pseudogeny according to J-S Liu and Schardl (1994), Jobes and Thien (1997), and Bailey et al. (2003). Chloroplast indels, which have been shown to contain phylogenetic information in *Scrophularia* (Scheunert and Heubl 2011, 2014), were coded as binary states for the ingroup only, using the simple indel coding algorithm (Simmons and Ochoterena 2000) as implemented in SeqState v. 1.4.1 (Müller 2005).

In order to make optimal use of the information contained in ITS sequence data, all sequences were examined for the presence of polymorphic sites (PS). Then, two methods were applied (with minor adaptations), which are intended to incorporate information from PS into phylogenetic analyses. Using the ad hoc 2ISP-informative maximum likelihood (ML) approach from Potts et al. (2014), all IUPAC codes including polymorphic sites (there termed 2ISPs, intra-individual site polymorphisms) are treated as unique characters, by recoding the complete alignment as a standard matrix, which is then analyzed using the multistate analysis option for categorical data in RAxML (see below). This method is similar to some approaches described in Grimm et al. (2007); we complemented the Potts et al. (2014) method by the application of Bayesian inference (BI) to our coded dataset as well, based on Grimm et al. (2007). Detailed information on the coding procedure and the original methods can be found in Online Resource 1.

A different approach, pursued by Fuertes Aguilar and Nieto Feliner (2003), concentrates on "Additive Polymorphic Sites" while ignoring the remainder of intra-individual polymorphisms. According to their definition, a site is referred to as an "APS" when both bases involved in the polymorphism can each be found separately at the same site in at least one other accession. To investigate the usefulness of APS in phylogenetic reconstruction, these were also extracted from the dataset. A subset of 17 alignment positions with high numbers of APS, here termed "highly polymorphic alignment positions" ("HPPs"), was then recoded according to the procedure described above and added to the original DNA alignment. This data matrix was likewise analyzed using ML and BI. More detailed explanations are available in Online Resource 1; used codes for all HPPs are listed in Online Resource 4.

Phylogenetic inference

Five datasets were used, one containing the combined *trnQ-rps16* and *trnL-trnF* region data and coded indels from both markers ("cp dataset"), one based on uncoded nr DNA sequence data ("uncoded dataset"), one with nr sequence data alongside coded HPPs ("APS-coded dataset"), one with the complete nr sequence alignment recoded following the 2ISP-informative approach ("2ISP-coded dataset"), and one corresponding to the 2ISP-coded dataset, but also comprising cloned sequences ("nr+clones dataset"). Datasets were analyzed separately using ML and BI. For comparison purposes, additional analyses of the uncoded dataset were conducted, once excluding the 17 highly polymorphic positions themselves and once excluding 17 accessions with high amounts of APS in their sequences (see "Results" section). Appropriate nucleotide substitution models were selected using MrModelTest v. 2.3 (Nylander 2004), which suggested GTR+Γ with four rate categories as best fit to the data according to the Akaike information criterion, adding a proportion of invariant characters for the ITS dataset only (GTR+I+Γ). Bayesian analyses were performed with MrBayes v. 3.2.2 (Ronquist et al. 2012) on a local PC. Maximum likelihood analyses were carried out with RAxML v. 7.4.2 (Stamatakis 2006) on a local PC using raxmlGUI v. 1.3 (Silvestro and Michalak 2012), and RAxML v. 8.0.24 (Stamatakis 2014) on the CIPRES Science Gateway (MA Miller et al. 2010; available at https://www.phylo.org/portal2/login!input.action, accessed 02 December 2015). Settings for all runs and the different datasets are described in Online Resource 1.

Identifying phylogenetic conflict and uncertainty within and among datasets

The amount of ambivalent signal contained within the ITS raw data was illustrated using SplitsTree v. 4.12.3. (Huson and Bryant 2006). To this end, a Neighbor-Net splits graph (NN; Bryant and Moulton 2004) was created, based on a pairwise distance matrix obtained from the ITS sequence alignment (non-excluded characters, see Table 1). In order to incorporate information from polymorphic sites, polymorphism *p*-distances (see Potts et al. 2014) were calculated for the ingroup. Online Resource 1 provides information on required software and optimal settings. Additional networks were computed excluding columns containing gaps and/or accessions with high numbers of "?" and "N" in their sequences, to assess the impact of missing data on the results.

For the visualization of incongruence among datasets, consensus networks (CN; Holland and Moulton 2003; Holland et al. 2004) were constructed in SplitsTree, from each 1001 trees of both BI runs of the cp dataset and the 2ISP-coded dataset. Importantly, edge lengths in these CNs are proportional to the split frequency within the sampled topologies ("COUNT" option). A trees threshold of 0.33 was used for displaying splits, and splits were transformed

Table 1 Alignment characteristics and Maximum Likelihood-based tree statistics for the plastid *trnQ-rps16* and *trnL-trnF* markers and nuclear ITS. Average G+C contents and parsimony-(un)informative characters calculated excluding outgroups, other characteristics include outgroup values. Percentage of parsimony-informative characters referable to non-excluded characters; the latter inclusive of "highly polymorphic alignment positions." Alpha, tree score, and length in ITS given for the uncoded and 2ISP-coded dataset. Values marked with *asterisks* are based on the nr+clones dataset; values with *circles* refer to results from the concatenated cp dataset. *Alpha* the alpha value of the gamma shape parameter, *avg* average, *bp* basepairs, *no* number

	trnQ-rps16	trnL-trnF region	ITS	ITS (clones)
No. of taxa (including outgroups)	180	94	181	62
Sequence length (avg.)	427–1584 bp(961 bp)	100–818 bp(725 bp)	247–667 bp(580 bp)	436–602 bp (571 bp)
Aligned length	2063 bp	946 bp	763 bp	763 bp
Non-excluded characters	2046 bp	930 bp	738 bp	738 bp
Parsimony-uninformative characters	139 bp	51 bp	69 bp	98 bp*
Parsimony-informative characters	134 bp (6.55%)	32 bp (3.44%)	125 bp (16.94%)	150 bp (20.33%)*
Average G+C content	27.00%	33.3%	60.12%	60.87%
ML tree score	−15,802.897°	–	−9001.089/−15,043.732	–
ML tree length	1.835°	–	6.273/26.544	–
Alpha	0.979°	–	0.718/1.089	–

as outlined in Online Resource 1. Congruence among the sequence datasets was also tested with the Incongruence Length Difference (ILD) test (Farris et al. 1995) implemented as the Partition Homogeneity Test in PAUP v. 4.0b10 (Swofford 2003). Accessions or clades exhibiting hard incongruence (HI) were identified by visual inspection of the cp and (2ISP-coded) nr phylogenetic trees for well-supported conflicting placements (Mason-Gamer and Kellogg 1996), using a threshold of ≥0.90 Bayesian posterior probability (PP) in both topologies. Further information can be found in Online Resource 1.

Molecular clock analyses

Divergence times were estimated for the cp dataset using BEAST v. 1.8 (Drummond and Rambaut 2007). For information on input matrices and how information from coded binary indels was incorporated see Online Resource 1.

Divergence dating using fossils was impossible for this dataset of *Scrophularia* (possible reasons for the failure of fossil-based dating and additional information on the used fossils are given in Online Resource 1). Thus, rather than completely relying on secondary calibration constraints for relaxed clock analyses, an approach which is generally error-prone (see e.g., Hipsley and Müller 2014), we decided to resort to a first-step strict clock analysis in order to obtain information about a reasonable age for the ingroup which could be used as a starting point for further analyses. For the strict clock, a fixed substitution rate of $8.1E^{-4}$ per site per million years was used (Lavin et al. 2005). Then, divergence times of the ingroup and the closest outgroup genus only (reduced dataset) were inferred under a relaxed clock with log-normally distributed rates, using the estimated ingroup age from the strict clock run as secondary calibration point. Analyses were performed with BEAST v. 1.8 on the CIPRES Science Gateway; exact procedures, prior values, and settings are provided in Online Resource 1. The relaxed clock analysis was repeated without data (prior-only) on a local PC, to review effective prior distributions and assess the decisiveness of the data.

Biogeographic analyses

Ancestral area optimization relied on the Bayesian Binary Markov Chain Monte Carlo (BBM) algorithm as implemented in RASP v. 2.0b (Yu et al. 2010, 2014). Biogeographic areas are mapped on the world map in Fig. 1b; detailed definitions can be found in Online Resource 5. Distributions of species (including those of known synonyms) were then assigned to the respective areas. Further information on the classification of areas, the determination of species distributions, and the RASP analyses is available from Online Resource 1.

"Maxareas," the maximum number of ancestral areas inferred at each node, was constrained following Ronquist (1997). We assumed equal dispersal ability for ancestors and their present-day descendants (Sanmartín 2003) and therefore set maxareas to two (83% of the *Scrophularia* ingroup species occur in one or two areas only). The number of maximum areas was kept at five during one additional run for comparison purposes. Inferred ancestral distributions were mapped on the majority-rule consensus tree from Bayesian analysis in Fig. 1a, using a threshold of 25% marginal probability (frequency of occurrence of the respective range over the Bayesian sample of trees).

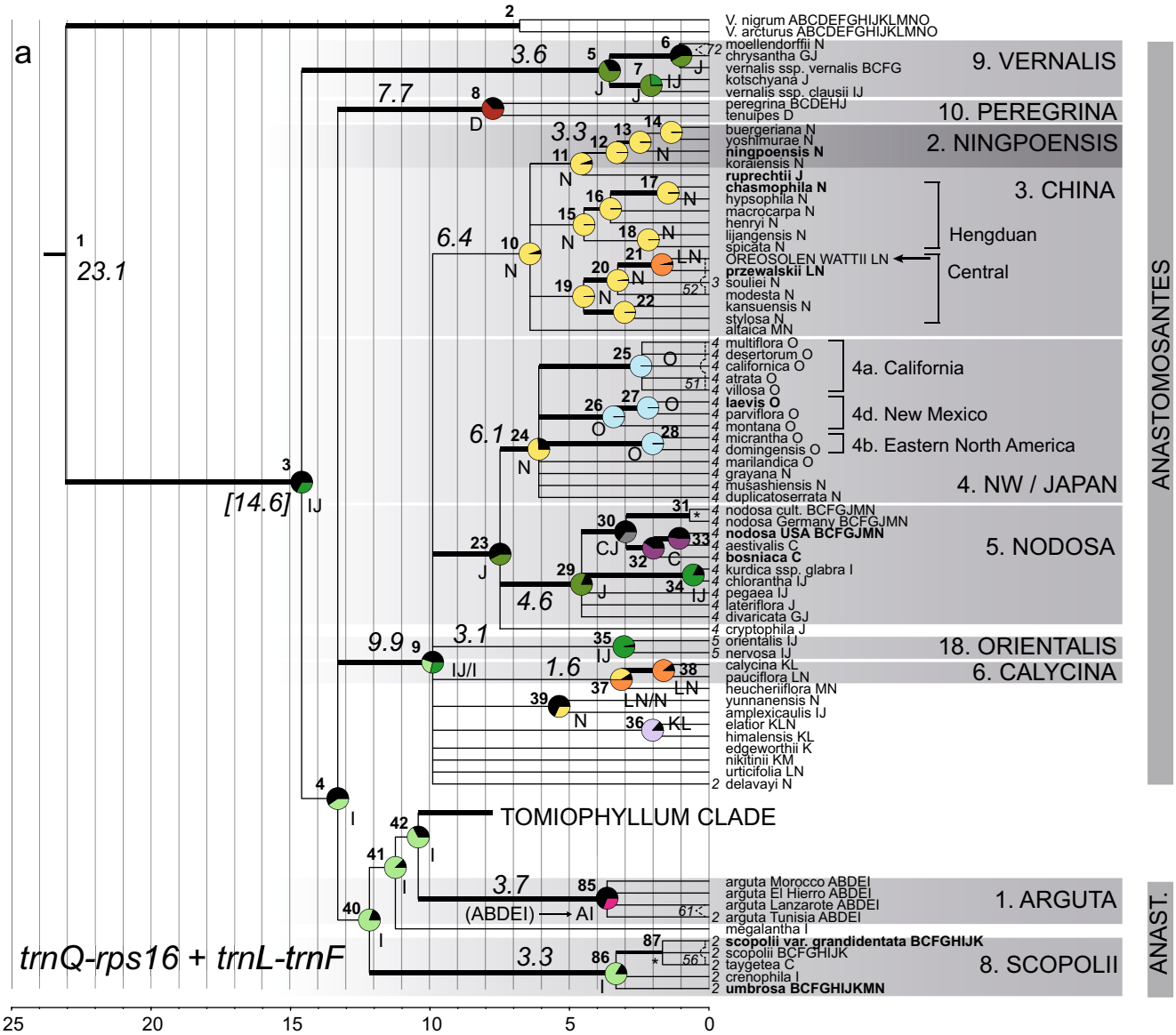


Fig. 1 **a** Dated phylogeny and ancestral area reconstruction for 147 *Scrophularia* species, on a majority-rule consensus tree obtained from Bayesian analysis of combined plastid *trnQ-rps16* intergenic spacer and *trnL-trnF* region alongside coded indels. Branches indicate levels of support, based on posterior probabilities (*PP*) and plotted bootstrap support values (*BS*) from Maximum Likelihood optimization; *bold* PP ≥ 95 or BS ≥ 85, *semi-bold* PP ≥ 90 or BS ≥ 75, *thin* PP < 90/BS < 75. Seven additional nodes only supported by ML (BS ≥ 50) were added manually but not incorporated into further analyses. *Gray bars on the right* denote Clades 1–18 and main species groups as discussed in the text. An *arrow* indicates the position of the Himalayan-Tibetan endemic genus *Oreosolen*. Single accessions displaying hard incongruence among (2ISP-coded) nuclear and plastid trees are marked in *bold*; Clades 7 and 5 (excluding *S. chlorantha*; plus *S. cryptophila*) as a whole are also hardly incongruent. The occurrence of large indels as defined in Table 2 is indicated next to each accession with the respective length type number; *no number* means length type = 1. *Node heights* represent mean ages and were inferred under a Bayesian relaxed clock with log-normally distributed rates, using one calibration point at node 3. Important clade crown ages are given in million years. Ancestral area optimization is based on 9002 trees from the Bayesian analysis, distribution ranges of single taxa are provided after the respective names, area codes and colors are as defined in (**b**) and Online Resource 5. *Pie charts* at nodes indicate inferred distributions of MRCAs from run four of four RASP runs (maxareas = 2); *asterisks* mark nodes where no ancestral distribution reached a marginal probability of 25%. At nodes 9, 37, 61, and 63, separate runs differed with respect to the most probable ancestral area. Alternative distributions shown in brackets derive from an additional analysis with maxareas = 5. Exact values for each node including highest posterior density intervals for inferred ages are listed in Online Resource 7. *NW* New World, *V Verbascum*, *cult* cultivated. **b** World map showing areas defined for ancestral area reconstruction analyses

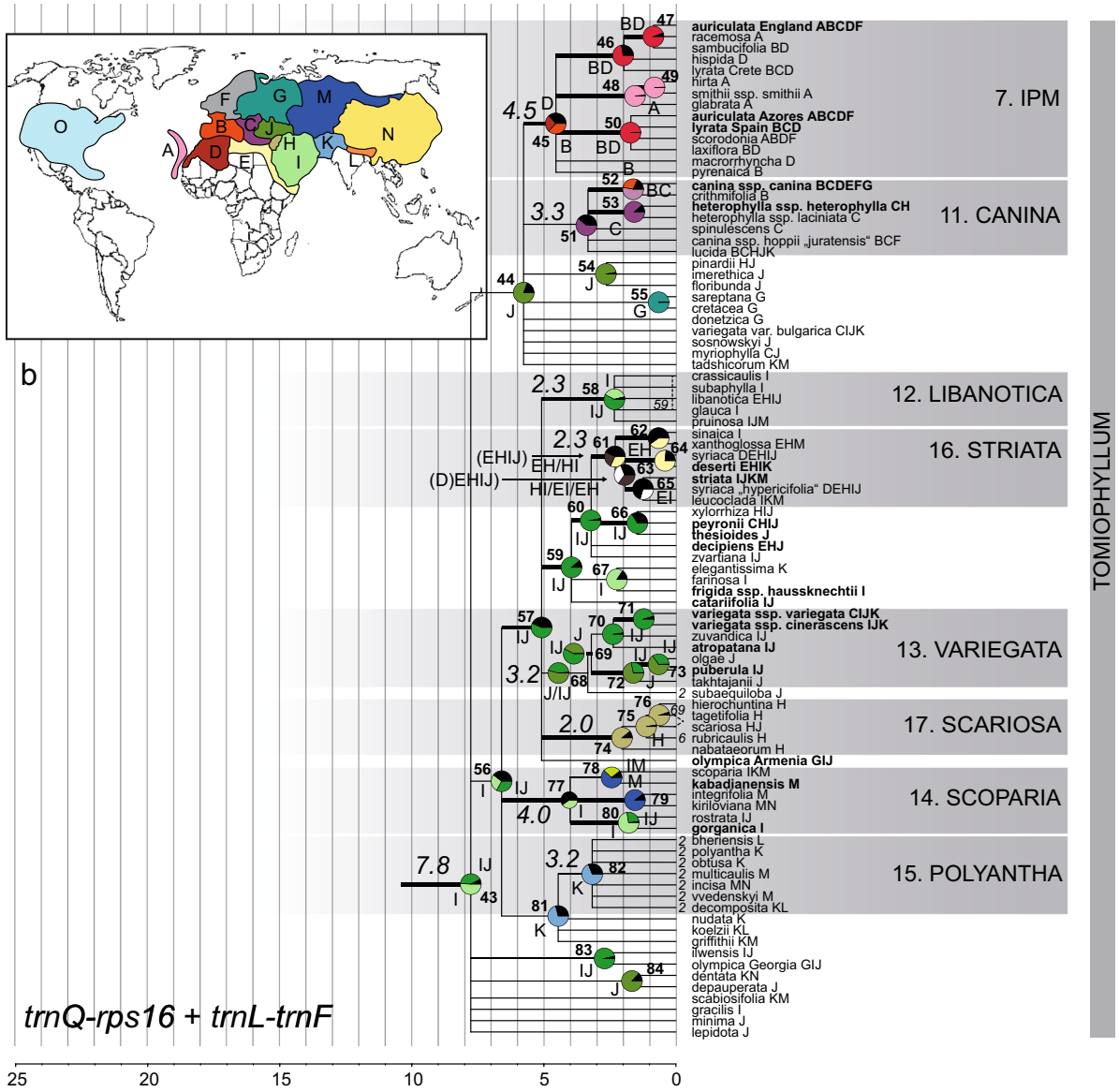


Fig. 1 (continued)

Results

Alignment characteristics

Altogether, 281 sequences were generated for this study, 125 for *trnQ-rps16*, 123 for ITS, and 33 for the *trnL-trnF* region. A total of 62 cloned ITS sequences was obtained from six accessions (available from GenBank under accession numbers KY067709–KY067770) and included into phylogenetic reconstructions, raising the number of ITS sequences to 243 in the nr+clones dataset. Data coverage for *trnQ-rps16* and ITS is complete for all accessions except *Aragoa*, where sequencing of *trnQ-rps16* failed and no sequence was available in GenBank; the genus was coded as missing for the respective marker but contributed a *trnL-trnF* sequence to the plastid alignment. For the *trnL-trnF* region, which was used as supplementary marker, only selected accessions were sequenced; the remainder was likewise coded as missing for *trnL-trnF*. Detailed information on sequence and alignment statistics including average G+C contents and proportions of parsimony-informative characters is given in Table 1. Twenty-nine ingroup indels were coded for the *trnL-trnF* region and 84 for *trnQ-rps16*. The latter is characterized by the occurrence of larger indels ranging from 312 to 839 characters in length; Table 2 shows the positions of all observed indels >300 characters (the respective accessions are marked in Fig. 1a).

PCR products of ITS showed clear single bands in most cases. However, polymorphic sites were present in almost three quarters of the ingroup accessions (121 of 163). Additive polymorphic sites (APS) as defined above were recorded in 95 accessions (58%), from which 17 had five or

more APS in their sequence ("APS-rich accessions," see Fig. 2a). Fourteen of these APS-rich accessions are members of the "Tomiophyllum" clade (see below). Generally, an unequivocal differentiation between artificial double peaks, PS and APS, was not always possible, which means that the reported numbers rather represent best possible estimates.

Phylogenetic relationships

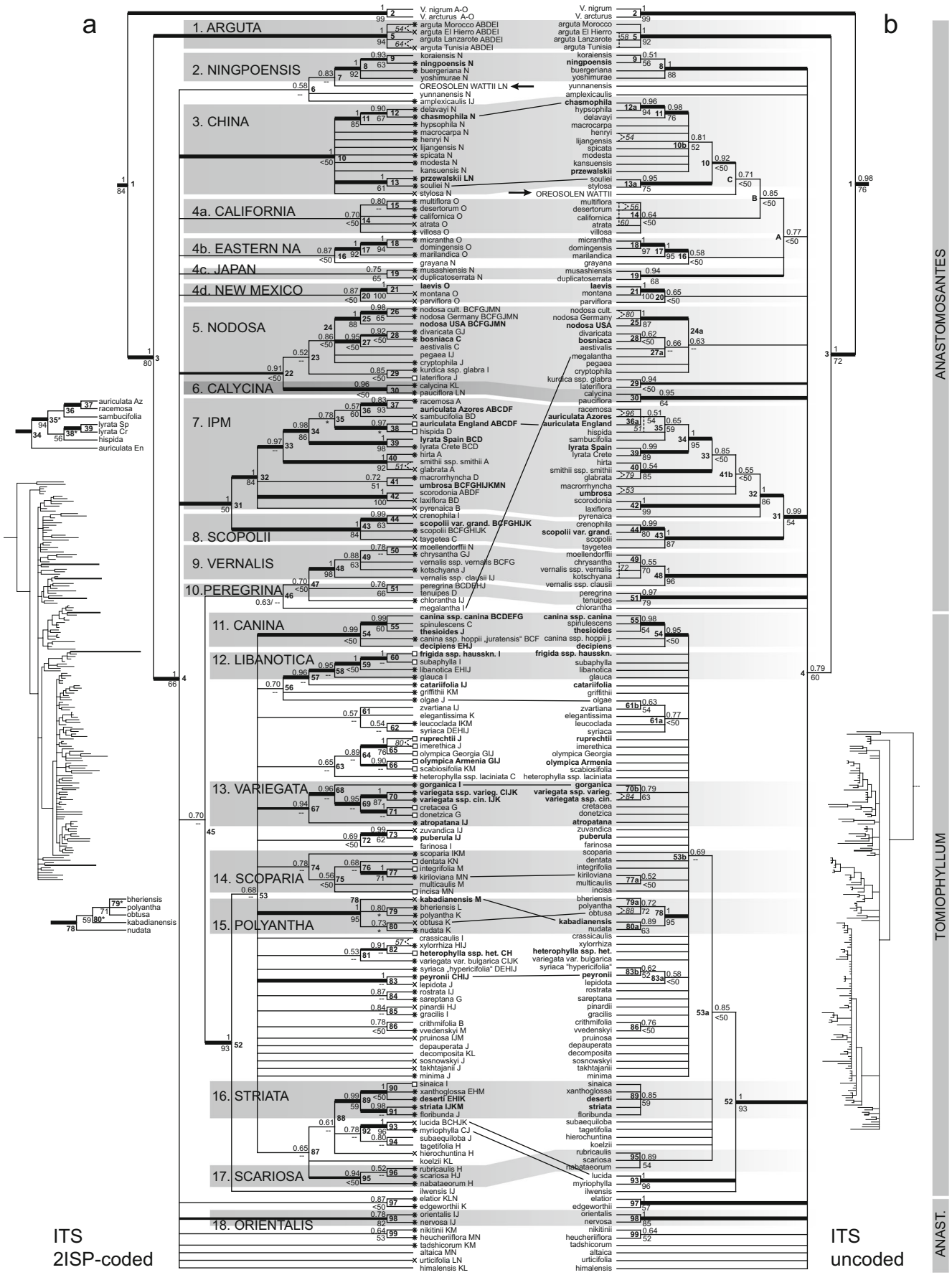
Bayesian analyses of the cp and nr datasets had reached convergence at the end of the runs (standard deviation of split frequencies below 0.01). The majority-rule consensus topologies, with all outgroups except *Verbascum* L., 1753 pruned, are shown in Figs. 1a and 2. Statistics on ML analyses are given in Table 1. The best ML trees only very rarely contradicted the Bayesian consensus trees with respect to nodes with bootstrap support ("BS") ≥ 50; supports were generally lower when using ML. Relative to the Bayesian tree from the uncoded ITS dataset (Fig. 2b, 64 nodes with PP ≥ 0.5), removal of the 17 HPPs yielded a tree with 18 nodes collapsed and weakened support values in 22 cases; removal of the APS-rich accessions from the uncoded dataset had less impact but led to generally lower supports in basal nodes (results not shown). Conversely, including the coded HPP matrix into calculations (APS-coded dataset) resulted in a tree with 19 new nodes (PP ≥ 0.5; not shown); using the 2ISP-informative approach and coding all double peaks present in the sequences (2ISP-coded dataset, Fig. 2a) yielded a tree with 35 new nodes relative to the uncoded phylogeny. By contrast, resolution did not change much in ML trees and was even slightly reduced.

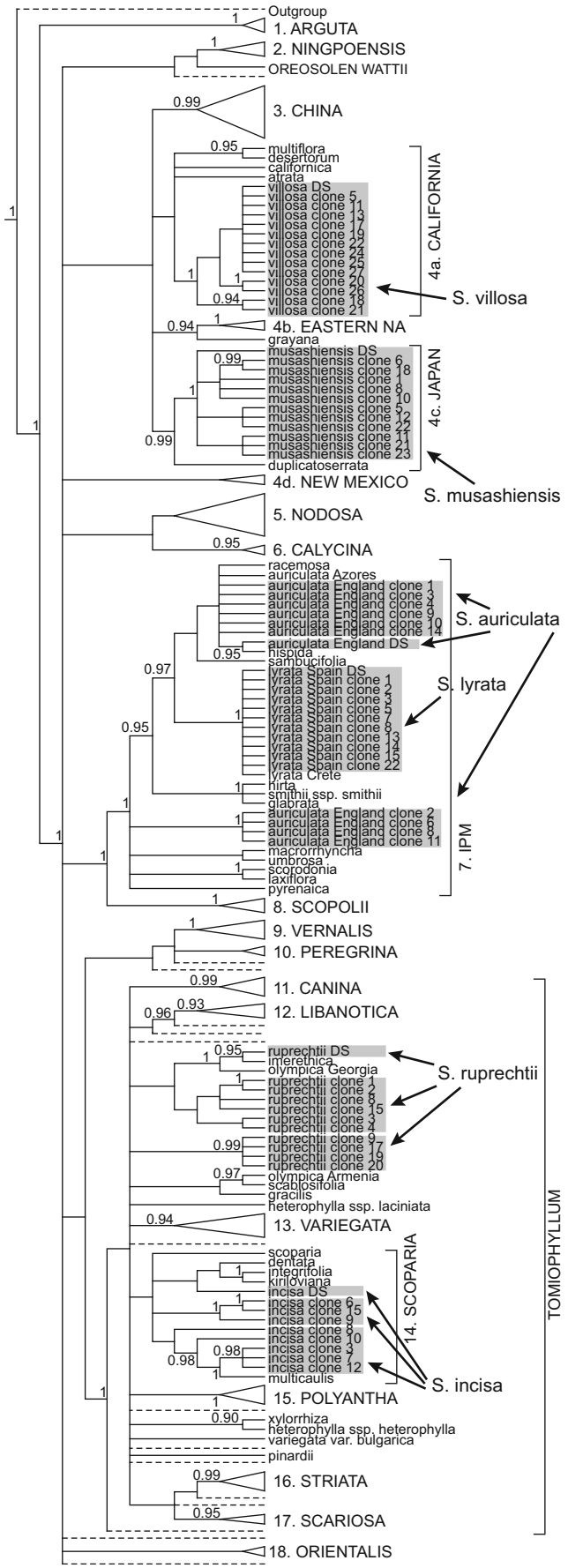
Both 2ISP-coded nuclear and plastid tree (Figs. 2a and 1a) support a monophyletic clade of all accessions from *Scrophularia* with moderate to maximum support (cp PP 1.00, BS 100/nr PP 1.00, BS 80). However, the accession from *Oreosolen wattii* Hook.f. is deeply nested within the ingroup at similar positions depending on the dataset, rendering *Scrophularia* paraphyletic with respect to this genus. For convenience and to avoid confusion with other clade names, Stiefelhagen's (1910) section names are adopted here to name the two main phylogenetic entities: the highly supported Tomiophyllum clade (cp PP 1.00, BS 99/nr PP 1.00, BS 93) largely corresponds to, but is not exactly identical with, *Scrophularia* sect. *Tomiophyllum*. The remainder of the

Fig. 2 Majority-rule consensus trees (cladograms) for 147 *Scrophularia* species, obtained from Bayesian analysis of the nuclear internal transcribed spacer region (ITS), with **a** intra-individual site polymorphisms (IUPAC codes) treated as informative using 2ISP coding or **b** using uncoded, unmodified sequences. Downsized phylograms are shown beside each tree, with six taxa having exceptionally long branches in the 2ISP-coded dataset marked in *bold*. Outgroup taxa are reduced to the closest genus. Posterior probabilities (*PP*) are given above branches, plotted bootstrap support values (*BS*) from Maximum Likelihood (*ML*) optimization below. Double dashes state that the node was not present in the fully resolved best-scoring ML tree. *Asterisks* denote cases with deviating ML topologies illustrated beside the tree; five/twelve additional nodes only supported by ML (BS ≥ 50) were added manually. Branches indicate levels of support as defined in Fig. 1. *Gray bars* denote Clades 1–18 and main species groups. *Arrows* indicate the position of the Himalayan-Tibetan endemic genus *Oreosolen*. Single accessions displaying hard incongruence among (2ISP-coded) nuclear and plastid trees are marked in bold; Clades 7 and 5 (excluding *S. calycina* and *S. pauciflora* Benth., 1835) as a whole are also hard incongruent. Accessions obtaining different positions in both phylogenies are connected across trees. Amounts of intra-individual polymorphism are indicated to the left of each accession using the following symbols: *no symbol* no polymorphic sites (*PS*), *cross* PS but no APS (additive polymorphic sites, see "Materials and methods" section), *star* APS present in the sequence, *square* ≥ 5 APS present, "APS-rich accession." Distribution ranges of single taxa are provided after the respective names, area codes are as defined in Fig. 1b and Online Resource 5. *NA* North America, *V Verbascum*, *cult* cultivated, *grand grandidentata*, *hausskn haussknechtii*, *varieg variegata*, *cin cinerascens*, *het heterophylla*

Table 2 Nine characteristic indels, corresponding to eight sequence length types, in the *trnQ-rps16* intergenic spacer alignment created from sequences of 162 *Scrophularia* accessions. Length type "1" no larger indels present, full alignment length 3325 basepairs. "Indel position" is referable to aligned length. Clade numbers as defined in the main text. Assessment of phylogenetic value as diagnostic character is given for each indel type. *No acc* number of accessions possessing the respective indel, *bp* basepairs

Length type	Indel position	Indel length	No acc	Species/clade	Diagnostic?
1	–	–	118	–	No
2a	485–796	312 bp	7	Clade 15	Yes
2b	518–845	328 bp	1	Delavayi	No
2c	527–845	319 bp	1	Subaequiloba	No
2d	528–845	318 bp	5	Clade 8	Yes?
	527–844	318 bp	1	Arguta El Hierro	No
3	688–1118	431 bp	1	Souliei	No
4	551–1147	597 bp	25	Clades 4+5	Yes
5	251–1014	764 bp	2	Clade 18	Yes
6	207–1045	839 bp	1	Rubricaulis	No


a
b
1. ARGUTA
2. NINGPOENSIS
3. CHINA
4a. CALIFORNIA
4b. EASTERN NA
4c. JAPAN
4d. NEW MEXICO
5. NODOSA
6. CALYCINA
7. IPM
8. SCOPOLII
9. VERNALIS
10.PEREGRINA
11. CANINA
12. LIBANOTICA
13. VARIEGATA
14. SCOPARIA
15. POLYANTHA
16. STRIATA
17. SCARIOSA
18. ORIENTALIS
ANASTOMOSANTES
TOMIOPHYLLUM
ANAST.
ITS
2ISP-coded
ITS
uncoded



◀ **Fig. 3** Majority-rule consensus tree (cladogram) for 224 accessions of 147 *Scrophularia* species, including 62 cloned sequences from six accessions, and obtained from Bayesian analysis of 2ISP-coded nuclear ITS. Detailed relationships are shown for clades which include clones and where deviating from Fig. 2a; *dashed lines* represent one or more accessions removed for clarity. Posterior probabilities ≥ 0.90 are given above branches. Clones and corresponding direct sequences (*DS*) are highlighted in *gray*; *brackets on the right* denote the clade they belong to. Distinct positions of clones and DS for each species are indicated by *arrows*. *NA* North America

species mainly belong to *Scrophularia* sect. *Anastomosantes*; these species do not form a monophyletic clade, but are paraphyletic with respect to the Tomiophyllum clade. Nevertheless, they are united by a number of shared characteristics and are therefore referred to as the "Anastomosantes group."

Several clades can be recognized in both trees (see boxes in Fig. 1a and 2a): for example, "Arguta" (Clade 1) comprises all four accessions of this annual species and receives high to maximum support in the analyses. A clade of mainly Chinese endemics ("China," Clade 3) receives maximum support by BI in ITS only. In the cp tree, its composition is slightly different and includes the "Ningpoensis" clade (Clade 2). The most widespread representative of the genus, the type species *S. nodosa*, forms a monophyletic clade with seven to nine other taxa ("Nodosa," Clade 5) and is moderately to highly supported by BI. In the cp tree, it includes the Southwestern Asian/Turkish - Caucasian *S. chlorantha* Kotschy & Boiss., 1879, in ITS the Turkish endemic *S. cryptophila* Boiss. & Heldr., 1853, and the mainly Southern Asian Calycina clade ("Calycina," Clade 6). Clade 7, the "IPM" clade ("Iberian Peninsula–Macaronesia" as introduced by Scheunert and Heubl 2014), is supported in all analyses and differs between cp and nr only with regard to *S. umbrosa* Dumort., 1827. Within the Tomiophyllum clade, fewer clades are present in both trees, and consistency regarding their members is much less pronounced. Taxa from the New World group differently in the analyses: the plastid tree supports monophyly of all New World species and three "Japanese taxa" (*S. grayana* Maxim. ex Kom., 1907; *S. duplicatoserrata* Makino, 1906; *S. musashiensis*) in a "New World ("NW")/Japan" clade (Fig. 1a, Clade 4). Within the clade, three subclades from the New World are supported, one with mainly lowland taxa centered in California ("California," 4a), one with subalpine taxa distributed in New Mexico and Arizona ("New Mexico," 4d), and one comprising species from the Greater Antilles, and in the nr tree the mainly lowland Eastern North American *S. marilandica* L., 1753 ("Eastern North America," 4b). In the nr trees, the NW/Japan clade collapses into its subclades, with *S. grayana* being associated with Clade 4b and two of the Japanese taxa forming Clade 4c ("Japan"). Clades 4a–d remain unconnected using the 2ISP-coded dataset, but based on

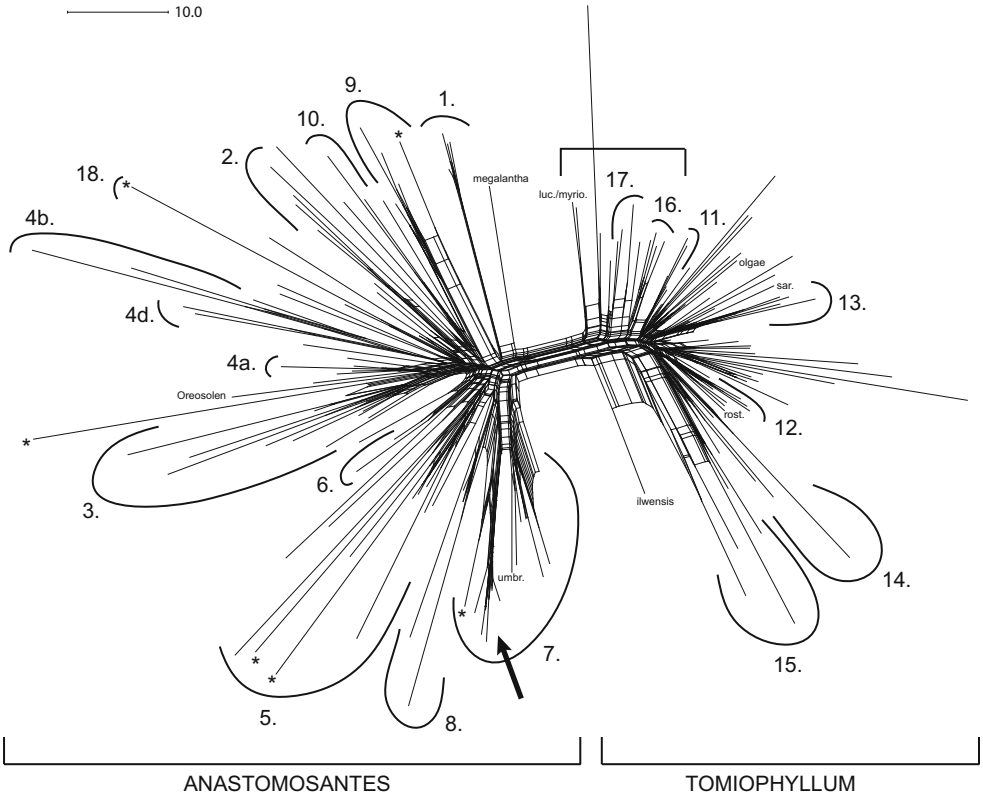


Fig. 4 Neighbor-Net splits graph for 163 accessions of *Scrophularia*, based on polymorphism *p*-distances (i.e., treating intra-individual site polymorphisms as informative) inferred from the ITS sequence data set. *Scale bar* corresponds to split weights based on ordinary least squares estimates. Clades 1–18 are marked, *square brackets* below denote main species groups. *Asterisks* highlight six taxa having exceptionally long branches in the 2ISP-coded dataset, e.g., *S. auriculata* from England, the accession with the highest number of polymorphic sites in this study, within Clade 7. The Auriculata subclade of Clade 7 is indicated by an *arrow*, an assemblage of taxa (node 87 in Fig. 2a) which also includes the Striata (16) and Scariosa (17) clades is marked by the square bracket above. *sar sareptana*, *rost rostrata*, *luc/myrio lucida/myriophylla*, *umbr umbrosa*

the uncoded dataset, three of them form a weakly to very weakly supported grade leading towards the China clade (Fig. 2). By contrast, in the cp tree the NW/Japan clade is sister to the Nodosa clade and *S. cryptophila* with moderate to maximum support. Altogether, the members of 11 of the 18 main clades vary due to low resolution and/or incongruence. Relationships among clades and the backbones of the trees are only poorly resolved.

Bayesian analysis of the nr+clones dataset resulted in the majority-rule consensus presented in Fig. 3. The topology is largely similar to the 2ISP-coded ITS tree, with generally equal or slightly lowered support values in clades without clones. The clade containing *S. ruprechtii* (Fig. 2a, node 63) collapsed into its subclades when clones were included.

ITS raw data network and incongruence among chloroplast and nuclear markers

The Neighbor-Net splits graph computed from polymorphism *p*-distances (Fig. 4) is extremely non-treelike; high complexity and substantial ambiguity are present throughout the whole ingroup. Its dumbbell-shaped structure traces the two main phylogenetic groups also seen in tree reconstructions. Accessions recovered in well-supported clades in Fig. 2a mostly group together in the NN. Although distance-based methods are often sensitive to missing data, their exclusion did not improve the resulting network distinctly; rather, negative effects were observed as the amount of discarded data increased. Thus, the NN in Fig. 4 is based on the full ingroup dataset.

Although several clades are found in both plastid and nuclear trees, their composition is often considerably incongruent. Considering only cases of well-supported incongruence (among the plastid and 2ISP-coded nr dataset) however, only 28 single accessions display hard incongruence as defined above (HI accessions; highlighted in bold in Figs. 1a and 2). These conflicts are based on switches within the same clade in almost 60%; swaps among Clades 1–18 are observed in *S. umbrosa, S. kabadianensis* L.Fedtsch., 1913, and *S. gorganica* Rech.f., 1955. In *S. ruprechtii*, incongruence involves a switch from a clade within Tomiophyllum in ITS towards the Ningpoensis clade within a large clade of Anastomosantes taxa in cp. In addition to the single HI accessions, the IPM clade (Clade 7) as a whole is incongruent as well: it is embedded in the Tomiophyllum clade (cp) while being sister to the "Scopolii" clade (Clade 8) of Anastomosantes in ITS (see also its position in Fig. 5a). Similarly, *S. cryptophila* and the Nodosa clade as shown in the cp tree (excluding *S. chlorantha*) are also hardly incongruent. The detected HI accessions thus sum up to a total of 47.

The ILD test did not detect significant incongruence between the two chloroplast markers at the $p = 0.05$ confidence level ($p = 0.180$), so combined analysis of the plastid data was justified. However, as expected from visual inspection of the trees, the test revealed severe incongruence among nuclear and plastid datasets ($p = 0.002$). Deleting the 47 HI accessions

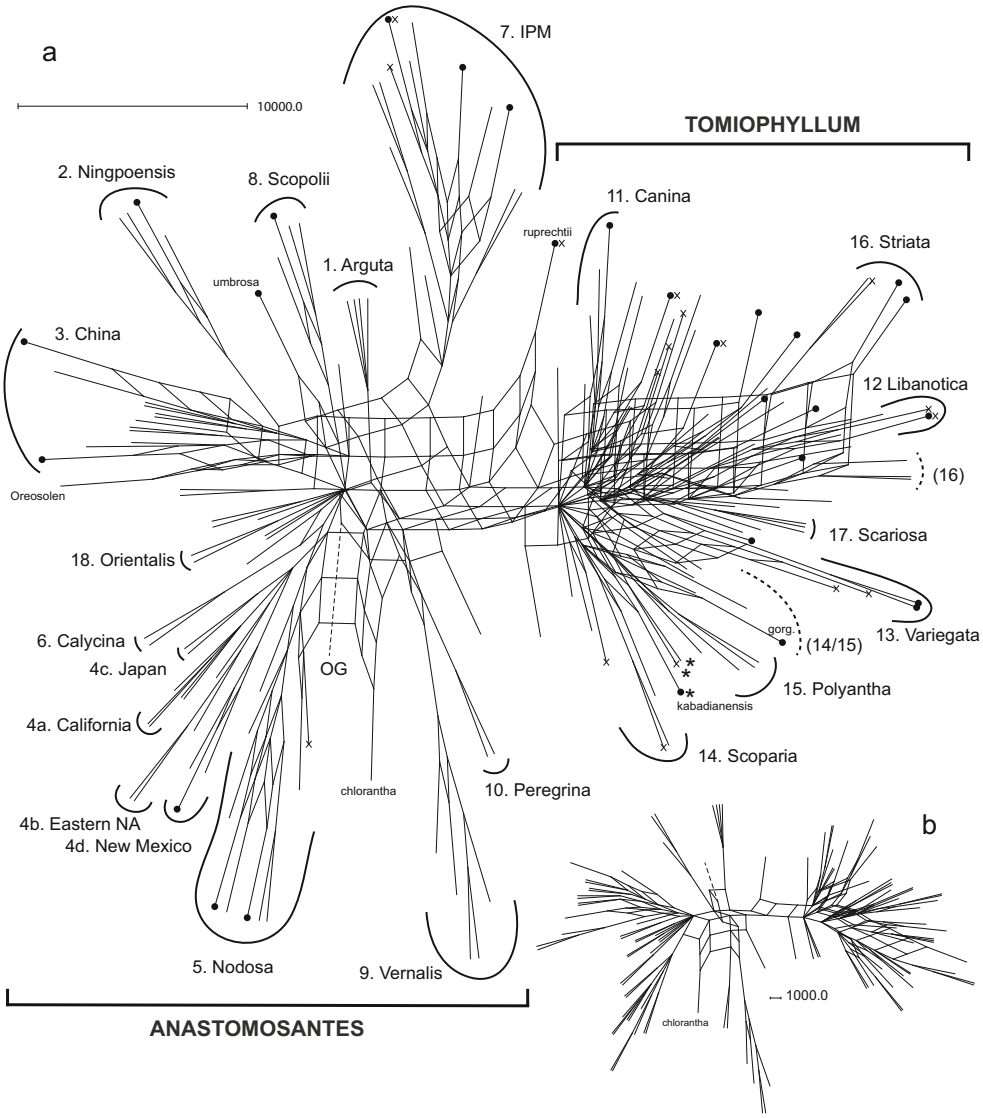


Fig. 5 Consensus networks (CNs) based on each 1001 trees from both runs of both single marker Bayesian analyses of the 2ISP-coded nuclear ITS and plastid datasets (which yielded the consensus trees shown in Figs. 2a and 1a). *Scale bar units* reflect the frequency of splits within the entered trees (COUNT option), trees proportion threshold for displaying splits = 0.33. **a** CN containing all accessions. *Square brackets* denote main species groups, Clades 1–18 are marked. *Dashed lines* highlight exemplary taxa supported within the indicated clade by one (the plastid) dataset only. *Asterisks* mark three species with intermediate positions between Clades 14 and 15. Hard incongruent accessions are highlighted by *dots*, APS-rich accessions by *crosses* (terms as defined in "Materials and methods" and "Results" sections). **b** CN with 28 hard incongruent accessions, the IPM clade, the Nodosa clade without *S. calycina*/*S. pauciflora*/*S. chlorantha*, and 17 APS-rich accessions removed using the "exclude selected taxa" option. *gorg gorganica*

from the matrix did not change the result ($p = 0.002$); incongruence between datasets apparently is not limited to well-supported conflicts. This is evident also from the consensus network constructed with Splitstree (Fig. 5a). Although the clades defined above can, at least partly, be traced in the CN, the relationships among them are inextricably entangled, often with similar numbers of trees supporting each of the conflicting splits. Bundles of parallel edges indicate conflicting signals or uncertainty also within several clades, and some are even split in the CN (examples see dashed lines in Fig. 5a). When HI accessions (labeled by black dots) were removed from the network, the number of parallel edges decreased considerably; the effect was much less pronounced when APS-rich accessions (indicated by black crosses) were excluded (not shown). But even upon exclusion of all marked accessions, a large number of reticulations remained in the consensus network (Fig. 5b, 99 non-trivial splits left compared to 167 in Fig. 5a).

Divergence time estimation

The chronogram from the strict clock analysis is available in Online Resource 6. Compared with the results from the BEAST run with the prior only, posterior distributions differed from prior distributions in most cases, indicating that the priors did not illegitimately influence the result. The effective prior distributions of the prior-only run matched the expected ones without any conflicts among priors. Figure 1a shows the respective chronogram, inferred by relaxed clock analysis of the reduced dataset, on the majority-rule consensus topology derived from the MrBayes runs. Node ages for all nodes alongside 95% highest posterior density (HPD) intervals are provided in Online Resource 7. The mean substitution rate was calculated at $7.8592E^{-4}$. The standard deviation of the uncorrelated log-normal clock and the coefficient of variation were 0.47 and 0.49 (95% HPD 0.26–0.71), indicating medium rate heterogeneity

within the dataset. The origins of the genus and its basal lineages can be traced back to the Miocene (23.1–11.2 million years ago, "mya", according to Fig. 1a); however, 11 of the 18 clades did not start to diversify before the beginning of the Pliocene (4.6–3.1 mya), and four clades even date to the Pleistocene (2.3–1.6 mya). Three of those belong to the Tomiophyllum clade, while old clades with crown ages in the late Miocene (7.7–6.1 mya) include the "Peregrina" (Clade 10), China and NW/Japan clades of the Anastomosantes group.

Ancestral area optimization

Results of the four independent RASP runs were largely similar; all runs, including that with maxareas set to five, reached convergence (standard deviations of split frequencies 0.0013–0.0018). In Fig. 1a, pie charts at nodes represent the inferred distributions; exact marginal probabilities for each range are available from Online Resource 7. Southwestern Asia, Turkey, and the Caucasus were inferred as the most likely ancestral areas of *Scrophularia* (I and J, Fig. 1a, node 3) with low marginal probability (32.70). The same ancestral distribution was inferred for the Tomiophyllum clade (node 43) with high probability. Some clades within the Tomiophyllum clade have other most probable ranges of origin, including the Levant which is here defined as present-day Lebanon, Syria, Israel, and Jordania ("Scariosa" clade 17, node 74), or Afghanistan to the Western Himalayas ("Polyantha" clade 15, node 82) with high frequencies of occurrence (89.22/68.72, respectively). The ancestor(s) of the New World taxa were reconstructed as distributed in Eastern Asia (node 24) with high marginal probability. The uncertainty in ancestral areas at nodes 61 and 63 argues for a more widespread occurrence of the most recent common ancestor, as also inferred by the five maxareas analysis (results in brackets in Fig. 1a).

Discussion

Implementation of ITS intra-individual polymorphism and chloroplast indel information

The genus *Scrophularia* provides a striking example of incongruence and ambiguity among, but also within, gene trees and the corresponding DNA sequences. Although tree reconstruction per se seems appropriate to adequately illustrate the tree-like parts of the genus' phylogenetic history, it is hampered by the widespread abundance of intra-individual site polymorphisms in the ITS sequences, which introduces considerable conflict into the dataset. The problem of reduced tree resolution when analyzing polymorphic sequences has been known for some time and has been noticed by several authors (e.g., Eidesen et al. 2007; Grimm et al. 2007), especially in cases where hybrids (or more generally, taxa bearing APS) were included into tree reconstructions (McDade 1995; Campbell et al. 1997; Whittall et al. 2000; see also the weakly resolved ITS phylogeny from dataset B in Fuertes Aguilar and Nieto Feliner 2003). Several suggestions have been made on how to deal with intra-individual polymorphisms. Among the most-used is pruning the respective taxa (e.g., Whittall et al. 2000; Fuertes Aguilar and Nieto Feliner 2003); another possibility is exclusion of polymorphic alignment positions (e.g., Scherson et al. 2008). However, in *Scrophularia*, attempts to infer a stable backbone using a representative subset of completely monomorphic sequences did not improve the result (not shown). Neither did the removal of APS-rich accessions and exemplary deletion of the 17 HPPs considerably reduced the resolution of the resulting phylogeny (see "Results" section). Further strategies include the replacement of polymorphisms by missing data or the most common nucleotide, their resolution in favor of the stronger signal (Fehrer et al. 2009), statistical haplotype phasing methods (e.g., Stephens et al. 2001, employed in Lorenz-Lemke et al. 2005), or cloning (see Nieto Feliner and Rosselló 2007; however, cloning all accessions may not be feasible in species-rich genera).

Rather than discarding or substituting sequence site variabilities, including them as phylogenetically informative characters seems to be a better solution. This can be achieved by the approach employed here, using 2ISP coding and the ad hoc implementation in ML from Potts et al. (2014) with additional adaptation of the method for BI. The procedure is straightforward to apply, does not require the use of step matrices, and allows the choice of appropriate DNA substitution models. As in Grimm et al. (2007) and Potts et al. (2014), recoding resulted in considerably increased resolution and enhanced support values in the phylogenetic tree: for instance, the "Libanotica" clade (Clade 12), corroborated by morphological similarities of its taxa (Boissier 1879; Grau 1981) is only recovered in the Bayesian topology using 2ISP coding (see Fig. 2a, node 58). Furthermore, the results obtained only very rarely contradict the topology generated without additional coding, but mostly strengthen existing clades. This corroborates the applicability of the method to the *Scrophularia* dataset.

The latter seems to be characterized by polymorphisms derived from several processes, including hybridization resulting in APS according to Fuertes Aguilar and Nieto Feliner (2003) but also others like e.g., inherited ancestral polymorphism (which might lead to ILS). Furthermore, homoeologous rDNA arrays might be subjected to differential silencing after interspecific hybridization. This can produce pseudogenes (Bailey et al. 2003; Volkov et al. 2007) recognizable by certain characteristics (Mayol and Rosselló 2001; Grimm and Denk 2008). Although G+C contents were slightly lowered in nine uncloned accessions, only one of them (*S. nervosa* Benth., 1846) showed a long edge in the NN

indicating increased distance (Fig. 4, Clade 18, see asterisk), and no ingroup accession had substitutions or length changes in the conserved parts of ITS1 or the 5.8 rDNA. However, it is still possible that incipient pseudogeny accounts for some of the observed sequence ambiguities. Finally, the rDNA marker used here can be affected by recombination (see Álvarez and Wendel 2003). No clear evidence of recombination was found in the sequences; however, respective patterns would be difficult to detect in a complex dataset like this (see below), which is additionally characterized by low sequence divergence. Consequently, an influence of recombination processes cannot be ruled out.

Due to the large number of polymorphisms, the processes involved in their formation cannot be distinguished in the *Scrophularia* dataset: informative 2ISPs are scattered across the alignment, with very few coherent mutation patterns identifiable among them. This also excludes the explicit detection of hybrids by comparing APS patterns as done by Fuertes Aguilar and Nieto Feliner (2003). Instead, APS were tentatively used for character coding, to assess their influence on the result. However, as reliable detection of all APS could not be achieved, only selected putative APS within 17 HPPs were coded. Analysis of the APS-coded dataset (results not shown) yielded an intermediate topology with respect to those from the 2ISP-coded and uncoded dataset. Supports of individual nodes matched those of one or the other dataset (e.g., nodes 10, 20, 68, 95, see Online Resource 7), were intermediate between them (nodes 19, 28, 40) or were worse or better (nodes 4, 33, 61b, 80a). This suggests that the approach is biased, and the results are dependent on the chosen subset of alignment columns and their APS. We thus conclude that restricting the coding procedure to APS only is not possible in *Scrophularia*. By contrast, the 2ISP-informative approach is particularly suitable for such datasets, as it does not discard polymorphisms based on their origin. The improvement in phylogenetic results seen here suggests that the information contained in intra-individual polymorphisms can be used even in cases where their sources are not exactly known, as also emphasized by Potts et al. (2014).

An inherent disadvantage of the approach is that if artificial ambiguities due to bad read quality are present, these are also coded and could possibly blur relationships. In the present study, discrimination of artificial double peaks from "real" polymorphism cannot be assumed to be completely reliable (although low-quality sequences are not too frequent), which was why all ambiguous bases were subjected to coding. In such cases, results regarding accessions with potential data quality issues, which have long branches in the coded tree compared to the uncoded tree, should be regarded with caution. From the six long-branch accessions marked in Fig. 2 (thumbnail trees; the respective accessions are marked by asterisks in the NN in Fig. 4), four can be assumed to be influenced by artificial ambiguities, while long branches seen in *S. lateriflora* Trautv., 1866, and *S. auriculata* from England rather also reflect real polymorphism. However, although cautionary interpretation regarding taxa with many artificial characters seems justified, the fact that both trees are largely congruent and that nodes strongly supported in the uncoded tree are only rarely weakened using 2ISP coding, indicates that the method is robust to a certain amount of "noise." Thereby, the tips of the phylogeny seem to benefit most from coding, while more basal relationships remain unchanged or may even collapse (Fig. 2b, nodes A and B, node 53b).

It is important to note that although 2ISP coding improves the resolution of the phylogenetic tree, it will not solve the problem of inherent conflict present within the data, among others incompatible signals after hybridization (Potts et al. 2014). Here, examination of the NN is useful as it represents all information and all conflict contained in the sequences (Bryant and Moulton 2004; Morrison 2010). Its highly interwoven structure as presented in Fig. 4 suggests that irrespective of the method applied, any bifurcating tree will suffer from an insufficiently resolved backbone as well as an amount of weakly supported nodes regarding certain relationships. Clades showing high amounts of ambiguity in the NN (Fig. 4) are likely to be sensitive to the type of analysis conducted (BI vs. ML), and will often remain insufficiently resolved (see Fig. 2a with inserts: Clade 15, Clade 7 with the "Auriculata" subclade, Clade 3, clade node 87, highlighted by a square bracket in Fig. 4, *S. ilwensis* K. Koch, 1844). This also means that no conclusions should be made based on weak Bayesian support values in these cases (compare e.g., the weakly supported sister clade of *S. rostrata* Boiss. & Buhse, 1860, and *S. sareptana* Kleop. ex Ivanina, 1972, to their positions in the NN, or the weak association of *S. olgae* Grossh., 1932, with the Libanotica clade 12).

It is remarkable that generally, results from ML are much less resolved (see Fig. 2); many nodes supported by BI are not found in the best-scoring ML tree or are supported by BS < 50. Furthermore, 2ISP coding does not improve the situation, it seems to have only very little effect. One possible reason for this is that ML reconstruction in RAxML per se is not completely naive concerning polymorphic sites as outlined in Potts et al. (2014), and that coding thus does not make much difference. The different way site ambiguities are handled are also visible from the different positions of the highly polymorphic *S. auriculata* from England. Another explanation might be that in a dataset with not too many, but clearly structured informative polymorphisms, bootstrapping more often produces deviating topologies while BI quickly achieves one "optimal solution."

Apart from the coding of ITS polymorphisms, additional information can also be drawn from plastid *trnQ-rps16* indels. This marker is particularly suitable for *Scrophularia*, regarding both its high information content in terms of parsimony-

informative characters (Table 1) as well as the occurrence of diagnostic indels as already described in Scheunert and Heubl (2011). Both NW/Japan (Clade 4) and Nodosa (Clade 5) clades are exclusively characterized by one particular indel of 597 bp length (Table 2). The same accounts for Clade 18, the "Orientalis" clade (764 bp), while a shorter indel (312 bp) occurs in Clade 15 (Polyantha). These length differences constitute a reliable diagnostic tool for the respective clades; quick discrimination can be achieved by a simple PCR reaction (see Fig. 2 in Scheunert and Heubl 2011). Other indels might be of limited phylogenetic value: the Scopolii clade is characterized by a 318-bp indel, however, in the respective region of the alignment (length types 2b–d, ranging roughly from position 518 to 845), indels also seem to arise spontaneously in single unrelated taxa or even accessions (Table 2, Fig. 1a).

Utility of gene tree discordance, cloned sequences, and Neighbor-Net splits graphs for tracing reticulate events in *Scrophularia*

In addition to intra-individual variability, many taxa obtained conflicting positions among nr and cp phylogenies. Moreover, the number of incongruent accessions is probably underestimated. As hard incongruence is by definition dependent on high supports of the associated nodes, it must be assumed that lowered resolution, caused by large amounts of PS in certain ITS sequences, prevented taxa from being recognized as hard incongruent. This is corroborated by the fact that the removal of HI accessions did not render the ILD test insignificant. Accordingly, the consensus network of both markers showed a considerable amount of reticulation even after excluding all HI accessions and APS-rich accessions (Fig. 5b). In consequence, weakly supported cases of incongruence should not be ignored completely (see for example the position of *S. chlorantha* between Clades 5 and 9/10).

Conflicts among datasets can be dealt with in various ways: ignoring the incongruence altogether (concatenation approach; Gadagkar et al. 2005; L-Y Chen et al. 2014; but see Rokas et al. 2003; Kubatko and Degnan 2007; Weisrock et al. 2012), pruning conflicting taxa prior to combined analysis (Huelsenbeck et al. 1996), or duplicating them (Pirie et al. 2008, 2009). While incongruence in the present dataset is far too widespread for pruning or using the taxon duplication approach, it is also quite obvious from the CN from ITS and plastid trees that combining both datasets in a concatenation approach would yield a rather uninformative tree. Comparison of individual plastid and nuclear gene trees including phylogenetic relationships of cloned sequences, and additional examination of the corresponding networks seems more suitable in this case.

Similar to the ambiguity present within the ITS dataset, the complex relationships found among the phylogenetic trees suggest that a combination of different processes has influenced the evolutionary history of *Scrophularia*. With the exception of *S. arguta*, conspecific accessions are never monophyletic, and sequences from three of the six cloned accessions group with other taxa. Unequivocal identification of evolutionary events like ILS and reticulation is not possible based on the current dataset, but some inferences can nevertheless be made. For example, while earlier studies have determined the placements of *S. auriculata* and *S. lyrata* within the Auriculata subclade (as in Fig. 2a, node 34), two of the accessions unexpectedly group with *S. scorodonia* L., 1753 and *S. laxiflora* Lange, 1878, in the cp tree. Although lineage sorting effects cannot be ruled out in this relatively young group, the observed pattern is more likely to reflect geographic proximity: on the Azores, where the accession from *S. auriculata* was sampled, *S. scorodonia* occurs as an introduced species, and the accessions from *S. lyrata* from Spain and *S. laxiflora* were collected app. 150 km apart, while *S. lyrata* (Crete) was collected as far as 2500 km away. Together with the fact that hybridization of *S. scorodonia* and *S. auriculata* is possible (Grau 1976; Dalgaard 1979), this suggests that the observed pattern might be due to introgressive hybridization. Interestingly, no APS were found in the ITS sequences of both accessions, which might be explained by repeated backcrossing towards *S. auriculata*/*S. lyrata* (chloroplast capture). Geographic patterns in plastid phylogenies, as opposed to those in ITS which are often morphology-corroborated, have been found in several other plant genera, including *Phlomis* (Lamiaceae; Albaladejo et al. 2005), *Mitella* (Saxifragaceae; Okuyama et al. 2005), or *Antirrhinum* (Plantaginaceae; Vargas et al. 2009). However, analysis of plastid data may also correctly infer relationships which are blurred in ITS. This is the case in e.g., the Scariosa clade, which is expected to include *S. hierochuntina* Boiss., 1853 based on morphological evidence (Boissier 1879, *Flora Orientalis*; Eig 1944; Grau 1980). Correct interpretation of phylogenetic relationships in *Scrophularia* therefore requires careful comparisons of all results.

Recent or ancient hybridization can result in deviating ITS copies (see above) as well as incongruence among markers (Fuertes Aguilar and Nieto Feliner 2003; Vriesendorp and Bakker 2005; Peng and Wang, 2008; Vargas et al. 2009), and obviously had an important impact on the speciation process in *Scrophularia* (as demonstrated in Scheunert and Heubl 2014). In such cases, cloned sequences are particularly useful as they can provide information on putative parent lineages (Fig. 3). In the species studied here, clones in general contained all variation corresponding to the extracted APS from the "direct sequences" (i.e., obtained from direct sequencing), and often even more. This means that cloning should be favored over direct sequencing as far as possible, especially when high polyploids are present and polymorphic sites are more easily missed (Joly et al. 2006). *Scrophularia auriculata* (with 2n = 84 chromosomes) was already proposed

to be an allopolyploid resulting from hybridization between *S. lyrata* or *S. hispida* Desf., 1798 (both 2n = 58) and *S. umbrosa* (2n = 26) or their ancestors (Grau 1979; Scheunert and Heubl 2014). The English voucher specimen of *S. auriculata*, with its chromosome number determined to match the typical number for the species, has as much as 13 PS in the direct sequence. Cloned sequences are separated into two distinct clades: six clones obtain a position similar to that of the accession in the cp tree; they are part of a clade also containing both direct sequences of *S. auriculata*, *S. racemosa* Lowe, 1831, and *S. hispida*. The fact that the direct sequence of *S. auriculata* England is sister to *S. hispida* using the 2ISP-coded dataset, and that none of the clones is found within the monophyletic clade of clones from *S. lyrata*, argues for *S. hispida* as potential parent. The remaining ITS clones are situated in a monophyletic clade at the basal polytomy of the IPM clade, but without depicting a sister relationship to *S. umbrosa*. This might suggest that the hybridization event is more ancient, with more time for accumulation of autapomorphisms in *S. auriculata*. In such cases, parental lineages would be more difficult to trace due to a greater accumulation of autapomorphies (Wolfe and Elisens 1994; Baumel et al. 2002; Vargas et al. 2009). The distinct status of *S. auriculata* England is also illustrated in the NN, where it is isolated in the Auriculata subclade and positioned closer towards the Scopolii clade (Clade 8), another potential parental lineage related to *S. umbrosa* (Fig. 4, species marked by an asterisk within the subclade). It remains unclear why traces of the hybrid ancestry of *S. auriculata* can be found in only one of the sampled accessions. However, the variable, intermingled phylogenetic relationships and weakly defined species boundaries among the closely related taxa of the IPM clade have not been fully recovered yet.

The Caucasian endemic *S. ruprechtii* is part of a predominantly Turkish-Caucasian clade in ITS. It was cloned to elucidate a possible hybrid origin concerning the Ningpoensis clade, with which it is closely associated in the cp tree, resulting in a clearly intermediate placement in the CN (Fig. 5a). However, although two diverging ITS ribotypes were found, neither of them bear signs of relationships with lineages from outside Tomiophyllum. Close relationships with *S. olympica* and *S. imerethica* Kem.-Nath., 1955, are corroborated by six of the clones, a clade of four clones remains unresolved.

The six species of the "Scoparia" clade (Clade 14) in ITS occur in partly overlapping distribution areas ranging from Central Asia across Afghanistan and the Western Himalayas to China and Siberia. They represent a rather homogeneous assemblage of subshrubs featuring few-flowered cymes, exserted stamens, linear staminodes, and leaves which are divided to various extents. *Scrophularia multicaulis* Turcz., 1840, a perennial species with included stamens, does not fit into this pattern, and also is the only species without any PS in the ITS sequences, while two (in *S. kiriloviana* Schischk., 1955) to eight (in *S. incisa*) PS are found in all other members. The clones from *S. incisa* reveal two main ribotypes, one of which is associated with *S. multicaulis*. Three of the clones remain unresolved within the clade. It seems reasonable to assume that *S. incisa* was the result of a hybridization event that involved *S. multicaulis* and a second member from the Scoparia clade, which would explain the morphological similarities. Shared PS in the ITS sequences connect *S. incisa* to *S. scoparia* Pennell, 1943, but also to *S. dentata* Royle ex Benth., 1835. Further evidence can be drawn from the occurrence of a sequence length polymorphism within ITS2, which is characteristic for the Scoparia clade and was excluded from calculations. The morphologically very variable *S. scoparia* (as all other sampled *Scrophularia* accessions) has a clear sequence containing a GTG motif, while *S. multicaulis* shows a clear sequence with a GTGTG motif at the respective position. *Scrophularia incisa* (as well as all other members of the clade) features a length polymorphism, with some ITS copies having GTG (here present in clones 6 and 15) and others having GTGTG. This might support the hypothesis that *S. scoparia*, which is likewise unresolved in Fig. 3, acted as second parent for *S. incisa*. Apart from the complex relationships within the clade, the Scoparia clade is also entangled with the Polyantha clade, whose species according to the ITS tree are distributed from Central Asia and Afghanistan southeastwards as far as the Eastern Himalayas. Three taxa (among those *S. incisa* as well as *S. multicaulis*) switch positions between the two clades in plastid and nuclear reconstructions, resulting in their intermediate position in the CN (Fig. 5a, marked by asterisks).

The origin of *Scrophularia*

Although divergence dating should rely on results from different cell compartments whenever possible, we refrained from performing molecular clock analyses on the ITS dataset. In *Scrophularia*, the respective sequences are highly polymorphic and considerably influenced by reticulation. This means that they cannot provide an entirely tree-like signal, which makes them unsuitable for molecular clock or ancestral area inferences. Results are therefore based on one, the plastid, marker only. However, it still should be kept in mind that estimation of divergence times might be impaired by the presence of a reticulate history. For example, for a hybrid lineage, one distinct time of divergence may not represent its true history which might have involved several periods of gene flow (Payseur and Rieseberg 2016) or several independent hybridization events. Furthermore, gene flow between two species might lead to underestimation of their divergence time, as demonstrated by Leaché et al. (2014) for species trees.

According to the molecular dating analysis, *Scrophularia* diverged from *Verbascum* around the Oligocene/Miocene

boundary (approximately 23 mya), with diversification of major lineages starting in the Miocene, within approximately the last 15 my. These results are comparable to those of a recently published time-calibrated phylogeny based on *ndhF* sequence data of Lamiales, where the authors used a combination of several fossil and secondary calibrations (Navarro Pérez et al. 2013). Ages as inferred here are in clear contrast with considerably older divergence times obtained in a small-scale study on New World species based on secondary calibration of the root only (Scheunert and Heubl 2011). This is probably due to too small taxon sample size and the choice of method and information source for obtaining secondary calibration points. Ancestral area reconstructions revealed that *Scrophularia* originated in a region comprising Southwestern Asia and Turkey (Fig. 1a, node 3), which corresponds to its present-day primary center of diversity. This contradicts Stiefelhagen (1910), who promoted the Himalaya as ancestral region for the genus.

In the Miocene, Southwestern Asia was under strong influence of processes initiated by the collision of the Arabian with the Eurasian plate, which had started in the late Eocene and led to the formation of the main mountain ranges within the region (Djamali et al. 2012a). During that period, Asian biota also were affected by substantial climatic and environmental changes, including a shift towards increased seasonality (An et al. 2001) and the aridification of interior Asia (Guo et al. 2002, 2004; Fortelius et al. 2006; Miao et al. 2012). A combination of these processes is likely to have triggered the divergence and diversification of *Scrophularia*. At the time inferred for the origin of the genus, the final closure of the Tethyan seaway and the retreat of the Paratethys ocean (Ramstein et al. 1997; Mouthereau et al. 2012) created additional suitable land area for plant colonization in Southwestern Asia, e.g., in the Zagros region. While the Greater Caucasus was still largely submerged at that time, the Alborz and Anatolian highlands already existed for a longer time (Popov et al. 2004). Also, according to time estimates presented here, diversification of *Scrophularia* into its main lineages began during a period where new suitable habitats for species adapted to montane environments came into existence in its ancestral region; by the emergence of the Greater Caucasus and its transformation into a mountain chain (approximately 14–13 mya and 9 mya; Meulenkamp and Sissingh 2003; Popov et al. 2004; Olteanu and Jipa 2006), the uplift of the Iranian Plateau (from 15–12 mya; Mouthereau et al. 2012; Djamali et al. 2012a and references therein), and the formation of the Western Alborz (approximately 12–10 mya) and Zagros (approximately 15–10 mya) mountains (Dercourt et al. 1986; Popov et al. 2004; Guest et al. 2007; Mouthereau et al. 2012). Additionally, the establishment of continental climate conditions (Berberian and King 1981) with increasing aridity (Ballato et al. 2010) during this period could have triggered the colonization of more moderate, humid habitats of higher elevations (Roe 2005).

Establishment of the genus in mountainous regions might have been accelerated by its potential for frequent and successful interspecific hybridization and (allo-)polyploidization. Both evolutionary processes enable adaptation to new or hitherto unsuitable environments by the rapid formation of new phenotypic combinations, promoting adaptive radiation and colonization, and thus have been associated with speciation events and diversification (Stebbins 1959; Seehausen 2004; Mallet 2007; Estep et al. 2014). While *Verbascum* resembles its sister *Scrophularia* in many respects including habitat requirements and a Southwestern Asian center of diversity, the former genus apparently lacks the aforementioned peculiarities. Although species easily hybridize wherever they grow together, the resulting offspring is always sterile according to Murbeck (1933) and Huber-Morath (1978). Reported chromosome numbers within *Verbascum* mostly range between 2n = 30–36, with the highest count being 2n = c. 64 (Goldblatt and Johnson 1979–). In contrast, *Scrophularia* features numbers from 2n = 18 (in *S. altaica* Murray, 1781) up to 2n = 96 (in the NW/Japan clade), with several clades being characterized by (high) polyploidy (Shaw 1962; Goldblatt and Johnson 1979–; Scheunert and Heubl 2014). It seems worth mentioning that the highest polyploids within *Scrophularia* are concentrated in regions where only few or no *Verbascum* species occur (i.e., China and the New World). Carlbom (1969) concluded that polyploidy in *Scrophularia* has triggered migration into the rough environments of higher altitudes as well as regions of higher latitudes. The predisposition for polyploidy and hybridization found in *Scrophularia* is shared by other lineages within Scrophulariaceae (e.g., *Diascia* or *Nemesia*; Steiner 1996; Datson et al. 2006) and related families (e.g., Clay et al. 2012 and references therein; Rojas Andrés et al. 2015).

Apart from that, mountainous regions themselves promote plant species diversification: diversification rates have been shown to be elevated in regions subjected to active orogeny (for a short overview see Hoorn et al. 2013 and references therein), and mountain ranges provide complex, heterogeneous habitats on small geographical scales, which facilitate adaptive speciation and allopatric divergence (Lobo 2001; Djamali et al. 2012a and b and references therein; Wen et al. 2014), and can also stabilize new hybrids by isolating them from their parents. These conditions might be assumed to also have produced the high levels of endemicity in *Scrophularia* as mentioned by Vaarama and Hiirsalmi (1967). Even in the absence of detailed distribution mapping, it seems likely that a combination of geographic isolation/habitat fragmentation and successful hybridization has been the key factor in the diversification of *Scrophularia*. This has also been suggested for other genera occurring in the Tibetan Plateau or mountainous regions of the Mediterranean (e.g., Senecioneae,

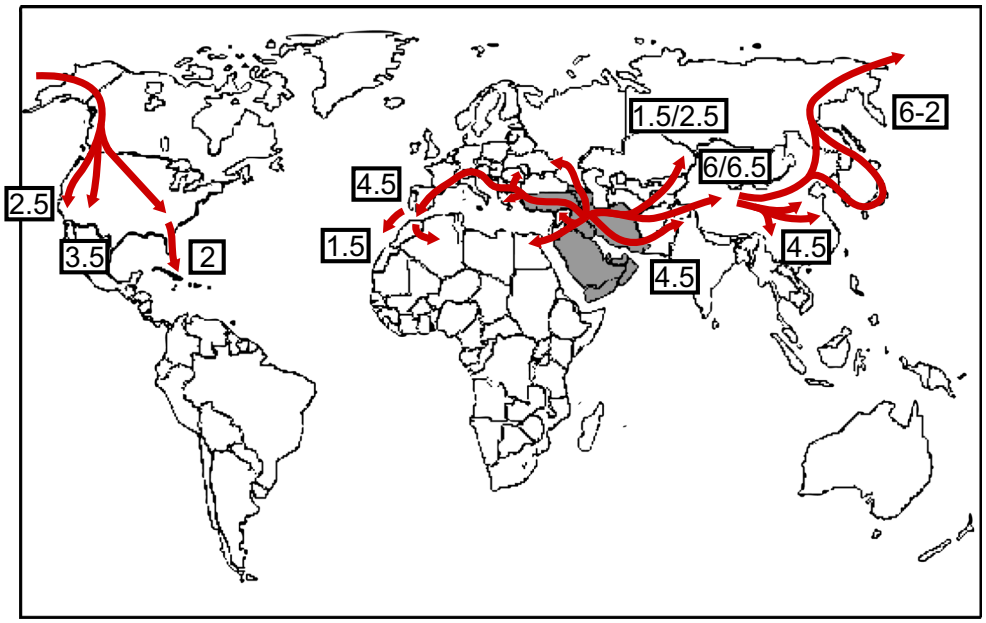


Fig. 6 Biogeographic history of *Scrophularia*. The ancestral region of the genus is marked in *gray*; *red arrows* indicate main dispersal routes as inferred from plastid DNA data. Note that arrows in some cases may illustrate more than one dispersal. *Boxes next to arrows* denote the approximate time of the migration event in million years ago, drawn from crown ages of the respective clades

Asteraceae, J-Q Liu et al. 2006; *Linaria*, Plantaginaceae, Blanco-Pastor et al. 2012; *Meconopsis*, Papaveraceae, Yang et al. 2012; *Rhodiola*, Crassulaceae, Zhang et al. 2014).

A remarkable result in the ITS tree is the basal position of the annual *S. arguta* as sister to the remainder of the generally persistent *Scrophularia* (Fig. 2a, node 3). However, upon a closer look, this position might likely be artificial. Potentially higher evolutionary rates in annuals can result in support of a sister relationship of the former to their perennial relatives (e.g., Laroche and Bousquet 1999; Andreasen and Baldwin 2001; Tank and Olmstead 2008; Müller and Albach 2010; J-X Yue et al. 2010). But, although rarely, other annual species are found in *Scrophularia* which obtain inconspicuous placements, e.g., in Clade 6 or 10 (*S. calycina* Benth., 1835, *S. peregrina* L., 1753). More importantly however, and unlike most of the other *Scrophularia* species, self-pollination is common in *S. arguta*, and the species is unique within the genus in possessing a mixed mating system of chasmogamous flowers occasionally complemented by small cleistogamous flowers near the ground. High substitution rates have been correlated with a selfing breeding system (Glémin et al. 2006), although this is subject of debate (Wright et al. 2002; Müller and Albach 2010). Moreover, mating shifts seem to create strong interspecies isolation barriers which effectively prevent hybridization (Wright et al. 2013). The Arguta lineage might thus owe its isolated position to the accumulation of mutations due to breeding system effects. It is likely that the particular reproductive traits of *S. arguta* have enabled colonization of habitats otherwise unsuitable for *Scrophularia*. In the hot and dry environments of e.g., the Sudan, Eritrea, Somalia and Oman, *S. arguta* consequently is the only representative of the genus. In the plastid tree, the species is shown as an earlier diverging lineage and is sister to the Tomiophyllum clade. Its ancestral area could not be satisfactorily determined; analyses with a maximum of five areas yielded an ancestral range corresponding to its present-day distribution (areas ABDEI; Fig. 1a, node 85). A recent study on *S. arguta*, based on representatives from several populations although not covering the whole distribution range, found evidence for a westward expansion from the east of its distribution range (Valtueña et al. 2016), which reaches its limit on the Arabian Peninsula.

Reconstruction of major evolutionary events

Based on individual clade ages and ancestral areas (Fig. 1a), several expansions of the genus in various directions can be hypothesized (Fig. 6). Eastward migration events to China and the Tibetan Plateau region are inferred at approximately 6 mya (nodes 10 and 24) and later. Given the preference for mountainous habitats in *Scrophularia*, dispersal along higher mountain chains as corridors for colonization seems reasonable. Evidence for growth of the Kunlun Shan in the northern part of the Tibetan Plateau has been reported since Eocene times (see Yuan et al. 2013), while uplifts of the Tian Shan located to the northwest presumably occurred from the late Oligocene through the Miocene until approximately 7 mya or later (Abdrakhmatov et al. 1996; Sobel et al. 2006; citations in Miao et al. 2012). The Hindu Kush, which spreads farther west into Afghanistan, underwent uplift around the Oligocene-Miocene boundary (Hildebrand et al. 2000), with tectonic processes in the region occurring much earlier (Dercourt et al. 1986; Hildebrand et al. 2001). The Kopet Dag finally, roughly situated between the Hindu Kush in the east and the Alborz of Iran in the west, only re-emerged as a mountain

chain at approximately 10 mya or later, after submergence following higher-altitude phases from the late Oligocene until the early Miocene (Dercourt et al. 1986; Popov et al. 2004). Altogether, this means that by the time inferred for the first eastward migrations of figworts, a more or less continuous mountain belt should have existed, which connected the Southwestern Asian Alborz to the Himalayas and the Tibetan Plateau, and presumably provided a suitable pathway for dispersal of *Scrophularia*. Transitions of this kind from Western/Central Asia to Eastern Asia (or vice versa) have been reported in several other genera, including *Incarvillea* (Bignoniaceae; S-T Chen et al. 2005), *Rhodiola* (Zhang et al. 2014), or *Solmslaubachia* (Brassicaceae; J-P Yue et al. 2009); the latter genus inhabits alpine scree-slope habitats similar to several *Scrophularia* species.

China has been colonized at various times and by different lineages; the most important secondary diversity center of *Scrophularia* harbors, among others, species from the "Vernalis" (Clade 9) and Calycina clades, *S. umbrosa*, and species from the Scoparia/Polyantha clade of Tomiophyllum. These mainly alpine taxa have extended their distribution areas from Central Asia, Siberia, or Southern Asia into China. In contrast, the China and Ningpoensis clades almost exclusively consist of Chinese species. When disregarding altitude, Chinese *Scrophularia* have rather similar habitat requirements (especially within the China clade s.str. as shown in Fig. 2); they occur in humid conditions in mountainous forests or grasslands, often in crevices and among rocks.

The Ningpoensis clade (Clade 2), with its crown age estimated at approximately 3 my, constitutes an eastern group of mainly non-alpine taxa including the pharmaceutically important *S. ningpoensis* Hemsl., 1899. Its distribution extends from the eastern parts of China to Korea, Japan, and Taiwan; repeated contacts between those landmasses from the late Miocene onwards offered opportunities for dispersal (references in Qiu et al. 2011). In contrast, the taxa of the China clade s.str. (Clade 3 in the ITS tree) are mostly alpine species with mostly narrow distributions. Most species from the "Hengduan" subclade resolved in the plastid tree are restricted to the Hengduan mountains (located in parts of Sichuan and Yunnan provinces with adjacent Tibet), which are considered one of the world's biodiversity hotspots (Mittermeier et al. 2004). The "Central" subclade comprises high-alpine and subalpine species distributed in central parts of China.

Crown ages of both subclades were estimated at about 4.5 my during the Pliocene. Many authors have attributed diversification events in the region to the uplift of the Tibetan Plateau (see review by Qiu et al. 2011 and references in Y-S Sun et al. 2012), often without checking for exact spatial and temporal concordance among inferred divergence times and geological events. This however seems to be indispensible given the complex geological history of the Tibetan Plateau and the ongoing debates on appropriate models for its formation (Yuan et al. 2013; C-S Wang et al. 2014; J-J Li et al. 2015). Diversification of the Hengduan subclade from the early Pliocene on coincides with a period of uplift hypothesized for the Hengduan Shan by B-N Sun et al. (2011) and Ming (2007). However, it may not be necessary to invoke uplift as a cause for diversification when its result seems to be more important: the extreme topography with alternating high peaks and deep ridges and the variety of vegetation types and climatic conditions which characterize this biodiversity hotspot (see Boufford et al. in Mittermeier et al. 2004) effectively stimulates speciation and leads to high species diversity and endemism. Generally, high species numbers have been linked with the "extreme physiographical heterogeneity of temperate eastern Asia" by Qian and Ricklefs (2000). Finally, the present-day distribution patterns of the Hengduan and Central subclade species might also be the result of recolonization after the Last Glacial Maximum (approximately 24,000–18,000 years ago) from suitable refugial areas in the region; such have been recognized in the Hengduan Shan (e.g., for *Metagentiana*, Gentianaceae, S-Y Chen et al. 2008; *Angelica*, Apiaceae, Feng et al. 2009; *Lepisorus*, Polypodiaceae, L Wang et al. 2011) and, for the Central subclade, in the Qinling mountains or, more generally, the "Northeast Qinghai-Tibetan Plateau edge" (Qiu et al. 2011).

According to our reconstructions, North America was colonized up to three times independently from Eastern Asia (Figs. 1a, 2). The Japanese and most of the New World taxa are connected to the Nodosa clade (plastid tree) as well as taxa from the China clade, with whom they share a clade in the uncoded ITS tree (Fig. 2b, node A) and the coded ITS+clones tree (Fig. 3). Divergence from the Asian ancestors has happened approximately 6 mya at the earliest (Fig. 1a, node 24). This corresponds to a general Eastern Asian-North American disjunct pattern found in many plant genera, e.g., *Picea* (Pinaceae; Lockwood et al. 2013), *Gleditsia* (Fabaceae; Schnabel et al. 2003), *Triosteum* (Caprifoliaceae; Gould and Donoghue 2000), and also Scrophulariaceae (Hong 1983), among many others (see H-L Li 1972; Boufford and Spongberg 1983; Hong 1993; Xiang et al. 1998; Wen et al. 2010, 2014). Similar divergence times of North American from Eastern Asian lineages have been reported in *Kellogia* (Rubiaceae, 5.42 ± 2.32 mya; Nie et al. 2005) and *Rhodiola* (5.3 mya, 95% HPD 2.3–9.1 mya; Zhang et al. 2014). Both studies suggest long-distance dispersal for colonization of North America. Zhang et al. (2014) additionally hypothesize migration across the Bering Land Bridge (BLB; Tiffney and Manchester 2001); this was also proposed for *Angelica genuflexa* max. 4.3 mya, by Liao et al. (2012). During which periods and how long the BLB was available for plant migrations remains a matter of debate. Estimates for the opening of the Bering Strait range from 3.3 to 9 mya (Brigham-Grette

2001; Denk et al. 2011); however, even after the final flooding, intermittent short-time closures of the Strait have been assumed, among others at 4.9, 4.0, 3.3, and 2.5 mya (KG Miller et al. 2005). Cold-adapted *Scrophularia* taxa could have spread to the New World during times when landmasses were connected; long-distance dispersal however is likely to have played a role as well, especially during later periods (see Fig. 1a, nodes 25, 26, 28). *Scrophularia* seeds do not possess special adaptations favoring any mode of dispersal; however, they are easily dispersed by wind due to their small size and weight, and thus may not have been dependent on suitable land bridges.

In accordance with results obtained by Scheunert and Heubl (2011), the New World taxa of *Scrophularia* are divided into three geography-based clades, whose distribution ranges do not overlap much and which receive stronger supports in the plastid tree. Most of the North American species are closely related; they have been successfully hybridized (Shaw 1962) or even intergrade naturally in contact zones (e.g., *S. parviflora* Wooton & Standl., 1913 and *S. californica* Cham. & Schltdl., 1827; Kearney and Peebles 1951). Shaw (1962) emphasized that rather than reproductive isolation, geographic barriers seem to play an important role in maintaining the species (compare Carlbom 1969), an assumption that exactly fits the general diversification mechanisms discussed above and is corroborated by the characteristics of the three clades found here. A similar situation was reported for *Jamesbrittenia*, another Scrophulariaceae genus prone to successful interspecific hybridization, where geography helps to maintain species identity (Verboom et al. 2016). Species diversity is greater in the west (10 species) than in the east of the North American mainland (two species), possibly due to greater geographic heterogeneity in the former (Qi and Yang 1999) but also the California floristic province biodiversity hotspot located there (Mittermeier et al. 2004).

Apart from eastward migrations by *Scrophularia*, resulting in the China and New World clades, westward movements from the ancestral region led to the colonization of the Mediterranean, Northern Africa, and Europe. Scheunert and Heubl (2014) recently found that the IPM clade (Fig. 2a, node 32/Fig. 1a, node 45), which comprises the majority of Iberian and Macaronesian species, is of hybrid origin, involving progenitors both of the Scopolii clade or *S. umbrosa*, and the "Canina" clade (Clade 11) or allies (Fig. 2a, node 31 or 32/Fig. 1a, node 44). According to reconstructions using the plastid dataset, these ancestors were distributed in Southwestern Asia and in the Turkey-Caucasus region, respectively (Fig. 1a, node 40 or 86/44). The approximate time of the hybridization event, limited by divergence from the parental lineage and diversification of the hybrid lineage, is assumed at around 5 mya; the ancestor of the IPM clade was most likely distributed in the Western Mediterranean and diversified from about 4.5 mya (Fig. 1a, node 45; Scheunert and Heubl 2014). The role of the Irano-Turanian floristic region as a key source for colonization of the Mediterranean has been emphasized (Comes 2004; Djamali et al. 2012b), especially for temperate elements (Quézel 1985; Thompson 2005; Mansion et al. 2008).

The Tomiophyllum clade

The relative ages of the two main lineages within *Scrophularia* have been discussed by various authors. *Scrophularia* sect. *Tomiophyllum* might be regarded as "primitive" due to putatively ancestral traits like the often xerophytic, subshrubby habit of its members and the general lack of polyploid chromosome numbers (Carlbom 1969). The section is centered in the Caucasus, Iran, Iraq, and Turkey, with approximately half of the sampled taxa distributed there, and is completely absent from Macaronesia and the New World. On the other hand, the mainly herbaceous, richly foliated, often meso- or hygrophytic members of *S.* sect. *Anastomosantes* (Stiefelhagen 1910) are characterized by a large number of polyploid species, a wide ecological amplitude and a geographic distribution which exceeds that of *S.* sect. *Tomiophyllum* by far. Regarding molecular results, the Tomiophyllum lineage is highly supported as a distinct clade in both analyses and is nested within clades of Anastomosantes taxa (Figs. 1a and 2a). This reveals *S.* sect. *Tomiophyllum* to be derived from within *S.* sect. *Anastomosantes*.

The factors leading to the evolutionary success of the Tomiophyllum lineage remain uncertain. One might speculate that changes in aridity in its ancestral region during the second half of the Miocene and later (Ballato et al. 2010) had an influence on its divergence and diversification (which started approximately 8 mya). While ecological preferences of members of *Scrophularia* sects. *Anastomosantes* and *Tomiophyllum* overlap, their habitats reveal a certain shift from moist sites on riverbanks and in forests (in the former) towards rock crevices and gravelly substrates with low humidity in the latter, illustrating a greater tolerance of dry conditions. This is also reflected in differences in their respective distributions, with Tomiophyllum species predominantly inhabiting the dry parts of e.g., Iran and Turkey, while not necessarily being absent from other areas. Several of the xerophytic species also take advantage of the lower temperatures at higher altitudes; the few desert representatives tend to flower during early spring, with their rhizomes persisting in crevices during hot periods.

It is noteworthy that the phylogenetic (and also morphological) boundaries between the Anastomosantes and Tomiophyllum groups are also not altogether strict. For example, the position of *S. megalantha* Rech.f., 1955 in the NN, with no unequivocal connection to any of the Anastomosantes clades and closer to the center of the graph (Fig. 4), indicates a certain affinity to the Tomiophyllum clade. This is also supported by the trees in Figs. 1a and 2a, however, with

consistently weak supports. On the other hand, similarities to *S.* sect. *Anastomosantes* can be found in some members of the Tomiophyllum clade, e.g., in the mainly Turkish and Caucasian *S. ilwensis*, which was considered part of *S.* sect. *Anastomosantes* by Stiefelhagen (1910) and occurs in more humid habitats like forests or near water. While plastid reconstructions of the Tomiophyllum clade support a main split into two, once more geography-based, main clades, this species is sister to the remainder of Tomiophyllum in the ITS trees (Fig. 2). Its distinct status is reflected also in its intermediate position in the NN (Fig. 4). Apart from *S. ilwensis*, 14 further taxa (including the "Striata" (Clade 16) and Scariosa clades) obtain basal positions within the Tomiophyllum clade in the uncoded ITS tree. In the 2ISP-coded tree, these species form a clade; both placements are however weakly supported (Fig. 2a, node 87; Fig. 2b, nodes 52 and 53a) due to inherent ambiguity in the data as discussed above. The NN (Fig. 4, square bracket) clearly depicts the complex relationships of this assemblage and also a certain shift towards Anastomosantes. *Scrophularia nabataeorum* Eig, 1944 from the Scariosa clade indeed features morphological traits typical for *S.* sect. *Anastomosantes* as well: Eig (1944) described its ambiguous characteristics in between the two main sections and stated himself being "undecided as to the affinity of this species." Interestingly, the Striata clade comprises some of the few species that have colonized truly arid environments, occurring in steppe and desert habitats.

Both Striata and Scariosa clade give evidence for migration into areas west and southwest of present-day Iran, which was facilitated since the dry-up of the Mesopotamian Basin in the late Miocene (Popov et al. 2004). This has led to the colonization of the Levant, the Arabian Peninsula south of Iraq, and also Eastern North Africa by members of these clades. Notably, while several IPM clade species also extend into (or are endemic for) western regions of North Africa, the three species confined to its eastern part are found within the Striata and Libanotica clades. Other species occur throughout Northern Africa (*S. canina*, *S. peregrina*, *S. syriaca* Benth., 1846, *S. arguta*).

Conclusions

The present paper represents the first comprehensive phylogenetic study of the genus *Scrophularia*, based on a broad taxon sampling including representatives of all sections. This study has confirmed the monophyly of the genus but has also provided evidence for significant phylogenetic incongruence and ambiguity among and within sequence datasets. Exemplary cloning of taxa showed that intra-individual site polymorphism in ITS is widespread. Our study suggests that conflicting signals in *Scrophularia* derive from a variety of sources, most importantly reticulation, due to frequent hybridization and introgression. The methodical workflow as presented here is suitable for any plant group where similar problems are encountered and laborious search for (potentially likewise problematic; Nieto Feliner and Rosselló 2007) low-copy nuclear markers or cloning of all taxa cannot be considered.

The molecular phylogenies revealed two large groups of species (of which only one is monophyletic) corresponding to previously described taxonomic entities. The emergence of *Scrophularia* in the Miocene and its diversification are closely linked to geological and climatic events in the Irano-Turanian region and Central/Eastern Asia. Most diversification events as well as further successful dispersals to other regions were dated to the colder Pliocene-Pleistocene period.

The inferred spatio-temporal framework will provide a solid basis for future studies focusing on specific clades or morphological questions. It can be assumed that the considerable morphological variability is linked to the complex evolutionary history of the genus; this is relevant also regarding previous taxonomic concepts, which need to be re-evaluated. A survey of the relevant morphological traits, together with karyological analyses, will complement the present study from a more taxonomic perspective, also with respect to the small Himalayan genus *Oreosolen*, which has to be transferred to *Scrophularia* (Scheunert and Heubl in preparation).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

This article does not contain any studies with human participants or animals performed by any of the authors.

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5. General Discussion

5.1. Phylogenetic relationships in Rhinantheae and taxonomic implications

Our analyses of tribe Rhinantheae yielded two phylogenetic trees partially supporting differing relationships. However, pruning of five incongruent taxa (see below) and subsequent combination of the data resulted in a better resolved phylogeny with a highly supported backbone. The phylogenies of Rhinantheae, as presented in Article I (and summarized in Fig. 9), corroborated previous findings in several ways. *Melampyrum* was sister to all other taxa within Rhinantheae in accordance with Bennett and Mathews (2006), and *Rhinanthus*, *Lathraea* and *Rhynchocorys* were grouped together in what we referred to as the "RRL clade". *Bartsia alpina* was the first-branching taxon within the core group of Rhinantheae (in agreement with Těšitel et al., 2010). This result was consistent among four accessions covering the geographic range of the species. It has already been known for some time that the New World species of *Bartsia* s.l. do not cluster with the generic type, but with species of *Parentucellia* (see below). However, our analyses for the first time showed that the East African species of *Bartsia* s.l. are distinct as well: they shared a clade with the monotypic *Hedbergia abyssinica*, far from *B. alpina* and New World *Bartsia* s.l.

As a consequence, some taxonomic rearrangements were necessary for the polyphyletic *Bartsia* (changes are mapped in Fig. 9). In the circumscription of Molau (1990), the genus contained 49 annual or perennial, herbaceous to rarely suffrutescent species: *Bartsia alpina* in *Bartsia* sect. *Bartsia*, two afromontane species within *B.* sect. *Longiflorae* Molau, *B. trixago* in *B.* sect. *Bellardia* (All.) Molau, and 45 South American species in four sections, *B.* sects. *Orthocarpiflorae* Molau, *Strictae* Molau, *Laxae* Molau and *Diffusae* Molau. According to our taxonomic concept, the generic type *Bartsia alpina* (photo see Fig. 9) is left alone in the traditional genus *Bartsia*, which is now defined as a monotypic genus containing one perennial, obligate hemiparasite with the following characteristics: geophytic, possessing a persistent subterraneous rhizome and annual aerial shoots, distributed in alpine and subarctic regions of Europe and Northeastern North America. The two afromontane species of *Bartsia* s.l., native to Eastern Africa from Ethiopia to Tanzania, are clearly connected to *Hedbergia* Molau by their vegetative morphology and palynological characters. *Hedbergia abyssinica*, a perennial hemiparasitic subshrub distributed in montane Western (Nigeria and Cameroon) and Eastern Africa (from Ethiopia to N-Zambia and Malawi), was separated from *Bartsia* s.l. by Molau (1988) due to its distinct flower morphology (rotate instead of bilabiate corolla, see photo in Fig. 9). However, this characteristic trait might simply represent an adaptation to a special pollinator. Species of *B*. sect. *Longiflorae* sensu Molau (1990) were therefore transferred to *Hedbergia*, which now consists of perennial hemiparasitic subshrubs with a rotate or bilabiate corolla and an afromontane distribution.

The third group within the polyphyletic *Bartsia* s.l. is unequivocally associated with *Parentucellia* and *Bellardia* as discovered earlier (Bennett and Mathews, 2006; Těšitel et al., 2010): the chiefly Mediterranean *Parentucellia latifolia* (L.) Caruel is sister to a clade of *Bartsia* s.l. from the Andean montane habitats of Colombia, Bolivia, Peru, and Chile to Northern Argentina. Species of *Bellardia* and *Parentucellia* are annual, facultative hemiparasites with a native distribution range predominantly in the Mediterranean (with *Parentucellia* also reaching farther east into Asia). Apart from that, both genera are introduced as noxious weeds to e.g. Australia and America. The

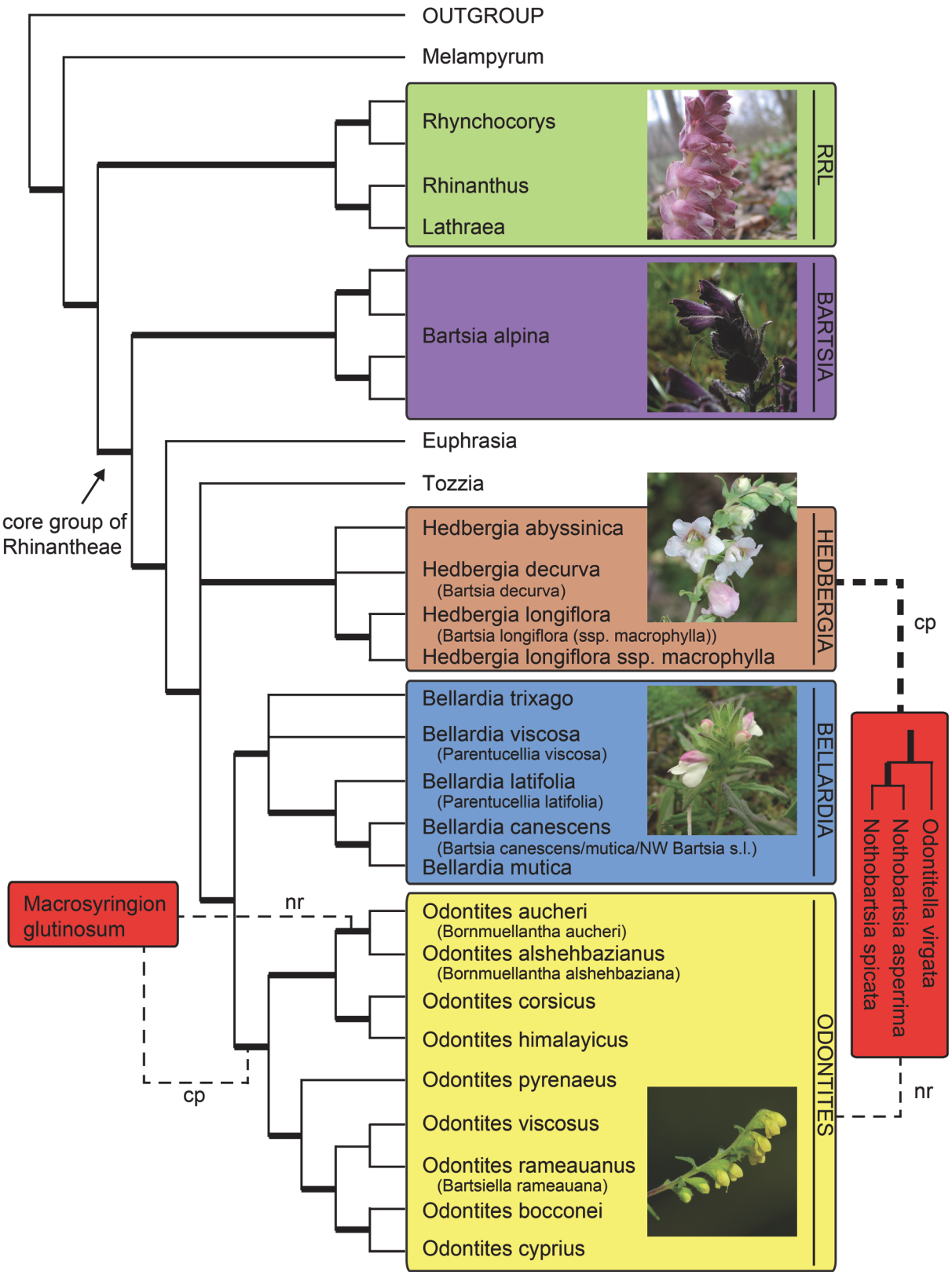

Fig. 9. Phylogenetic relationships in Rhinantheae. Consensus tree yielded from analysis of a combined plastid and nuclear dataset. Only nodes with supports PP ≥ 0.80 or BS ≥ 75 are shown. Bold lines represent nodes with supports of PP ≥ 95 or BS ≥ 85. Divergence of the core group of Rhinantheae is marked by an arrow. Species which were subject to taxonomic changes are given with their former names in brackets. Four accessions showing plastid-nuclear marker incongruence are connected to their respective sister groups in the chloroplast (cp) or nuclear (nr) trees by dashed lines. Photos of representative species, from top to bottom: *Lathraea squamaria* L. (photo by Andreas Fleischmann), *Bartsia alpina* L. (photo by Andreas Fleischmann), *Hedbergia abyssinica* (Benth.) Molau (photo by Jakub Těšitel), *Bellardia trixago* (L.) All., *Odontites viscosus* (L.) Clairv. (photo by Andreas Fleischmann). RRL, Rhynchocorys-Rhinanthus-Lathraea clade

monotypic *Bellardia* itself used to be part of *Bartsia* s.l. sensu Molau (1990; see above), but later again was regarded different because of a deviating corolla, calyx and seed morphology. Although not visible from the phylogenetic tree, consensus networks reveal a certain relationship of *Bellardia* and *Parentucellia viscosa* (L.) Caruel (see Fig. 4 of Article I). This is corroborated by morphological similarities, overlapping distribution ranges, reports of putative hybrids among the two species, and also by results of Uribe-Convers and Tank (2016). As there seems to exist a continuous pattern of shared characteristics leading from *Bellardia* to *P. latifolia*, we included *Parentucellia* into *Bellardia*. To avoid paraphyly of *Bellardia*, Andean *Bartsia* s.l. were also transferred into this genus (however we limited taxonomic combinations to the species actually analyzed). This classification implies a long-distance dispersal of the Mediterranean ancestor of the Neotropical lineage with subsequent adaptive radiation, resulting in a disjunct pattern which is not uncommon (see e.g. Calviño et al., 2008; Bräuchler et al., 2010; Kadereit and Baldwin, 2012) and has its counterpart in the establishment of an African lineage from Mediterranean ancestors (or, more general, European ones, as put by Uribe-Convers and Tank, 2015) in the Hedbergia clade, as inferred by Těšitel et al. (2010). *Bellardia* thus now comprises annual as well as perennial hemiparasites. A different approach was taken by Uribe-Convers and Tank (2016), who accepted *Bellardia viscosa* (L.) Fisch. & C.A.Mey but transferred *Bellardia latifolia* (L.) Cuatrec. back to *Parentucellia*. For the Andean species of *Bartsia* s.l., they proposed a new genus, *Neobartsia* Uribe-Convers and Tank, with 47 species. Although this classification encompasses all of the New World species (while Article I only included two of them), it leaves important questions regarding the considerable similarities of the involved taxa unanswered.

In relation to *Bornmuellerantha*, which was found to be nested in *Odontites* by Těšitel et al. (2010), our results revealed that another two of the small genera segregated from *Odontites* by Rothmaler (1943) and Bolliger (1996), based on divergent corolla morphology and pollen characters, are in fact part of *Odontites*: *Bartsiella* and *Macrosyringion*. *Bornmuellerantha* and *Bartsiella* were re-included into a broad circumscription of *Odontites*; however, no final decision was made on *Macrosyringion*: although *M. glutinosum* (M.Bieb.) Rothm. was nested in *Odontites* as sister to *Bornmuellerantha* in ITS, its placement was incongruent regarding the plastid markers, where it was weakly supported as sister of *Odontites* s.l. (Fig. 9). Several cases of reticulate evolution have been reported in Rhinantheae, so hybridization (possibly also involving a parent from outside *Odontites* s.l.; see Pinto Carrasco et al., accepted) could explain the observed pattern. The fourth *Odontites*-like genus, *Odontitella*, surprisingly was found to be sister to *Nothobartsia*, a genus consisting of two perennial species from the Iberian Peninsula and neighboring regions, which was separated from *Bartsia* s.l. by Bolliger and Molau (1992). (Ancient) Hybridization likely played a role in the origin of this clade; it is incongruently placed as sister to the Hedbergia clade in the chloroplast tree but sister to *Odontites* s.l. in ITS (Fig. 9). A hybrid origin of the Nothobartsia-Odontitella clade from those two lineages is corroborated by morphology: *Nothobartsia* shares characters with *Odontites* (e.g. the elongate-spicate inflorescence, the obovoid capsules, pendulous ovules, and relatively narrow-winged seeds) as well as *Bartsia* s.l. (e.g. the broad ovate stem leaves and the entire galea) according to Bolliger and Molau (1992). Shared characters are also found in *Odontitella*.

Interestingly, besides these common morphological traits, which are shared by *Nothobartsia* and *Odontitella*, but also other lineages, there are few characters which are exclusively synapomorphic for the two genera; rather, there is actually a number of

morphological differences (Pinto Carrasco et al., accepted). This illustrates a general problem linked to the analysis of morphological characters in Rhinantheae: while individual genera have accumulated autapomorphic features which make them easily diagnosable (e.g. *Euphrasia*, *Hedbergia*, *Macrosyringion*, *Bornmuellerantha*), synapomorphies for groups of species are often missing (a problem which is also observed in Rhinantheae as a whole). Plesiomorphic characters resulting in the "'*Bartsia*-like' general morphology" found in many lineages (Těšitel et al., 2010) impede the correct assignment of taxa to their respective lineage, with hybridization (possibly resulting in intermediate morphologies) further complicating the situation. In consequence, morphological characters as traditionally used (like corolla shape, anther indumentum or pollen types, see Rothmaler, 1943; Bolliger & Wick, 1990; Bolliger, 1996) are not entirely suitable for generic classification in Rhinantheae. Regarding phylogenetic reconstruction based on molecular markers, our study clearly shows that a careful assessment of topological incongruence is essential in the group, with respect to correct inference of the relationships of taxa or lineages affected by reticulate evolution (Nothobartsia-Odontitella, *Macrosyringion*), but also to node supports in combined marker trees which might be affected if pruning of incongruent taxa is omitted. Altogether, according to the updated classification presented in Article I, the core group of Rhinantheae now consists of four monophyletic, distinct lineages (Hedbergia, Bellardia, Odontites s.l., and Nothobartsia-Odontitella; Fig. 9) plus the monotypic genera *Bartsia* s.str. and *Tozzia*, as well as *Macrosyringion* and the large genus *Euphrasia*.

5.2. The biogeographic history of Scrophularia

The biogeographic history of the genus *Scrophularia* as a whole had never been studied in detail. Our comprehensively sampled phylogeny (Article IV) revealed that the genus originated around the Oligocene / Miocene boundary at app. 23 million years ago (mya), when it diverged from its sister genus *Verbascum*. Its most likely geographic region of origin lies in Southwestern Asia s.l. (including Iran, Iraq and part of the Arabian Peninsula but also Turkey and the Caucasus; depicted by the dashed line in Fig. 4), which largely corresponds to its present-day primary center of diversity. These results contradict Stiefelhagen (1910) who, in his monographic treatment of the genus, promoted the Himalayas as ancestral region for *Scrophularia*. From Southwestern Asia, several lineages of the genus spread to the east as well as the west, finally resulting in the broad northern hemispheric distribution observed today (see Fig. 4). A similar situation involving an Irano-Turanian (sensu Takhtajan, 1986) diversity center and clades with both more easterly (to Yunnan) and westerly (to the Canary Islands) distributions can be found in *Ferula* L. (Apiaceae; Kurzyna-Młynik et al., 2008). These patterns are indicative of some characteristic features of the Irano-Turanian region in general: especially the west-central part of the region, which comprises most of Iran, Armenia, Azerbaijan, Afghanistan, and parts of Turkey, Iraq, Turkmenistan and Pakistan, is "a major center of speciation and endemism" (review by Djamali et al., 2012b, there referred to as the "IT2 sub-region"). This seems to be due to its high diversity of habitats (forests, scrubs, alpine grasslands, steppes etc.), its heterogeneous topography (see chapter 5.3.2.) and its climatic distinctness (Djamali et al., 2012b). High species richness and endemism in the Irano-Turanian region are combined with a high tendency to spread elsewhere, which means a pronounced representation of Irano-Turanian elements in neighboring regions, especially to the west (Mediterranean and Saharo-Arabian region; Djamali et al., 2012b; see also Mansion et al., 2008 and references

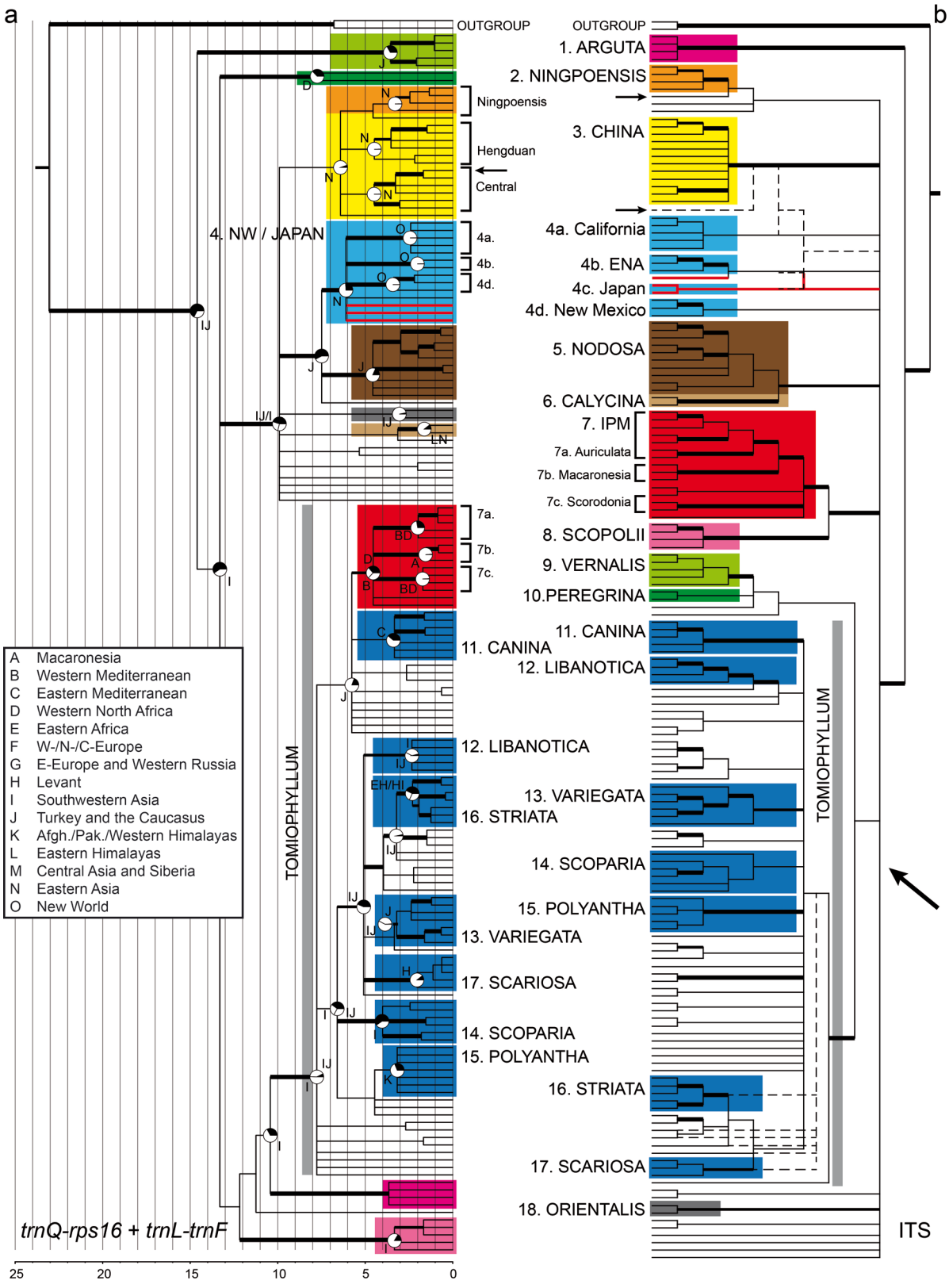

Fig. 10. Phylogenetic relationships in *Scrophularia*, based on (a) analysis of combined plastid data and (b) nuclear ITS data, including a dated phylogeny (in million years) and ancestral area reconstruction for the plastid tree. Nuclear tree computed with sequence polymorphisms treated as informative; important relationships supported by the uncoded tree added by dashed lines. Branches indicate levels of support according to Figs. 1 and 2 of Article IV (bold: PP≥ 95 or BS ≥ 85, semi -bold: PP ≥ 90 or BS ≥ 75, thin: PP < 90 / BS < 75). Small arrows indicate the position of *Oreosolen* Hook.f., a large arrow the large basal polytomy of the ITS tree. Pie charts represent marginal probabilities of inferred ancestral distributions; area codes at the nodes as explained on the left side. The three sampled Japanese taxa are highlighted by red branches. ENA, Eastern North America clade, IPM, Iberian Peninsula – Macaronesia clade

therein). However, plant migrations from Central Asia to the east have also been documented in several cases (see e.g. S-T Chen et al., 2005; Yue et al., 2009).

5.2.1. Eastward migrations

In *Scrophularia*, early eastward migrations to Eastern Asia were inferred at app. 6 mya (Fig. 10a, and Fig. 1a of Article IV) and were followed by diversification of the "China" clade into three lineages with several species in China and surrounding regions, at about 4.5 and app. 3 mya. This resulted in the genus' most important secondary center of diversity (Fig. 4). Our analyses presented in Article II and IV also revealed that the New World was colonized (possibly repeatedly and maybe also involving back-dispersals) from Eastern Asia, with the New World taxa diverging from their Asian ancestors app. 6 mya at the earliest. Colonization might have happened through migration via the Bering Land Bridge (BLB; Tiffney, 1985) as depicted in Fig. 4; during the later Tertiary, this passage was increasingly limited to cool-temperate taxa (Tiffney and Manchester, 2001), which would however not have posed a problem for *Scrophularia*. Even after the closure of the BLB (estimates range from 3.3 to 9 mya; Brigham-Grette, 2001; Denk et al., 2011), migration might have been successful, using short-time openings (assumed e.g. at 4.9 mya, 4.0 mya, 3.3 mya, and 2.5 mya; Miller et al., 2005). Furthermore, *Scrophularia* may also have reached the New World via long-distance dispersal: although the seeds do not possess special adaptations, they are easily dispersed by wind due to their small size and weight, and might have overcome the Bering Strait in this way. Generally, it can be stated that the Bering region was one of the most important connections between the two continents, with several lineages continously distributed from Eastern Asia to North America until the global climatic cooling of the late Pliocene and the Pleistocene (Xiang et al., 2000; Wen et al., 2016). Multiple dispersals in either direction and repeated back-colonizations often necessitate a broad sampling to identify numbers and times of dispersal correctly.

Divergence of North American from Eastern Asian lineages is known from several groups, e.g. *Kellogia* Torrey ex Benth. (Rubiaceae; Nie et al. 2005), *Rhodiola* L. (Crassulaceae; Zhang et al. 2014), *Angelica* L. (Apiaceae; Liao et al., 2012) or *Aralia* L. (Araliaceae; Wen et al., 1998). In *Scrophularia*, three North American lineages are supported. In several of the reconstructions, Eurasian and New World clades share sister relationships; the Japanese species (highlighted by red branches in Fig. 10) often even are more closely related to (Eastern) North American species than to other Eastern Asian taxa, a scenario discussed in Donoghue et al. (2001) and found in other lineages as well (see e.g. Li et al., 2003). These close relationships correspond to general Eastern Asian–North American disjunct patterns which are known for a long time and from many plant genera and can involve Eastern as well as Western North America (Boufford and Spongberg, 1983; Kadereit and Baldwin, 2012; Wen et al., 2016; for examples see e.g. Gould and Donoghue, 2000; Schnabel et al., 2003). In family Scrophulariaceae, both patterns are found (Hong, 1983).

5.2.2. Westward migrations

Westward expansion in *Scrophularia* led to the colonization of the Mediterranean, Northern Africa and Europe, which is typical for Irano-Turanian elements as outlined above. The Iberian Peninsula and Macaronesia host another secondary diversity center and are represented by a large clade of mostly Iberian taxa, which has a crown age of about 4.5 my (Article IV). Within this "Iberian Peninsula - Macaronesia" = "IPM" clade (Fig. 10), three subclades can be found (Article III). One is largely confined to the Iberian

Peninsula ("Scorodonia") while another reaches into Western North Africa with several species ("Auriculata"). Interestingly, while these species do not extend beyond Tunisia, other taxa are confined to Eastern North Africa. These belong to lineages from the Tomiophyllum clade ("Striata" and "Libanotica") which migrated into areas west and southwest of Iran and eventually colonized Eastern North Africa (Article IV); others today are found exclusively in the Levantine region ("Scariosa" clade). Some species again can be found throughout Northern Africa (*S. syriaca* Benth. from the Striata clade and *S. canina*, *S. peregrina* and *S. arguta* Sol.). Migration into more southern areas was largely precluded by the increasing aridity in these regions during the Pliocene and Pleistocene (Axelrod and Raven, 1978). An east-west split regarding plant lineages distributed in Africa was also observed in *Digitalis* L. (Bräuchler et al., 2004) and *Ranunculus* L. sect. *Ranunculastrum* (Paun et al., 2005).

The third lineage of the IPM clade exclusively consists of Macaronesian taxa ("Macaronesia" subclade). Members from the Auriculata, Scorodonia and "Arguta" clades are also represented in Macaronesia, summing up to a total of four phylogenetic lineages which have colonized the islands of Madeira, the Azores, the Cape Verde and the Canary Islands according to reconstructions in Article III (and IV). Madeira comprises representatives from all four lineages: two of them presumably have entered Madeira from the Western Mediterranean or also Western North African mainland (i.e., the Iberian Peninsula or Morocco), and one from the Canary Islands. The Canary Islands were colonized two times independently, including one dispersal from the Western Mediterranean (Fig. 5 of Article III). No conclusions were possible for the Azores (where *S. auriculata* L. is native) and the Cape Verdes (which host *S. arguta*). For *S. arguta* in general, no migration pathways could be determined; it is presently also found on Madeira and the Canary Islands. Valtueña et al. (2016), using a larger sampling, inferred three different colonization events to the Canaries, evidenced by different haplotypes on the western and eastern islands and Gran Canaria. Colonization possibly happened from Morocco in the latter two groups.

A second example for colonization of islands from the adjacent mainland (and subsequent diversification) in *Scrophularia* is found in the Greater Antilles, which constitute one of the very few examples where the genus advances into tropical regions. The species distributed on the islands were revealed to be the result of a dispersal event from the North American mainland (Florida) to the Caribbean (Cuba), by an ancestor of the Eastern North American *S. marilandica* L. (Article II); all species involved are part of the "Eastern North America" ("ENA") clade (Fig. 10). Dispersal is a frequent mechanism of Antillean colonization with several examples in flowering plants (amongst many others e.g. *Styrax* L. sect. *Valvatae*, Styracaceae, Fritsch, 2003; Lythraceae, Graham, 2003). Hedges (2006) stated that dispersal likely represents the key factor in Antillean colonization by terrestrial vertebrates and often leads to adaptive radiation on the islands. This seems to be true in *Scrophularia* as well: while only two species are native in the eastern parts of the USA (and only *S. marilandica* in Florida), the Greater Antilles harbor seven species, one widespread in forests of Cuba, Hispaniola, Puerto Rico and Jamaica, and six endemic to the Hispaniolan pine forests above 800 m; this underlines the status of these regions as biodiversity hotspot (Mittermeier et al., 2004; Francisco Ortega et al., 2007).

5.3. The influence of geography and topography on diversification

5.3.1. Origin and expansion of Scrophularia

The evolutionary and biogeographic history of *Scrophularia* has been heavily influenced by geologic processes since its divergence at the beginning of the Miocene. It can be hypothesized that the emergence of the genus was a result of substantial geological reorganizations, and climatic changes, in its ancestral region. The Miocene brought about a shift towards increased seasonality (An et al., 2001) and the aridification of interior Asia (Guo et al., 2002, 2004; Miao et al., 2012), with substantial environmental effects on Asian biota. Furthermore, the collision of the Arabian with the Eurasian plate, which had been initiated in the late Eocene (Berberian and King, 1981; Mouthereau et al., 2012), exerted strong influence on Southwestern Asia during the Oligocene and Miocene. Around the time inferred for the origin of the genus (Article IV), the final closure of the Tethyan seaway and the retreat of the Paratethys ocean (Ramstein et al., 1997; Mouthereau et al., 2012) created additional suitable land area for plant colonization, e.g. in the Zagros region. Highlands had been already existing for a longer time in Anatolia and the Alborz (Dercourt et al., 1986; Popov et al., 2004). The Arabia - Eurasia collision eventually led to the uplift of the Iranian plateau and the formation of the main mountain ranges of the region during the Miocene (see Djamali et al., 2012a). Diversification of *Scrophularia* into its main lineages consequently began during a period where new suitable habitats for plants adapted to montane environments were created; by the emergence of the Greater Caucasus and its transformation into a mountain chain (app. 14-13 mya and 9 mya; Popov et al., 2004; Olteanu and Jipa, 2006), the uplift of the Iranian Plateau (from 15-12 mya, Mouthereau et al., 2012; Djamali et al., 2012a and references therein) and the formation of the Western Alborz (app. 12-10 mya) and Zagros (app. 15-10 mya) mountains (Dercourt et al., 1986; Popov et al., 2004; Guest et al., 2007; Mouthereau et al., 2012). The establishment of continental climate conditions (Berberian and King, 1981) with increasing aridity (Ballato et al., 2010) during this period might additionally have promoted the colonization of more moderate, humid habitats at higher elevations (Roe, 2005); temperate elements additionally benefitted from the temperature decrease at app. 14-13.5 mya, after the mid Miocene climatic optimum (Zachos et al., 2001; Tiffney and Manchester, 2001). The pronounced emphasis on mountainous regions in the present-day distribution of *Scrophularia* lends support to this scenario.

Mountain formation also possibly triggered the spread of the genus into remote areas, by providing suitable migratory pathways. Given the habitat preferences mentioned above, dispersal along higher mountain chains seems reasonable. By the time inferred for the first eastward migrations of figworts at 6 mya, the rise of the Eastern and Central Asian mountain chains, the Kunlun Shan, the Tian Shan and the Hindu Kush from the Eocene through the Miocene (Dercourt et al., 1986; Abdrakhmatov et al., 1996; Hildebrand et al., 2000, 2001; Sobel et al., 2006; references in Miao et al., 2012; Yuan et al., 2013), together with the re-emergence of the more westerly Kopet Dag at app. 10 mya or later (Dercourt et al., 1986; Popov et al., 2004), had formed a more or less continuous mountain belt, which connected the Southwestern Asian Alborz to the Himalayas and the Tibetan Plateau. Migrations from Western or Central Asia to Eastern Asia or vice versa are also known from e.g. *Incarvillea* Juss. (Bignoniaceae; S-T Chen et al., 2005), *Rhodiola* (Zhang et al., 2014), or *Solmslaubachia* Muschl. (Brassicaceae; Yue et al., 2009).

5.3.2. Diversification in the Irano-Turanian region and the Mediterranean

The most important factor regarding diversification in *Scrophularia* is however that tectonic processes with associated mountain building generated habitats which themselves promote speciation. In the Iranian region, the center of *Scrophularia* species diversity, pronounced topographic barriers also act as boundaries separating biogeographical or climatic regions (e.g. the Alborz mountain system, see Djamali et al., 2012b). Furthermore, the tectonic history of Southwestern Asia created a complex topography of several mountain ranges, which provide heterogeneous habitats on small geographical scales. This facilitates adaptive speciation and allopatric divergence, and has led to the extraordinary species diversity and endemism found in the region (Djamali et al., 2012b), for example in *Astragalus* (Fabaceae; Podlech and Zarre, 2013) or *Cousinia* Cass. (Asteraceae), which has been found to be most diverse "in regions with the widest range of topographical variations and the highest frequency of elevations above 2000 m" (Djamali et al., 2012a). Geological heterogeneity in the Irano-Turanian region was also invoked as explanation for diversification of a large clade of *Haplophyllum* A.Juss. (Rutaceae; Manafzadeh et al., 2014). The connection of topographical complexity and high species richness is of course not confined to the Irano-Turanian region. Similar patterns are found in other parts of the world, and remarkably often coincide with diversity centers of *Scrophularia*. Indeed, all *Scrophularia* diversity centers comprise mountainous regions (Fig. 4), and high levels of endemism are found throughout the genus (Vaarama and Hiirsalmi, 1967).

A good example for "vicariance" caused by fragmentation of habitats is found in the Mediterranean, where plate movements in combination with marine regression / transgression events explain part of the large species diversity and number of endemics, through the repeated formation of contacts and barriers for plant lineages (e.g. Mansion et al., 2008). On a smaller scale, topographical complexity was suggested to promote diversification and speciation processes, often in combination with climatic changes during the Quaternary glaciations (Thompson, 2005). Lobo (2001) examined patterns of plant species richness in the Iberian Peninsula and found that diversity is significantly related to maximum elevation and altitude range (= environmental heterogeneity). This might also explain the secondary diversity center of *Scrophularia* found in the Iberian Peninsula with 22 species, 12 of those endemic to the Peninsula including the Pyrenees. While the origin of the IPM clade (by ancient hybridization, see chapter 5.4.3.) was inferred at around 5 mya and its crown age at app. 4.5 mya (Article IV), i.e. well before the establishment of the Mediterranean climate rhythm (around 3 mya, Suc, 1984), diversification of the main lineages was dated to have happened from 2 mya on (see also Navarro Pérez et al., 2013). This implies that speciation in the fragmented habitats was additionally reinforced by expansions, contractions or shifts of distribution ranges created by climatic fluctuations (Thompson, 2005). In *Scrophularia*, two Iberian alongside two Mediterranean endemic species were found to obtain isolated positions in a haplotype network (Fig. 3 of Article III) and were unresolved in the respective tree. These species are restricted to only small areas, and exclusively or predominantly inhabit regions classified as refugia within the Mediterranean bioclimatic region (Médail and Diadema, 2009). These, putatively more ancient, lineages might have got isolated while retreating into the favorable conditions of the climatically stable refugial areas. Many examples for this mode of speciation are known from the Iberian Peninsula, e.g. from *Erodium* L'Hér. (Geraniaceae; Fiz Palacios et al., 2010) or *Reseda* L. (Resedaceae; Martín Bravo et al., 2010). Hybridization of lineages in secondary contact zones has been another important factor, see chapter 5.4.

Apart from topography and climate, a spatial structuring of genetic variation might also be generated by habitat islands due to different soil types. An example is found in the Iberian gypsophyte *Gypsophila struthium* L. (Caryophyllaceae; Martínez Nieto et al., 2013). Habitat fragmentation by soil types is also a driver of speciation in the Strait of Gibraltar region. The climatically stable Algeciras and Tanger Peninsulas are characterized by a landscape comprising deep gorges and a mosaic of limestone outcrops and siliceous sandstone patches producing large numbers of narrow endemics, mostly originating from recent speciation (Rodríguez Sánchez et al., 2008; Lavergne et al., 2013). Some narrow endemic *Scrophularia* species are found in the region, amongst those *S. fontqueri* Ortega Oliv. & Devesa (not sampled for this thesis), native to calcareous substrates in the Rif mountains of Northern Morocco; *S. viciosoi* Ortega Oliv. & Devesa (see chapter 5.4.2.) of Málaga province, Spain which prefers similar habitats in calcareous outcrops; and *S. laxiflora* Lange (Fig. 10, Scorodonia subclade), distributed on sandstone soils on both sides of the Strait of Gibraltar, in Southern Cádiz province of Spain and the Tanger region, Morocco; the latter also houses another narrow endemic, *S. papillaris* Boiss. & Reut. (not sampled), which is closely related to *S. scorodonia* (Ortega Olivencia and Devesa Alcaraz, 1996, 1998; Ibn Tattou, 2007; Ortega Olivencia, 2009). As yet, no evidence for an association of these species with the edaphic characteristics of the Strait of Gibraltar region has been provided. However, Lobo (2001) found general evidence for an (albeit subordinate) influence of bedrock geology variables on Iberian vascular plant diversity.

5.3.3. Diversification in Eastern Asia and the New World

In Eastern Asia, another secondary diversity center of the genus, where more than half of the 36 *Scrophularia* species listed in the Flora of China (Hong et al., 1998) ascend to alpine levels of 3000 m or more, the extreme relief found in mountain regions also limits gene flow effectively. Qian and Ricklefs (2000) attributed considerably higher species numbers in Eastern Asia compared to Eastern North America (the Eastern Asian - Eastern North American species diversity bias) to "the extreme physiographical heterogeneity of temperate eastern Asia". Apart from that, similar to the Mediterranean region, climate fluctuations could have enabled repeated fragmentation and extensions of distribution areas, further pushing diversification (Qian and Ricklefs, 2000). In a review on plant diversification on the Tibetan Plateau, Wen et al. (2014) highlighted the "extremely complex topography with diverse habitats", which fosters allopatric divergence, as a main diversification mechanism on the plateau. Like for other mountain systems (Hughes and Atchison, 2015), many authors have attributed diversification events in the region to the uplift of the Tibetan Plateau (examples e.g. in Wen et al., 2014), although not always legitimately so (Renner, 2016). In *Scrophularia*, diversification of the Hengduan subclade (Fig. 10), with most of its members restricted to the Hengduan mountains and adjacent areas, might possibly have been triggered by a phase of uplift of the Hengduan Shan. However, the general great uncertainty in dating both divergence times and geologic events makes conclusions based on the present data somewhat questionable. Diversification of the Hengduan and also the Central subclade in China seems to be more obvious to relate to the extreme topography and the variety of vegetation types and climatic conditions which characterize the Mountains of Southwest China biodiversity hotspot (Mittermeier et al., 2004; Boufford, 2014). Apart from that, the distribution patterns seen today might also have been generated by survival of plant lineages in suitable refugia and recolonization of China from these refugia after the Last Glacial Maximum (app. 24,000-18,000 years ago; Liu et al., 2012). The two predominantly alpine Hengduan and Central subclades can both be associated

with important refuges, which have been recognized in the Hengduan Shan (see S-Y Chen et al., 2008; Feng et al., 2009; L Wang et al., 2011) and the Qinling mountains or, more generally, the "Northeast Qinghai - Tibetan Plateau edge" (Qiu et al., 2011), respectively (Article IV). Unfortunately, unequivocal identification of the underlying diversification mechanisms is not possible based on the sampling used here. The third Chinese (sub)clade, "Ningpoensis", predominantly comprises non-alpine species distributed from the eastern parts of China to Korea, Japan and Taiwan and seems clearly separated from the Hengduan and Central subclades mostly consisting of alpine taxa with narrow distributions. An arid zone separating the two regions throughout the Miocene (Tiffney and Manchester, 2001) could possibly have constituted a barrier for *Scrophularia*, resulting in vicariant speciation; this was also proposed for *Thuja* L. (Cupressaceae) by Peng and Wang (2008).

The Eastern Asian - Eastern North American species bias as investigated by Qian and Ricklefs (2000) and Xiang et al. (2004) is found in many plant groups, e.g. in *Lespedeza* Michx. (Fabaceae; Xu et al., 2012), *Panax* L. (Araliaceae; Wen and Zimmer, 1996) and also *Scrophularia* (36 species in China compared to 19 species in entire North America plus the Caribbean). In the New World itself, species diversity in the U.S.A. plus Canada is again lower in the east than in the west. Only one species is distributed throughout the region (*S. lanceolata* Pursh, see chapter 5.4.2.). The eastern part harbors only one additional (also widespread) species; its possible phylogenetic relationship to a recently discovered narrow endemic Northeastern Mexican species (Mayfield and Nesom, 2012) still needs confirmation. On the other hand, of the nine species confined to the west, only two are distributed in more than two states, and four are limited to one to three counties only. A clade of five taxa (Fig. 10, "California" clade) is distributed in the California floristic province, a Mediterranean-type hotspot (Mittermeier et al., 2004). Qi and Yang (1999) analyzed plant diversity in California and found a positive correlation of mean elevation and speciation capacity, as well as a relation of spatial variability of elevation to plant diversity. However, only one *Scrophularia* species from the California clade ascends to alpine elevations. Diversity related to spatial heterogeneity is rather found in the "New Mexico" clade, which includes one alpine and three subalpine species. Notably, distribution ranges of the markedly geography-based, two western and one eastern clades of North American *Scrophularia* do not overlap much. Here, geographic isolation seems to obtain another role, in maintaining already established species: most North American species are closely related - they can be artificially crossed (Shaw, 1962) or intergrade naturally in contact zones (Kearney and Peebles, 1951). This means that rather than reproductive isolation, geographic barriers might be important in sustaining the identity of the species (Shaw, 1962; see also Carlbom, 1969, who however advocates a different species concept for *Scrophularia*). A similar effect has been observed in the Scrophulariaceae genus *Jamesbrittenia* Kuntze, which is also capable of successful interspecific hybridization (Verboom et al., 2016).

5.4. The influence of hybridization on diversification

5.4.1. Phylogenetic tree incongruence and intra-individual polymorphism

The genus *Scrophularia* is characterized by a high level of natural hybridization and hybrid speciation as revealed in Articles II, III and IV. This process can be detected using molecular sequence data, e.g. by examining gene trees for well-supported incongruence. Usually, a combination of uniparentally (plastid) and biparentally

(nuclear) inherited molecular markers is used, and the respective position of a hybrid lineage next to one or the other of its parents might allow conclusions on the hybrid speciation event (e.g. Soltis and Kuzoff, 1995; Marhold and Lihová, 2006; Fehrer et al., 2007). This approach led to the successful detection of hybrids in Rhinantheae and *Scrophularia* (Article I-III; see the examples given below). When incongruence is not too widespread, conflicting taxa can also be revealed using special software (Aberer et al., 2013; Pérez Escobar et al., 2016). In cases where nuclear ribosomal DNA (ITS) is used for reconstruction and concerted evolution of rDNA units (e.g. Arnheim et al., 1980) is slowed down or non-operational, intra-individual polymorphisms in sequence data (which might result from a hybridization event) can be extracted and interpreted in order to detect possible parent sequences (Fuertes Aguilar and Nieto Feliner, 2003). In *Scrophularia*, concerted evolution seems to be incomplete in some, but not all species, resulting in both monomorphic and polymorphic ITS sequences (Article IV).

However, both tree incongruence and intra-individual nucleotide polymorphism can result from a variety of sources other than hybridization / introgression. Long branch attraction will lead to artificial relatedness of taxa which are subtended by long branches in phylogenies (Felsenstein, 1978). This will ultimately cause gene tree discordance; inappropriate sampling or model settings during calculations might also generate incongruent trees. Both tree incongruence and intra-individual polymorphism can be created by Incomplete Lineage Sorting (ILS; the persistence of ancient polymorphism / deep coalescence; Degnan and Rosenberg, 2009), recombination, or the presence of paralogous sequences (Álvarez and Wendel, 2003; Wolfe and Randle, 2004). The latter two, together with processes of differential silencing and pseudogenization are additionally intensified in the presence of hybridization (Álvarez and Wendel, 2003; Volkov et al., 2007). The situation is particularly complex when the investigated lineages are of recent divergence. Several authors have attempted to disentangle the different processes leading to gene tree incongruence, especially to distinguish between ILS and hybridization (Maureira Butler et al., 2008; van der Niet and Linder, 2008; Joly et al., 2009; Konowalik et al., 2015). However, when a combination of different processes has repeatedly influenced a phylogenetic history, single events might become difficult to discern.

Furthermore, these phenomena can severely impact phylogenetic tree reconstruction itself, e.g. by reduced node resolution in datasets containing intra-individual polymorphism. To overcome this situation, affected accessions have been pruned from analyses, or polymorphisms were excluded or variously replaced (Fuertes Aguilar and Nieto Feliner, 2003; Lorenz-Lemke et al., 2005; Scherson et al., 2008; Fehrer et al., 2009). The method employed in Article IV, treating polymorphims as informative (see Methodology, chapter 3.), proved to be most suitable as a maximum of data was incorporated into the analyses; it resulted in considerably increased tree resolution while only exceptionally contradicting the uncoded phylogeny (Fig. 2 of Article IV compares both topologies). However, it must be stressed that any kind of reticulation will fundamentally interfere with traditional approaches based on building dichotomously branching trees; this problem of inherent conflict in the data cannot be solved using coding techniques (Potts et al., 2014). Consequently, a large basal polytomy remains in the nuclear tree in Fig. 10b (indicated by an arrow). In these cases, examination of phylogenetic networks is a suitable alternative (Huson and Bryant, 2006); these again can be supported by incorporating information from polymorphisms (see Article IV). Fig. 11 shows the consensus network of all trees leading to the consensus tree in Fig. 10b. It illustrates the highly entangled relationships (resulting from uncertainty and conflict) among and also within clades, which are concealed when forced into a single bifurcating tree, and result in the aforementioned polytomy.

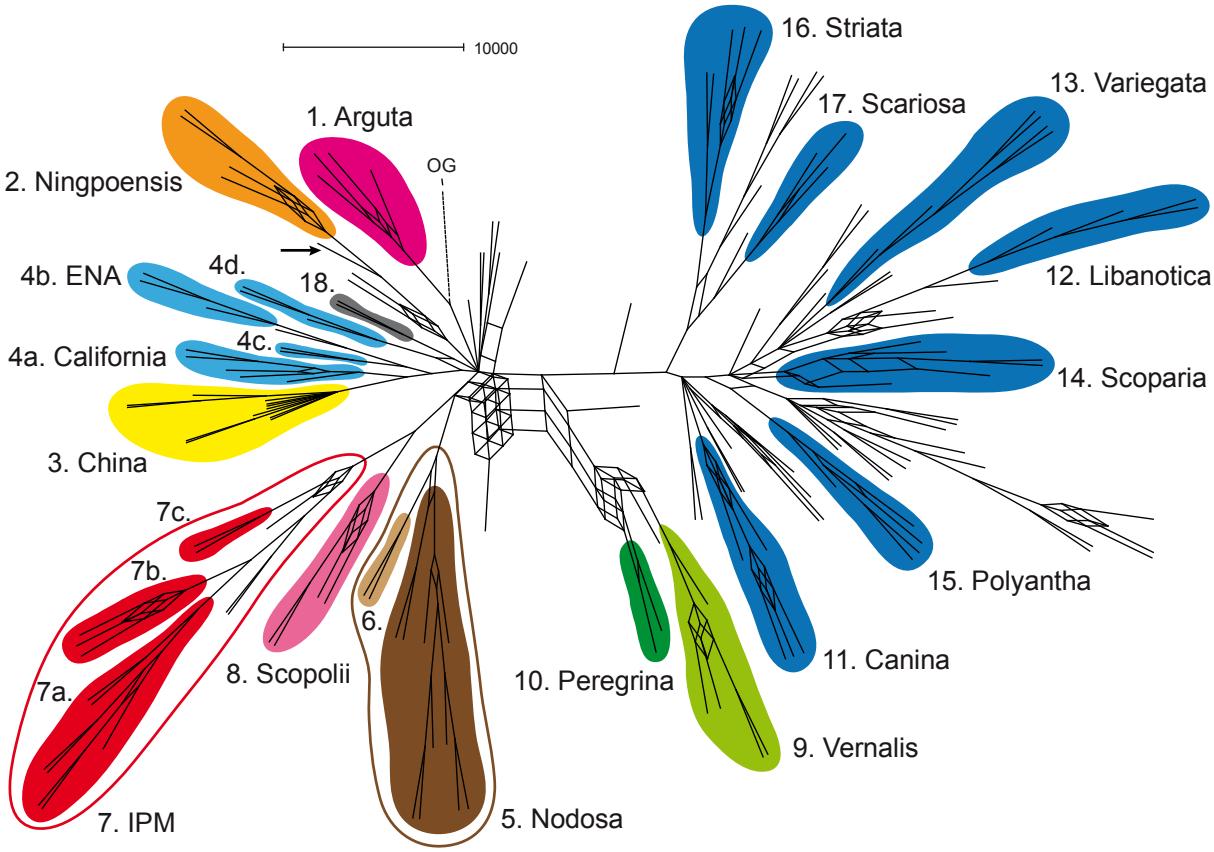

Fig. 11. Consensus network based on trees from both runs of the Bayesian analysis that resulted in the ITS consensus tree shown in Fig. 10b. Clades are highlighted, colors correspond to those in Fig. 10b. The large basal polytomy observed in the consensus tree can be explained in part by the highly networked part on the left side (Anastomosantes group, see chapter 5.5.) of the consensus network

In the uniparentally inherited chloroplast, extensive reticulation (e.g. by repeated introgression) can ultimately lead to a phylogenetic pattern that is no longer based on taxonomic relationships but rather traces geographic proximity. Examples are found in *Antirrhinum* L. (Plantaginaceae; Vargas et al., 2009), *Phlomis* L. (Lamiaceae; Albaladejo et al., 2005), and many others. In *Scrophularia*, this effect is not too pronounced, with taxonomic relationships still being visible in plastid trees (Articles II-IV). Some clades ("Nodosa", "NW (= New World) / Japan", "Orientalis", "Scopolii", "Polyantha") are characterized by the occurrence of large diagnostic indels (see Fig. 7), which however are not exclusive among clades (Article II, IV).

5.4.2. Hybrid speciation in Scrophularia - homoploid hybrid species

Detection of hybridization in *Scrophularia* is complicated by the fact that corresponding patterns are often overlain by other processes. DNA sequences and phylogenetic trees are likely influenced by hybridization, introgression, ILS, incipient pseudogenization, and even recombination and paralogy among sequences cannot be completely ruled out. To unravel all of these effects would require a different kind of sampling and lies beyond the scope of this thesis. However, despite the abovementioned difficulties, several examples of hybrid speciation within *Scrophularia* could be revealed or confirmed in Articles II-IV. Hybridization is likely to have played a prominent role in the evolution of the genus and has surely promoted its diversification, given the occurrence of natural hybrids, its comparatively young age and its flower and pollination biology (see Introduction, chapter 2).

In the rather homogenous group of New World *Scrophularia* representatives, most species have restricted distributions (see chapter 5.3.3.), with only *S. marilandica* being more widespread in the eastern parts of North America. One species however, *S. lanceolata*, the lanceleaf figwort, represents an exception, in being widely distributed throughout the U.S.A. and Canada. I revealed this species to have a hybrid origin, involving progenitors from the New Mexico clade (see Fig. 10) and *S. marilandica* from the Eastern North America clade (Article II). Karyological evidence cannot confirm this hypothesis (all species involved are high polyploids of variable chromosome numbers). However, *Scrophularia lanceolata* is very similar to *S. marilandica* morphologically, and also shares some characters with *S. parviflora* Wooton & Standl. from the New Mexico clade, e.g. a similar pattern of corolla color and a spatulate staminode. Some morphological traits (like the shape of the capsule) are intermediate between those of the parental lineages; flowers and capsules are also larger than in the latter, which could be interpreted as a sign of hybrid vigour. It seems possible that its reticulate origin is the reason for the success of *S. lanceolata*, which has spread more widely than any other *Scrophularia* in the New World.

The heterogeneous topography, different soil types, and the climatic history with several glacial refugia across the Iberian Peninsula have resulted in several endemic species as outlined above. Furthermore, we revealed that five *Scrophularia* Iberian endemics are likely to be the result of homoploid hybrid speciation (Article III). This mode of speciation, which does not involve a change in ploidy in the offspring, is still regarded as unusual and rare, although several cases have been documented (Schumer et al., 2014; Vallejo Marín and Hiscock, 2016). *Scrophularia sublyrata* Brot., *S. oxyrhyncha* Coincy, *S. reuteri* Daveau, *S. valdesii* Ortega Oliv. & Devesa, and also the already mentioned *S. viciosoi* from Málaga, Southern Spain, are morphologically related to the Auriculata subclade, and, according to our analyses in Article III, originated from hybridization of ancestors or members from the latter (acting as female parent in all but *S. viciosoi*) and the Scorodonia subclade. As in the case of *S. lanceolata*, species from the putative parental lineages can be successfully crossed, supporting the hypothesis of a hybrid origin. In contrast to endemic species from the Strait of Gibraltar region (see chapter 5.3.2.), *S. sublyrata*, *S. oxyrhyncha*, *S. reuteri* and *S. valdesii* are confined to granite substrates; their distribution areas lie within the western floristic zone of the Iberian Peninsula as defined by Moreno Saiz et al. (2013). The species are of very recent divergence, having probably originated in the late Pleistocene (Navarro Pérez et al., 2013). Diversification in the mountains of the Iberian Peninsula during Quaternary climatic oscillations has been already discussed in chapter 5.3.2. In this context, range shifts of species and secondary contact zones of course also provided ample opportunities for hybridization. The resulting hybrid then could easily have been isolated during subsequent periods of range contraction. Isolation from their parents is regarded as essential for the establishment of newly formed homoploid hybrid species (Rieseberg and Willis, 2007). Evidence for hybridization caused by Pleistocene climate fluctuations coupled with heterogeneous topography has been found in many plant lineages from the Mediterranean and the Iberian Peninsula, amongst others *Armeria* Willd. (Plumbaginaceae; Gutiérrez Larena et al., 2002), *Antirrhinum* (Plantaginaceae; Vargas et al., 2009) or *Linaria* Mill. (Blanco Pastor et al., 2012). It might have been particularly frequent in mountainous areas of Southern Europe, where temperature change effects were less serious and species could avoid them by altitudinal instead of latitudinal migrations (Nieto Feliner, 2011), thus staying in relative proximity to other populations. However, it has to be mentioned that the rapid formation of species suffering secondary contacts should also have produced ILS (Blanco Pastor et al., 2012); the latter authors tested for ILS as alternative explanation for phylogenetic

incongruence, which unfortunately was not possible with my *Scrophularia* dataset. Their results supported the occurrence of both ILS and hybridization, and possibly also homoploid hybrid speciation.

5.4.3. Allopolyploid hybrid species and hybrid lineages

In contrast to homoploid hybrid speciation, allopolyploidization is easier to confirm: the respective hybrid taxa have a different ploidy level compared to their parents, acquired by chromosome doubling after hybridization, by using a 'triploid bridge' (Mallet, 2007), or resulting from the fusion of two unreduced gametes. This way, chromosome numbers add additional evidence to that from tree incongruence and intra-individual polymorphism. The hypothesis of Ortega Olivencia and Devesa Alcaraz (1990), of *S. alpestris* J.Gay ex Benth. (2n = 68) being an allopolyploid of *S. scopolii* Hoppe ex Pers. (2n = 26; Scopolii clade) and *S. bourgaeana* Lange (2n = 42; Nodosa clade), was confirmed by our phylogenetic reconstructions in Article III. The putative hybrid taxon was incongruent in plastid and nuclear tree reconstructions, changing positions between the two parent lineages. *Scrophularia auriculata* (2n = 84) was proposed to have resulted from allopolyploid hybridization between (ancestors of) *S. lyrata* Willd. (2n = 58; Auriculata subclade of the IPM clade) and *S. umbrosa* Dumort. (2n = 26, 52; likewise incongruently placed within the IPM or Scopolii clade) by Grau (1979), based on morphological traits. The respective results in Article III were ambiguous with respect to *S. lyrata*; a relationship to *S. umbrosa* could not be found at all. In Article IV, a specimen from *S. auriculata* with a confirmed chromosome number turned out to be highly polymorphic in ITS; its clones revealed two ribotypes obtaining different positions in the tree (Fig. 3 of Article IV). We concluded that hybridization of the Algerian – Moroccan endemic *S. hispida* Desf. (2n = 58, Auriculata subclade, and morphologically close to *S. lyrata*) and *S. umbrosa* or their ancestors likely have generated the most widespread species within the Auriculata subclade, and probably also *S. racemosa* Lowe, a Madeiran endemic with likewise 2n = 84 chromosomes. In another accession from *S. auriculata* and one from *S. lyrata*, we found evidence for introgression (chloroplast capture), by *S. scorodonia* and *S. laxiflora* (Scorodonia subclade), respectively (Article IV).

Reticulation in *Scrophularia* is not limited to single taxa. Based on gene tree discordance, it was revealed that the whole IPM clade, composed of Mediterranean and Macaronesian representatives, likely originated by hybridization of members / progenitors of two nowadays widespread lineages, *S. umbrosa* or the Scopolii lineage, and the "Canina" lineage or allies, at around 5 mya (Articles III and IV). These ancestors were inferred to have been distributed in Southwestern Asia and the Turkey-Caucasus region, respectively, while the ancestor of the IPM clade had its range in the Western Mediterranean. The exclusive chromosome number of 2n = 58, which is typical for the latter, might have resulted from the merger of the two 2n = 26 chromosomes from both parents (and subsequent chromosome doubling), with subsequent ascending aneuploidy creating the number observed today. This does not seem unusual, as aneuploidy is encountered in several species of the IPM clade, e.g. in *S. sublyrata*, *S. glabrata* Aiton or *S. viciosoi*, and *S. canina* was exceptionally counted with 2n = 30 chromosomes (Ortega Olivencia and Devesa Alcaraz, 1990).

Even higher polyploidy is observed in the NW / Japan and Ningpoensis clades. North American species have 2n = 86 to typically 2n = 96 chromosomes (excluding *S. montana* Wooton which has 2n = 70-76), and one Japanese alongside some other Eastern Asian taxa have been counted with up to 2n = 96, but also much lower numbers. Generally, a high variability regarding chromosome counts is observed in Eastern Asian

and Southern Asian species, including diploid as well as polyploid chromosome numbers. The origin of high polyploidy in these lineages remains unclear. Phylogenetic reconstructions in Article IV suggest that hybridization might have been involved regarding the New World and Japanese taxa. They are related to the Nodosa lineage as well as the China clade, which is perfectly plausible given both their morphological characteristics and their biogeographic ancestry. Tentative cloning of a Japanese and a North American species however could not corroborate the hypothesis of a hybrid origin, but as the event is expected to be more ancient, this does not necessarily refute it either.

5.4.4. Combined effects of topography, hybridization and climate fluctuations

Altogether, it seems evident that a combination of geographic isolation, habitat fragmentation and successful interspecific hybridization and polyploidization has been the key factor in the diversification of *Scrophularia*. In general, reticulation and polyploidization are now seen as major driving forces in the evolution and diversification of plants (Soltis and Soltis, 2009; Abbott et al., 2013). A varied topography could additionally support hybrid speciation, by stabilizing newly formed hybrids through isolation from their parents; this might have been the case in the two Iberian homoploid hybrid species *S. oxyrhyncha* and *S. reuteri*, now distributed in the Cordillera Central and the Sierra Morena (Article III). Climatic changes however may have triggered extensive gene flow in mountainous and other regions and resulted in geographical structuring of haplotype relationships, as found in *Armeria* and also *Antirrhinum* (Gutiérrez Larena et al., 2002; Vargas et al., 2009). However, Vargas et al. (2009) and Blanco Pastor et al. (2012) also highlighted the role of geographical speciation in addition to hybridization, evidenced by limited distributions of several endemic, often endangered, species. This is also observed in *Scrophularia*; hybrid and non-hybrid endemics with narrow distributions thereby are not limited to Mediterranean taxa. On the other hand, the ability to generate polyploid lineages by hybridization in *Scrophularia* seems to constitute a fitness benefit compared to its closest sister genus *Verbascum*, where there is no evidence for hybrid speciation and which lacks high polyploids. Interestingly, high polyploid lineages of *Scrophularia* have colonized regions where few or no *Verbascum* species occur (China and the New World). The reticulation processes which enabled this success are mirrored in the large amount of incongruence and polymorphism encountered when reconstructing phylogenetic relationships in the genus.

5.5. Taxonomy and morphological traits

All accessions of *Scrophularia* which were analyzed in this thesis formed a monophyletic clade. Figworts therefore constitute a natural group, although the genetic distance (nucleotide divergence) to *Verbascum* is comparatively low, given several clear morphological differences (see pairwise distances and character differences in Supplementary Table S1 of Article III). Surprisingly, the Himalayan - Tibetan endemic genus *Oreosolen* was shown to be deeply nested within *Scrophularia* in all analyses of Article IV (see arrows in Fig. 10). The large Tomiophyllum clade largely corresponds to, but is not exactly identical with, *Scrophularia* sect. *Tomiophyllum* sensu Stiefelhagen (1910). The remaining species mainly belong to *Scrophularia* sect. *Anastomosantes*; they do not form a monophyletic clade, but one which is paraphyletic with respect to the Tomiophyllum clade. This means that phylogenetic relationships to some point reflect

the morphology-based subgeneric classification proposed in Stiefelhagen's (1910) monograph of the genus. Furthermore, they reveal *S.* sect. *Tomiophyllum* to be derived from within *S.* sect. *Anastomosantes*, despite the putatively primitive traits of the former (habit often subshrubby, xerophytic, general lack of polyploid chromosome numbers; Carlbom, 1969). On the other hand, the mainly herbaceous, richly foliated, often meso- or hygrophytic members of *S.* sect. *Anastomosantes* (Stiefelhagen, 1910) are characterized by a wide range in chromosome numbers, a wide ecological amplitude and a geographic distribution exceeding that of *S.* sect. *Tomiophyllum* by far; this might reflect a longer phylogenetic history.

The Tomiophyllum clade diverged from its sister clade at about 10.5 mya and diversified from app. 8 mya (Article IV; Fig. 10a). Changes in aridity in the Middle East during the second half of the Miocene may possibly have triggered these events (Ballato et al., 2010). Ecological preferences of members of the two sections overlap, but their habitats show a certain shift from moist sites on riverbanks and in forests (*S.* sect. *Anastomosantes*) towards rock crevices and gravelly substrates with low humidity in *S.* sect. *Tomiophyllum*; this indicates a greater tolerance of dry conditions. Furthermore, Tomiophyllum species predominantly inhabit the dry parts of e.g. Iran and Turkey, while not necessarily being absent from other areas. Interestingly, the first-branching lineages of the Tomiophyllum clade, as supported by some of the nuclear phylogenetic results (Fig. 10b, dashed lines; compare also Fig. 2b of Article IV), comprise some species with morphological affinities to *S.* sect. *Anastomosantes*, e.g. *S. nabataeorum* Eig from the Scariosa clade. On the other hand, the likewise basally branching Striata clade includes some of the few species that have colonized truly arid environments, occurring in steppe and desert habitats.

The lack of exclusive (albeit typical) morphological characteristics of *Scrophularia* sects. *Tomiophyllum* and *Anastomosantes* illustrates a general problem in the genus, which is encountered also in Scrophulariaceae and related families (see chapters 2.1.1., 2.2.3., and 5.1.): morphological synapomorphies for phylogenetic clades can hardly be found at all; many traits seem to have originated several times independently and occur in several phylogenetically unrelated groups. In addition, sequence divergence among *Scrophularia* species is often very low (Supplementary Table S1 of Article III); both phenomena could result from recent divergence in some clades, but are likely also due to hybridization processes. The occurrence of homoplastic characters is a problem also shared with the sister genus *Verbascum*, as is the lack of resolution in molecular phylogenetic trees (Ghahremaninejad et al., 2014).

Despite this apparent deficiency in morphological differentiation, several examples of conspicuous floral morphology have emerged, often from clades with otherwise 'ordinary' species. For example, the supposedly hummingbird-pollinated *S. macrantha* Greene ex Stiefelh. (Shaw, 1962), endemic to New Mexico (Fig. 10, New Mexico clade), possesses large, tubular, bright pink corollas, and was found to be sister to *S. laevis* Wooton & Standl. (Article II), a species of similarly narrow endemic distribution but with dull greenish corollas of about 12 mm (Martin and Hutchins, 1981), which was synonymized with *S. montana* by Shaw (1962). Similar flowers with tubular but yellow corollas are found in some Southern and Eastern Asian species (China clade); their pollinators are still unknown. The genus *Oreosolen* also features this type of flower, and thus fits smoothly into its position within *Scrophularia* as suggested by our molecular results in Article IV. Macaronesia has produced several unusual *Scrophularia* flowers (Fig. 3d, g, i), including the Gran Canaria endemic *S. calliantha* Webb. & Berthel. with its large, open, orange-red corollas of up to 23 mm. Passerine birds are the main pollinators of this eye-catching species, alongside insects and even a

lizard (Ortega Olivencia et al., 2012). However, this conspicuous morphology does not seem to be correlated with phylogenetic distinctness; the species remains unresolved among other endemics within the Macaronesia subclade of the IPM clade (Fig. 1 of Article III). On other archipelagoes, floral morphology seems to reflect the particular insular conditions. The Caribbean species *S. minutiflora* Pennell (Eastern North America clade) has very small, white corollas. These are typical for the flora of the Antilles and are thought to be adapted to pollination by minute, endemic insects (Borhidi, 1996). Altogether, pollinator shifts seem to be responsible for deviant corolla types in several cases (see Navarro Pérez et al., 2013).

A greater reliance on self-fertilization apparently has influenced *S. arguta* and the Arguta clade (Fig. 10). Flowers of the annual *S. arguta* are often self-pollinating and, in addition to the chasmogamous flowers, the species possesses smaller cleistogamous flowers, sometimes on particular shoots near the ground, and even subterranean inflorescences (Dalgaard, 1979). This is likely to have supported the spread of the species into areas otherwise unsuitable for *Scrophularia*, like the Sudan, Eritrea, Somalia and Oman, where *S. arguta* is the only representative of the genus. The species from the Arguta clade (which also includes *S. lowei* Dalgaard, Fig. 3g), occupy an isolated position within the genus. The basal position of the clade as sister to all other species of *Scrophularia* in the nuclear phylogeny (Fig. 10b) surely reflects its distinctness, but not necessarily an annual ancestry of the genus as a whole. It is possible that the position of *S. arguta* in ITS is artificially due to high substitution rates correlated with a mating system that emphasizes selfing (Glémin et al., 2006).

5.6. Conclusions and Outlook

The studies and publications presented in this thesis have clarified the fundamental phylogenetic relationships in the studied groups. Rhinantheae constitute a predominantly European, or more generally Eurasian group, which dispersed into the Southern Hemisphere with few representatives. The latter (*Bellardia* and *Euphrasia*) have radiated in South America and Africa as well as New Guinea, New Zealand, and Australia, respectively. The tribe now includes five main groups alongside five single genera (see also McNeal et al., 2013; Pinto Carrasco et al., accepted). The genus *Scrophularia* is divided into two main groups which largely correspond to those of the latest taxonomic treatment. Its evolutionary history and biogeography were substantially influenced by diversification in mountainous regions which constitute its preferential distribution areas. Centers of species diversity are likewise often correlated with global biodiversity hotspots, e.g. the Caucasus and the Irano-Anatolian hotspot, Macaronesia and the Baetic–Rifan complex within the Mediterranean Basin hotspot, the Mountains of Southwest China hotspot or the Caribbean Islands hotspot (Mittermeier et al., 2004; Médail and Diadema, 2009). While montane habitats have promoted allopatric speciation and in several cases preserve species integrity by providing spatial isolation, the genus' potential for widespread interspecific hybridization (leading to homoploid hybrid speciation and allopolyploidy), also in the context of historical climatic changes, has contributed essentially to the diversity observed today, with species distributed over most of the Northern Hemisphere.

The delimitation of phylogenetic lineages based on molecular data in *Scrophularia* is hampered by considerable incongruence and ambiguity in the data, accompanied by a variable morphology which lacks distinctive shared traits in many cases. The problem of convergence in morphological characters is encountered also within Rhinantheae, but while in the latter, the exclusion of incongruent specimens leads

to a reliable phylogenetic hypothesis, relationships are excessively interwoven in *Scrophularia*. Incongruence and ITS polymorphism are caused by various processes including hybridization, introgression and ILS, and often their effects overlap, making it impossible to distinguish single events. Simply analyzing a greater number of molecular markers does not solve the problem of conflict due to reticulation. Instead in this thesis, I have chosen to focus on methods which allow a maximum of information to be drawn from plastid markers and, more importantly, ITS, which as a biparentally inherited marker is particularly valuable to trace reticulation. Using these approaches, a phylogenetic framework of the genus has been built and is now available for further, more detailed study, which might aim at several different aspects:

Complex relationships in certain clades require studies involving multiple populations, a comprehensive geographic sampling and the according methodical approaches, including those intended to distinguish hybridization / introgression from ILS. Furthermore, extensive cloning could provide valuable insights into reticulation processes within and between clades and species; highly polymorphic markers like AFLPs or microsatellites might be helpful to assess intraspecific variation. Apart from that, whole genome analyses by next-generation sequencing as now widely applied, should be able to add to the understanding of *Scrophularia* evolution. Another important focus lies on chromosome evolution within the genus. Providing chromosome counts for poorly investigated groups, and assessing ploidy levels and genome sizes will help to further elucidate the role of allopolyploidization.

Ongoing research in *Scrophularia* concentrates on morphological evolution. The considerable variability of morphological traits is likely linked to its complex evolutionary history. In this regard, all previous taxonomic concepts are re-evaluated, relevant morphological characters extracted, and, together with karyological analyses, linked to the phylogenetic results presented in this thesis (Scheunert and Heubl, in prep.). Together with a formal assessment of the taxonomic status of the Himalayan - Tibetan endemic *Oreosolen*, this should provide the basis for a thorough taxonomic revision of the genus *Scrophularia*.

6. Literature

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