The Evolutionary Dynamics of Genes and Genomes:

Copy Number Variation

of the *Chalcone Synthase* Gene in the Context of Brassicaceae Evolution

Dissertation

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Abstract

The Brassicaceae (Mustards, Cruciferae) are a cosmopolitan family comprising 370 genera and around 3660 species assigned to lately 50 tribes. The tribal system was originally based on solely homoplasious morphological character traits and reaches back to the early 19th century. De Candolle introduced the first tribal classification of the family nearly 200 years ago (1821) containing 21 partly still utilised classifications nowadays. Although labelling seems to be up to date, generic delimitations have been under permanent significant substitution and replacement. The tribes are arranged in three major monophyletic lineages and some additional small groups. The relationships within and between these lineages have not been resolved very clearly yet, as the Brassicaceae are characterised by frequently occurring hybridisation and polyploidisation events. This could be either the result of early and rapid radiation events or perhaps be the product of reticulate evolution, lediang to conflicting gene trees (KOCH & AL-SHEHBAZ 2009).

This lack of resolution could in parts be resolved via the application of the nuclear encoded *chalcone synthase* gene (*chs*) on 39 of the Cruciferous tribes. Several small-scale tribal-specific duplication events, including age estimations, could be detected giving insight into the evolutionary history of this molecular single- or low-copy gene. Most definitely a tendency towards diploidisation is proven by purifying selection as well as accelerated synonymous substitution rates among this family resulting sooner or later in the reduction of preliminary multiplied *chs* loci. Supposedly, *chs* is single-copy in most diploid mustard taxa.

The determination of orthologous and paralogous gene copies exposed to be of essential cause as it could be proven that yet functional but fluctuating DNA sequences demonstrate a huge impact on divergence time estimates as well as on any other extrapolation applying nucleotide or amino acid data. However, all crown age estimations calculated with diverse approaches resulted in reasonable output, dating the most recent common ancestor (tmrca) of the family to the Late Miocene or Oligocene.

Adjustments of the DNA sequences resulted in a well-resolved thoroughgoing gene tree phylogeny facilitating established taxonomic as well as phylogenetic achievements and do, moreover, hint to further ambiguities which have to be clarified by the commitment of additional marker systems.

Zusammenfassung

Die Kreuzblütler (Brassicaceae, Crucifereae) sind eine kosmopolitische Familie bestehend aus etwa 370 Gattungen und 3660 Arten, die 50 Triben zugeteilt werden. Das Tribensystem basierte ursprünglich auf morphologischen Charaktereigenschaften und kann bis ins 19. Jahrhundert zurückdatiert werden. De Candolle stellte die erste Tribenklassifikation der Kreuzblütler bereits vor fast 200 Jahren (1821) vor, welche aus 21 Klassifikationen bestand, die teilweise bis heute noch in Gebrauch sind. Obwohl es aussehen mag, als wären die Benennungen aktuell, waren gattungsbezogene Abgrenzungen stets von maßgeblichen Änderungen und Neuerungen betroffen. Die Triben sind in drei große monophyletische Linien und einige zusätzliche kleine Gruppen unterteilt. Die Beziehungen sowohl innerhalb als auch zwischen diesen Linien konnten bis heute nicht befriedigend abgegrenzt werden, was der Tatsache geschuldet ist, dass die Kreuzblütler durch das regelmäßige Auftreten von Hybridisierungen und Polyploidisierungen betroffen sind.

Dieser Mangel an Auflösung konnte teilweise durch die Verwendung des nukleär kodierenden Chalkonsynthase-Gens (*chs*) bei 39 Triben behoben werden. Verschiedene triben-spezifische begrenzte Duplikationsereignisse, inklusive deren Altersdatierung, konnten aufgedeckt werden und liefern damit Einblick in die evolutionäre Geschichte dieses molekularen Markers, der eine einzelne Kopie oder maximal wenige Genkopien aufweist. Höchstwahrscheinlich kann eine Tendenz zur Diploidiesrung attestiert werden, die sowohl durch negative Selektion als auch durch eine beschleunigte synonyme Substitutionsrate innerhalb der Familie belegt wird, die früher oder später zur Reduktion des vorübergehend vervielfachten *chs* Locus führen wird. Wahrscheinlich liegt die Chalkonsynthase als singuläre Kopie in den meisten diploiden Kreuzblütlern vor.

Die Bestimmung der Orthologie und Paralogie der Genkopien stellte sich als essentiell heraus, da nachgewiesen werden konnte, dass die noch funktionalen DNA Sequenzen, welche in absehbarer Zeit fluktuieren werden, großen Einfluss sowohl auf Altersabschätzungen als auch auf alle weiteren Hochrechnungen haben, die auf Nukleotid- oder Aminosäuresequenzen basieren. Jedoch resultieren alle Altersberechnungen, die mit verschiedenen Ansätzen den jüngsten gemeinsamen Vorfahren datieren, in einer Schätzung, die ins späte Miozän beziehungsweise das Oligozän fällt.

Die Bereinigung der DNA-Sequenzen führte zu einer gut aufgelösten Genbaum Phylogenie, die bereits nachgewiesene taxonomische und phylogenetische Erkenntnisse bestätigt. Außerdem konnten verschiedene Ungereimtheiten aufgezeigt werden, welche es nun durch die Anwendung weiterer Markersysteme aufzuklären gilt.

Introduction

1 The Mustard Family

The mustards (Brassicaceae), are a large plant family of a high awareness level not only in research but also in everyday life. They are widely distributed in cuisine as oil and vegetable plants (cauliflower, Brussels sprouts, kohlrabi, radish, rapeseed oil etc.) and are grown in gardens, both for consumption and as ornamental plants (*Aubrieta* ADANS, *Erysimum cheiri* (L.) Crantz, *Hesperis* L., Matthiola R.BR.) all over the world. So they are meanwhile of major economic and, partly therefore, scientific interest and importance. The probably best known species among the family is *Arabidopsis thaliana* (L.) Heynh, proposed by Friedrich Laibach in 1943, and is assigned to the tribe Camelineae. *A. thaliana* still is the model organism of choice, with an increasing importance and impact in respect to systematical, taxonomical, developmental and evolutionary research due to the tremendous level of knowledge about genes and their putative functions.

During the last few years, more wild allies and close relatives of *Arabidopsis*, *Arabis* and *Brassica* are focused on for several acquirements like their resistance to extreme environmental stress or their capability to attune to the accumulation of heavy metal as “extremophytes” (AL-SHEHBAZ 2012, AMTMANN 2009, BRESSAN et al. 2001, INAN et al. 2004, KRAMER 2010). The later named acquirement was investigated in *Arabidopsis halleri* (L.) O'Kane & Al-Shehbaz (AL-SHEHBAZ et al. 1999) and *Noccaea caerulescens* (AL-SHEHBAZ & O'KANE 2002, INGROUILLE & SMIRNOFF 1986, KOCH & GERMAN 2013, KRAMER 2010), while stress tolerance (salt, drought, cold) in *Eutrema s.l.* and *Schrenkiella* as well as accumulation (BARKER et al. 2009) have already been subjects to research. The potential of mustards to provide insight into genetics of flowering time (SCHRANZ et al. 2002), hybridisation, polyploidisation or gene silencing (PIRES et al. 2004) also supplied the research with an enhanced insight into evolutionary procedures on many different levels.

Even more information is and will be gathered from the achievement of sequencing full genomes at a defensible financial and time effort. During the last decade, a magnitude of fully annotated genomes was made available for research, e.g. *Arabidopsis lyrata* Reut. (HU et al. 2011), *Arabis alpina* (LOBREAUX et al. 2014), *Thellungiella parvula* and *Sisymbrium irio* (HAUDRY et al. 2013). Fully sequenced genomes improve the knowledge of functional variants and provide insight into evolutionary processes only theoretical before hands. As all this has drawn particular attention towards the family, the Brassicaceae have subsequently become a model for evolutionary biology (COUVREUR et al. 2010, FRANZKE et al. 2011, KOCH & AL-SHEHBAZ 2009).

The phylogenetic position of the Cruciferae, a cosmopolitan family, belonging to the order Brassicales, within the angiosperms is well established and approved. The Cleomaceae, for which a family-specific paleoploidisation [*Cleome spinosa* alpha (Cs-α)] could be confirmed (BARKER et al. 2009), are acknowledged as sister to the Brassicaceae and therefore serve as outgroup in various reconstructions (HALL et al. 2002, SCHRANZ & MITCHELL-OLDS 2006). Lately, 50 tribes are recognised (AL-SHEHBAZ et al. 2014) which are arranged in three major monophyletic lineages, introduced only in 2006, which are lineages 1, 2 and 3 (BEILSTEIN et al. 2006), and some additional small groups partly not assigned yet. BEILSTEIN et al. (2006) contributed therewith the first comprehensive phylogeny of the family.

Another group, expanded lineage II, was introduced later by FRANZKE et al. (2011) to assign several tribes in the family. Relationships between and within these lineages, especially concerning expanded lineage II, are not satisfyingly resolved yet. The internal classification of the Brassicaceae has long been and is still controversial. This expanded lineage, especially, is characterised by a lack of resolution. Lineage I to III are supported by several additional studies based on various marker systems like nuclear ITS (BAILEY et al. 2006), mitochondrial *nad*4 (FRANZKE et al. 2009) *trn*F, *adh* (alcohol dehydrogenase), *chs* (chalcone synthase), *mat*K (plastidic maturase) and ITS (internal transcribed spacer) combined in a supernetwork analysis (KOCH et al. 2007). This resulted in a largely congruent phylogeny, where expanded lineage II exacts further enquiries. The polytomy in the backbone phylogeny is not terminally resolved, yet. The avowal most frequently consulted is the happening of early and rapid radiation events resulting in minor genetic variation between the respective taxa. The duplication of the genome, known as whole genome duplication (WGD) or polyploidy, are widely accepted as a prevalent pathway for speciation in angiosperms and are traded as the event providing new genetic raw material for evolution of new lineages and species (CAO & SHI 2012, HURKA et al. 1989, KELLIS et al. 2004, WANG et al. 2011). Hence, WGDs are recognised as fundamental mechanisms in plant speciation and evolution (DOYLE et al. 2008, SOLTIS et al. 2009). All fully sequenced flowering plant genomes, including *Oryza* and *Sorghum*, contain evidence of paleopolyploidisation (BARKER et al. 2009).

The analysis of the *Arabidopsis* genome uncovered at least three ancient rounds of whole genome duplications within the Brassicaceae during their evolution. The most ancient known are At - γ (gamma) WGD or 1R, which occurred around 300 million years ago (mya), near the origin of all angiosperms (DE BODT et al. 2005). Lately, it was discovered that this paleopolyploidisation event is shared among other Rosids, including papaya (*Carica*); rape (*Vitis*) and poplar (*Populus*). The intermediate event, referred to as the *At*-β (beta) or 2R is supposed to have happen 170-235 mya (BOWERS et al. 2003, MING et al. 2008), near the radiation of the eudicots, and could be demonstrated by three groups of syntenic regions among their genome (JAILLON et al. 2007, MING et al. 2008). The most recent duplication event, occurring exclusively but independently in the Mustards and Cleomaceae (BHIDE et al. 2014, BHIDE et al. 2009) is known as *At*-α (alpha) or 3R. The age of that event which, in parallel, coincides with the radiation event of the core Brassicaceae, remains controversial. On the one hand due to the generation time hypothesis which suggests that the dating of all three duplication events seems to be a vast overestimation, settling *At*-γ as the synonymous duplication event detected in *Carica* and *Vitis*, shared by all Rosids and maybe even all eudicots. *Carica*, as a member of the Brassicales, did not undergo *At*-β, the constructive consequence is that the duplication event took place after *Carica* diverged from the rest of the Brassicales which dates the age of the intermediate WGD to a more recent point (LYONS et al. 2008). On the other hand, various approaches, concerning mostly the methodology, result in highly heterogeneous outcome. First family-wide studies used estimated rates of synonymous nucleotide substitutions (KOCH et al. 2001, KOCH et al. 2000) or, alternatively, the calculated age of the Arabidopsis duplication event (BELL et al. 2010, ERMOLAEVA et al. 2003, FAWCETT et al. 2009, SCHRANZ & MITCHELL-OLDS 2006) to deflect the age of the Cruciferae crown group including the most recent common ancestor.

These estimates concluded with evaluations to be between 15 to 60 million years and above (BEILSTEIN et al. 2010, COUVREUR et al. 2010, FRANZKE et al. 2009) depending on calibration approach, molecular data matrices and deviating sampling. But most estimations result in approximations that date the origin of the Brassicaceae to the Late Eocene or the Oligocene. However, there is still a debate going on concerning the method of secondary calibration via fossil constraints in the Brassicaceae. As mentioned later (see 2: The Tribal System of the Brassicaceae), morphological character traits in this family evolved independently several times (homoplasy), which, as a consequence, increases the effort to receive authentic anchor points representing the reliable phylogenetic placement intended to. For example, the putative Oligocene fruit fossil is assigned to the Thlaspideae. Assuming this assignment to the respective tribe is erroneous, as the estimates of divergence are biased (FRANZKE et al. 2011). Divergence time estimates still remain an open issue although a rough time frame is localised and affirmed to date.

But it is not only polyploidisation that shape the contemporary image of the Brassicaceae. Other important events as recombination, small scale duplications, hybridisation, convergent, parallel and reticulate evolution frequently occur and influence genome size, chromosome number, chromosome arrangement, number of gene copies, adaptational mechanisms, to name only a few consequences affected, are being examined extensively (LIHOVA et al. 2006, LYSAK & LEXER 2006).

2 The Tribal System of the Brassicaceae

"What looks alike is alike" used to be the guideline for earlier tribal classifications, totally embezzling parallel evolution. This system, which is more than one century of age (HAYEK 1911, JANCHEN 1942, SCHULZ 1936) and summarised in numerous reviews (AL-SHEHBAZ 2006, APPLE & AL-SHEHBAZ 2002, KOCH et al. 2003, MITCHELL-OLDS et al. 2004) was merely based on morphological characters which could be simply observed. Prior to 2005 only the tribe Brassiceae did and still does reflect the phylogenetic relationships of its component genera, while all other tribes, even Lepidieae, which were discussed to be monophyletic as well (ZUNK et al. 2000), were proven to derive from artificially delimited origin (AL-SHEHBAZ 2006). This resulted in an unclear artificial system which, since then, as a consequence, is under constant rearrangement. Due to the permanently increase of DNA techniques, methods and material in molecular biology, especially within the last 20 years, the tribal system is changing from a synthetic to a natural system, reflecting actual phylogenetic relationships within the Crucifer family. It became clear that most of the taxonomically relevant morphological characters evolved convergent, and only a small amount of taxonomic traits, that evolved uniquely, remains (FRANZKE et al. 2011).

This vivid changes are reflected by impressive numbers of research results with subsequent tribal reestablishments, recreations and renamings (AL-SHEHBAZ et al. 2011, AL-SHEHBAZ & O'KANE 2002, AL-SHEHBAZ & WARWICK 2007, GERMAN et al. 2009, GERMAN & AL-SHEHBAZ 2010, KOCH & GERMAN 2013, RESETNIK et al. 2013, WARWICK et al. 2010) published within considerably short lapses of time. The tribal classification of AL-SHEHBAZ et al. (2006) recognised 25 tribes. While only eight years later, in 2014, 49 tribes were actually approved, which is close to a duplication of this systematic unit. Just very recently, a new genus in the tribe Malcolmieae, namely *Marcus-Kochia*, was established (AL-SHEHBAZ et al. 2014) changing again the overall amount of genera in Brassicaceae. The actual achievements, mainly gathered within the last two decades, resulted in a delimitation comprising 320 genera with 3660 species organised in 49 (now 50) tribes, where only a small proportion of Brassicaceae taxa, namely less than 3%, (34 genera with 90 species) remains unassigned (Al-Shehbaz, 2012a).

Much effort was put in retracing and assembling the evolutionary history of branches or even the whole Mustard family. The majority of contributions combined morphological characters like trichomes (AL-SHEHBAZ & O'KANE 2002, AL-SHEHBAZ & WARWICK 2007, BEILSTEIN et al. 2008, FUENTES-SORIANO & AL-SHEHBAZ 2013, KOCH 2003) or seed morphology (AL-SHEHBAZ 2012, BROCHMANN 1992, KHALIK 2002, KOCH & MUMMENHOFF 2001), with molecular data resulting in phylogenies gaining incredible insight into the putative factual history and taxonomic relationships (AL-SHEHBAZ 2006, AL-SHEHBAZ et al. 2006, BEILSTEIN et al. 2006, BEILSTEIN et al. 2008, GERMAN et al. 2009, KOCH et al. 2007, WARWICK et al. 2010). The overall ambition is to establish a complete delamination of the Brassicaceae family comprising comprehensible definitions and boundaries of monophyletic tribes, which reflect the natural relationships within this plant family and to assign the last less than 5% of the remaining unassigned taxa to their respective position in phylogenies. A synopsis, covering the complete Crucifers, was recently provided by AL-SHEHBAZ (2012), which already is, in minor parts, not representing the latest findings.

3 Chalcone Synthase

The Chalcone Synthase gene (*chs*) is a nuclear gene that holds a central role in plant secondary metabolism. It is the branch point enzyme and serves as the initial step of the flavonoid pathway (WANG et al. 2007) and belongs to the class enzymes known as type III PKSs. It catalyses the condensation reactions of p-coumaroyl-CoA and three C(2)-units from malonyl-CoA to produce naringenin chalcone, the progenitor of all flavonoids.

This phenylpropanoid pathway produces secondary metabolites which are directly involved in the interaction between plants and environments (WINKEL-SHIRLEY 2001). In many plants flavonoids including anthocyanins, are thought to function against multiple biotic and abiotic environmental cues (COBERLY & RAUSHER 2003, IRWIN et al. 2003). In *Arabidopsis* CHS expression is known to up regulate in leaves due to environmental stress (WADE et al. 2001) and pathogen attacks. Genetic polymorphism of these essential genes may have ensured the survival and reproduction in *Arabidopsis*.

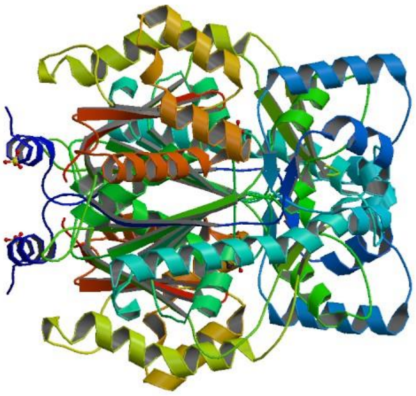


Figure 1 Assumed biological molecule of *chalcone synthase*, calculated with MODELLER v.9.11 (ESWAR et al. 2007). Homology of protein three-dimensional structure is modelled with known related structures employing the ModBase (PIEPER et al. 2011) database.

It has been demonstrated that enhanced flavonoid synthesis is highly associated with pigment biosynthesis (BOSS et al. 1996, KOES et al. 2005, SCHMELZER et al. 1988), which in turn affects the plant-pollinator interaction (IRWIN et al. 2003, SCHEMSKE & BIERZYCHUDEK 2001), disease resistance, UV protection in plant tissues (JOOS & HAHLBROCK 1992, KOOTSTRA 1994, WINKEL-SHIRLEY 2002), and alleviation of heat stress. All these adaptation traits affect the survival and reproduction of flowering plants. Therefore the products of this secondary metabolic pathway that control the flavonoid production, enable the plant to adapt more efficiently to a stressful environment (YANG et al. 2002).

To date not only CHS but also other enzymes involved in plant development and stress adaptation, like phenylalanin ammonia lyase (PAL), *chalcone isomearse* (CHI), stilbene synthase (STS) and flavanone 3-hydroxlase (F3H) have been isolated and studied in different model plants. It was shown through FLIM FRET imaging that CHS interacts with these enzymes (CROSBY et al. 2011). The CHS enzyme is encoded by a small multigene family in many plant taxa, varying in copy number (KOES et al. 1989). In most dicots, CHS form a family with six to twelve members, such as in *Populus* (TUSKAN et al. 2006), *Glycine max* (TUTEJA et al. 2004), *Viola cornuta* (FARZAD et al. 2005, VAN DEN HOF et al. 2008) and *petunia* (KOES et al. 1989). Only five functional *chs* genes have been described in morning glories (DURBIN et al. 2000), with special reference to the genus *Ipomea*, while nine gene copies exist in clover (HOWLES et al. 1995). It was suggested by (DURBIN et al. 1995), that new *chs* genes in flowering plants are recruited repeatedly resulting in increased nucleotide substitutions in newly duplicated genes. Providing further evidence of functional divergence appears to have occurred repeatedly in angiosperms (YANG et al. 2002).

In *Arabidopsis thaliana*, two active additional *chs*-like paralogous genes have been identified (WANG et al. 2007). Consequently, further copies are also often called "*chs*-like" regardless of their functionality. However, the *chs*-like genes in *Arabidopsis thaliana* (WANG et al. 2007) show a different expression pattern (ZHOU et al. 2013) in comparison to the one discovered first, and only one gene encodes chalcone synthase (OWENS et al. 2008). In the close relative *Arabidopsis halleri ssp. gemmifera* at least two additional *chs*-like copies have been described (WANG et al. 2007). Both sets of paralogs have been long diverged as the sequence identity is low, which suggests diverse evolutionary travelling. This indicates that the *chs* gene is single copy in nearly all *Arabidopsis* taxa and, moreover, in the whole Brassicaceae family (WANG et al. 2007).

Exon 1
Exon 2

Figure 2. Illustration of the *chalcone synthase* gene (5' to 3'). Promoter and intron are depicted by lines while the exonic regions are shown as blocks.

CHS exists as a homodimeric protein with each monomer approximately 42-45 kDa in size (AUSTIN & NOEL 2003). The gene serves as nuclear marker with a complete size of 1500 bp and a coding region of approximately 1200 bp, both depending on genus affiliations. This relatively suitable dimension provides convenient preconditions for convenient handling in the lab. Not only locations but also reference sequence of the gene are available. Moreover, CHS is highly conserved across diverse species at chromosome 5, which, most likely, is a forecast for function and supports the probability to obtain the demanded gene. *Chs*, like most genes, evolves relatively steady, which, in combination with the conservation, suggests to hold some functional significance (WANG et al. 2007). Chalcone synthase has additionally proven to be an appropriate marker (AUSTIN & NOEL 2003, HEMLEBEN et al. 2004, JOLY et al. 2009, KOCH et al. 2007, KOCH et al. 2000, LIHOVA et al. 2006) in regard to phylogenetic reconstructions concerning the Brassicaceae family and its suitability for studies in both gene duplication and investigations on the origin of gene families (DURBIN et al. 2000). This is due to the fact that *chs* is a single-copy gene in most of the mustards. Additionally, this nuclear gene is scarcely affected by any recombination event resulting expectedly in one copy per diploid taxon.

Based on several previous publications, phylogenies generated from *chs* and other nuclear genes like ITS are in conflict with other markers (BAILEY et al. 2006, BEILSTEIN et al. 2006, FRANZKE et al. 2009, KOCH et al. 2001, KOCH et al. 2000), unable to offer a suitable explanation for this contradiction. This lack of scientific performance demonstrates that the nuclear genome either underwent diverse discriminative developments in respect to the plastidic genomes, or the methods applied on the nuclear DNA are not powerful or technically mature enough to result in representative comparable results.

The aim of this study is *not* to once more re-draw the Brassicaceae phylogeny with another marker providing congruence among the genome and decide upon topology, but to test whether *chs* is a candidate locus for the construction of gene trees. The objective is to receive hints and unravel not only the evolutionary past of this gene, but also more recent events by sequence analysis.

The aim of this study is to generate hints of the occurrence of this gene, by pointing out specific evolutionary events like potential small scale duplication events and other dynamics that could have ended up in putative copy number variations in branches or lineages of the Brassicaceae, or even within the complete family.

Part 1: Trouble with the Outgroup

4 Material and Methods

Experimental and analytical procedures for the initial *chalcone synthase* (*chs*) data are specified in the first part of the work on hand. Within this part, the complete data set, holding 668 Brassicaceae sequences and one *Cleome* sequence, which serves as outgroup, are employed. Descriptive as well as experimental approaches are explained and applied on those 669 *chs* copies.

4.1Experimental set-up

4.1.1 Plant material and data composition

Representative taxa for 39 of the 49 tribes were utilised from cultivations in the greenhouses of the Botanical Garden in Heidelberg. The remaining ten tribes could not be included, as no appropriate seed material was available. The seeds were all collections from the wild to avoid crossbreeding as the natural habitat of the species is widespread. In most cases at least two species per tribe, if not monogeneric anyway, were included in analysis. Seed stocks stem from several rounds of seed increase with special emphasis on diploids to ensure single copies and a maximum of two alleles with the slightest genetic variability. After all, plant material from young leaves from 205 species appending to 104 genera, covering 44 tribes was employed and can be viewed in the appendix (supplementary material S10).

Tribe	monogeneric	lineage	n genera	n species	Reference
Aethionemeae	no	n/a	1/1	2 /35	Koch & Al-Shehbaz, 2008
Alysseae	no	exp lin II	5/18	5/170	Resetnik et al., 2013
Alyssopsideae	no	lin I	2/4	3/7	Al-Shehbaz et al., 2010
Anastaticeae	no	lin III	3/13	3/37	Al-Shehbaz et al., 2012
Anchonieae	no	lin III	2/10	4/51	Couvreur et al., 2010
Arabideae	no	exp lin II	4/	24/52	Karl & Koch, 2013
Biscutelleae	no	n/a	1/2	1/8	German & Al-Shehbaz, 2008
Bivonaeeae	yes	exp lin II	1/1	1/1	Al-Shehbaz et al., 2006
Boechereae	no	lin I	1/8	6/122	Al-Shehbaz et al., 2006
Brassiceae	no	lin II	14/47	28/235	Al-Shehbaz et al., 2006
Buniadeae	yes	lin III	1/1	1/3	Al-Shehbaz & Warwick, 2006
Calepineae	no	exp lin II	1/3	1/7	German & Al-Shehbaz, 2008
Camelineae	no	lin I	3/16	5/74	Koch & Al-Shehbaz, 2008
Cardamineae	no	lin I	4/9	15/35	Koch & Al-Shehbaz, 2008
Chorisporeae	no	lin III	2/4	2/57	German et al., 2011
Cochlearieae	no	exp lin II	2/2	21/24	Koch et al., 2012
Coluteocarpeae	no	exp lin II	3/6	4/14	Al-Shehbaz et al., 2012
Conringieae	no	exp lin II	1/2	1/7	German & Al-Shehbaz, 2008

Tribe	monogeneric	lineage	n genera	n species	Reference
Crucihimalayeae	no	lin I	2/2	4/11	German & Al-Shehbaz, 2010
Descurainieae	no	lin I	3/6	5/58	Al-Shehbaz et al., 2006
Dontostemoneae	no	lin III	2/2	4/17	Al-Shehbaz & Warwick, 2006
Erysimeae	yes	lin I	1/1	8/150	German & Al-Shehbaz, 2008
Euclidieae	no	lin III	8/25	27/68	Al-Shehbaz & Warwick, 2007
Eutremeae	no	lin II	2/4	4/24	Al-Shehbaz & Warwick, 2006
Halimolobeae	no	lin I	1/5	1/44	Bailey et al., 2007
Heliophileae	no	exp lin II	1/1	1/60	Al-Shehbaz et al., 2006
Hesperideae	no	lin III	1/2	1/47	Al-Shehbaz et al., 2006
Iberideae	no	exp lin II	1/2	2/30	Al-Shehbaz et al., 2006
Isatideae	no	lin II	4/9	5/32	Koch & Al-Shehbaz, 2008
Kernereae	no	exp lin II	1/2	1/2	Al-Shehbaz et al., 2010
Lepidieae	no	lin I	2/8	9/245	Koch & Al-Shehbaz, 2008
Malcolmieae	no	lin I	1/10	2/68	Al-Shehbaz & Warwick, 2007
Megacarpaeeae	no	exp lin II	2/2	2/12	Al-Shehbaz et al., 2009
Microlepidieae	no	lin I	1/16	8/56	Al-Shehbaz et al., 2010
Oreophytoneae	no	lin I	1/2	1/6	Al-Shehbaz et al., 2010
Physarieae	no	lin I	2/7	3/133	Fuentes Soriano & Al-Shehbaz, 2013
Schizopetaleae	no	exp lin II	1/6	1/31	Al-Shehbaz et al., 2006
Sisymbrieae	yes	lin II	1/1	4/43	Al-Shehbaz et al., 2007
Smelowskieae	no	lin I	2/2	4/25	Al-Shehbaz et al., 2010
Stevenieae	no	exp lin II	2/5	2/14	Al-Shehbaz et al., 2011
Thelypodieae	no	lin II	4/31	4/193	Warwick et al., 2009
Thlaspideae	no	lin II	2/12	2/78	Al-Shehbaz et al., 2006
Turritideae	yes	lin I	1/1	2/82	Al-Shehbaz et al., 2012
Yinshanieae	no	lin I	2/2	2/14	Al-Shehbaz et al., 2010
Unassigned		n/a	2/34	2/90	Koch & Al-Shehbaz, 2008
Cleomaceae	no	n/a	1/8	1/257	Sánchez-Acebo, 2005

Table 1. Overview on the number of tribes (COUVREUR et al. 2010), genera and species of the Brassicaceae employed in this study with latest reference revisions. Tribes are assigned to corresponding lineage. n/a means that this tribe is not (yet) assigned to any lineage.

4.1.2 DNA extraction and PCR amplification

Genomic DNA was generally extracted according to the standard CTAB-protocol of Doyle and Doyle (DOYLE & DOYLE 1987) with some slight modifications concerning time-spans and quantity of reagents which will be described below.

Green leaf tissue of an approximate size of 0.25 cm^2 was taken from each species which was homogenised in an automatic swing mill (PeqLab Precellys 24 homogeniser, Bertin Technologies, Erlangen, Germany) for 30 seconds. To each 2 ml tube two to three glass beads were added. The grinded leaf material was incubated with 800 µl CTAB extraction buffer [containing 2% (w/v) CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM Na-EDTA, pH = 8.0 and freshly added 0.2% β-Mercaptoethanol] each at 60°C on a thermo block (Eppendorf) for 30 minutes. Then, 500 µl cold chloroform-isopropanol (1:24, v/v) were added. The mixture was inverted and stored at 4°C for 15 minutes. Afterwards the samples were centrifuged at maximum speed (40,000 rpm) for 5 minutes at 4°C as well. The upper phase of about 600 µl was extracted and mixed with 400 µl ice-cold isopropanol and left on ice for 10 minutes. After 5 minutes of centrifugation the isopropanol-phase was separated. The remaining pellets were washed twice with 350 µl of 70% ethanol. After drying, 50 µl of low TE-buffer admixed with 2 U of RNAse were added for RNA digestion (60 minutes at 37°C on a thermo mix).

Concentration of extracted DNA was measured on a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies Inc., Wilmington, USA) and all samples were diluted to a final DNA concentration of 100 ng/µl for the following cloning procedure.

To amplify the respective markers, PCR were performed in a reaction volume of 25 µl each, which contained 10-50 ng template DNA, 5 µl PCR reaction buffer, a final concentration of 2.0 mM $MgCl_2$, 10 pmol of each primer, 2.5 pmol of each nucleotide, and 0.5 U of Mango Taq Polymerase (Bioline, Luckenwalde, Germany). Chalcone synthase sequences were initially amplified with the primer pair CHS-PRO1-fw [5'-CAT CTG CCC GTC CAT CAA ACC TAC C-3'] and CHS-EX2-TERM-rev [5'-TTA GAG AGG AAC GCT CTG CAA GAC-3'], as designed by Koch et al. (2000). The forward primer is situated in the promoter of the gene (CHS-PRO1-fw) leaving less than 200 nucleotides of the regulatory regions to make sure that, firstly, the beginning of the coding region is completely synthesised and secondly, that the remaining part of the promoter ensures the functionality of the gene. The reverse primer (CHS-EX2-TERM-rev) starts with the end of the second exon. Consequently, the complete gene was amplified at one stretch. Some accessions from the tribe Arabideae showed to be recalcitrant and could not be amplified although several attempts were conducted. Therefore, a new primer pair was designed (ARA-N-CHS-1-for [5'-GGC ACA RAG AGC TGA TGG A-3'] and ARA-N-CHS-5-rev [5'- AGA GAA GAT GAG AGC RAC WCG -3']) and used on those accessions indeed resulting in reliable amplifications, as they are more discriminative. PCR products from this primer combination result in shorter sequences, amplifying only a partial fragment of the gene.

All amplifications were run on a Peltier Thermal Cycler (MJ Research, Waltham, MA, USA) with the following programme. Initial denaturation step for 3 minutes at 95°C, followed by 30 cycles of 30 seconds at 95°C (denaturation) and 30 cycles at 58°C for 30 seconds, as well (annealing). Another 30 cycles were performed for 1 minute at 72°C for elongation and 5 minutes at the same temperature for the final elongation. The procedure was stopped with a hold at 10°C.

PCR products were run with electrophoresis in a 1.5% agarose gel in TAE-buffer which was stained with GelRed Nucleic Acid Stain (Biotium, Hayward, CA, USA). As reference for DNA fragment length, 1 µl smart ladder (Eurogentec; Cologne; Germany). To ensure the absence of contamination and the quality of the PCR run, one negative (PCR mix with added highly pure water instead of DNA) and one positive (already tested and amplified DNA fragment) reference were applied to each agarose gel. The complete fragment length, around 1,400 base pairs, was expected to be displayed on the gel.

4.1.3 DNA cloning and sequencing

Chs is reported to be single-copy (CAIN et al. 1997, KOCH et al. 2000), in most diploids or at least a low-copy gene. But two *chs*-like genes have been identified in *Arabidopsis* by Wang et al. (2007), and up to 13 copies can be identified in other angiosperms like *Ipomea* (DURBIN et al. 1995). To avoid accidental multiplication of pseudogenes, resampling or transferred genes, a colony PCR can be employed to test the plasmids, but only after the cloning procedure. Therefore, the respective fragments were cloned into chemical competent *E. coli* cells (strain JM109). DNA cloning does not only heighten the amount of the respective marker, it also stabilises the received products. Purified DNA (Wizard SV Gel and PCR Clean-Up System; Promega, Madison, WI, USA) was ligated into a vector (pGEM-T vector system, Promega, Madison, WI, USA). Afterwards the *E. coli* cells are transformed with the vector-insert construct. Recombinant bacteria were allowed to grow overnight in agarose plates, containing X-gal, for blue and white screening, selecting for further analysis only white (positive) colonies.

A colony PCR was conducted in order to detect as many clones as possible carrying the complete gene sequence The universal primer T7 [5'-TAA TAC GAC TCA CTA TAG GG-3'] and SP6 [5'-ATT TAG GTG ACA CTA TAG AA-3'] in order to detect potential DNA sequence variation.

Sequencing of all *chs* plasmids was performed at two commercial sequencing services (GATC, Konstanz, Germany; MWG Eurofins, Ebersberg, Germany). In case fragments were directly sequenced (i.e. without subsequent cloning), either the identical amplification primers or universal primers M13 (-21) [5'-TGT AAA ACG ACG GCC AGT-3'] and M13 (-29) [5'-CAG GAA ACA GCT ATG ACC-3'] were employed.

4.1.4 Internal validation with ITS

For internal taxonomic validation of the utilised genera, the internal transcribed spacer regions (ITS) of the nuclear ribosomal RNA were employed. ITS1 and ITS2 are the “most commonly used nuclear markers (EDGER et al. 2014)“ in phylogenetic studies. The GeneBank accession numbers for the synthesised ITS sequences referred to at the work on hand are LN589647-LN589719.

cycles	Temperature (°C)	Time (min)	Repetitions	Step
1	94	5	1	Initial denaturation
2-11	94 65	0.5 1	11	Annealing
12-22	65 (- 0.7 per cycle)	1	11	Denaturation
23	72	1	-	Elongation
24	10	∞	∞	Final elongation

Table 2. ITS specific PCR touchdown protocol as modified by DOBES et al. (2004).

The further proceeding is the same like described for CHS with the exception of directly sequencing the PCR products without a cloning procedure.

To amplify the marker, PCR reactions were performed in a reaction volume of 25 µl each, which contained 10-50 ng template DNA, 5 µl PCR reaction buffer (5 mM MangoTaq buffer), a final concentration of 1.5 mM $MgCl_2$, 0.2 mM of each primer, 0.1 mM dNTPs, and 0.5 U of Mango Taq Polymerase (Bioline, Luckenwalde, Germany). The primers used for ITS are the forward primer ITS-18F [5'-GGA AGG AGA AGT CGT AAC AAG G-3'], as modified by MUMMENHOFF et al. (1997) and the reverse primer ITS-25R [5'-TCC TCC GCT TAT TGA TAT GC-3'], as designed by WHITE et al. (1990). The amplifications were run on a PTC (MJ Research, Waltham, MA, USA). The programme run was a slightly modified by DOBES et al. (2004) touchdown PCR to ensure that only the explicitly desired products are multiplied.

4.2 Bioinformatical data analysis

4.2.1 Sequence editing and alignment

SeqMan II v.6.1 (DNAStar, Madison, WI, USA) was utilised for editing and contig assembly alignment of *chs*. Nucleotide sequences were aligned with the programme BioEditSequence Alignment Editor v. 5.0.6. (Hall, 1997-2001) initially using the implemented programme CLUSTAL-W (THOMPSON et al. 1994) with changed Multiple Alignment parameters to Gap Opening Penalty = 3 and Gap Extension Penalty = 1,8 to improve accuracy. Promoter/intron/exon boundaries were determined by comparing the genomic sequence to previously published *chs* exonic boundaries (KOCH et al. 2001). The alignment was visually examined and manually adjusted guided by identification of open reading frames, exon positions and termination codons. For revision and certainty, each alignment was double-checked with another programme, MUSCLE (EDGAR 2004), implemented in MEGA5 (TAMURA et al. 2011). Complete identical sequences (number of identical sequences) were identified and eliminated with the programme ElimDupes (KUIKEN et al. 2005) from the data set as they do not provide any additional or necessary information.

A study by THOMPSON et al. (1999) compared a number of alignment programmes and showed that there is a major correlation between the accuracy of the aligned sequences and the resulting phylogenetic trees. The calculated threshold for amino acid identity is 20% as this results in around 50% correctly aligned residues. Another study (OGDEN & ROSENBERG 2006) demonstrated that the tree accuracy varies only little with alignment accuracy as long as this is above 50%. Therefore the pairwise amino acid distance, which is the proportion (p) of amino acid sites at which two sequences are different, of the overall multi-sequence alignment was tested via a p-distance (1 – amino acid identity) based tool, implemented in MEGA version 5 (TAMURA et al. 2011), as well.

Afterwards, the complete data set was divided into the single tribes to receive a better impression of the closely related genera and their relationships within and between the taxa. Therefore the promoter and intron regions, as they were too variable to be aligned with confidence, were manually removed and the remaining sequences were aligned in individual alignments (upon request) following the same procedure as described above for the complete alignment. Alignment accuracy was calculated for each tribe, as well.

Nucleotide sequences have been deposited in the European Molecular Biology Laboratory (EMBL) GeneBank library (LK937201-LK937666 and LN623709- LN623711), available from European Nucleotide Archive (ENA). A large overall alignment (alignments are available on request) serves as the bases of any splitting and analysis of further subsets.

4.3 Comparative phylogenetic reconstructions and analyses

4.3.1 Tests applied on data set prior to phylogenetic analyses

Prior to phylogenetic inferences, the best-fit nucleotide substitution model and the parameter estimates used for tree reconstruction, according to the Akaike Information Criteria

(AIC), for the respective data set were identified via ML analyses. Therefore the Modeltest 3.7 (POSADA & CRANDALL 1998) in conjunction with PAUP (Phylogenetic Analysis Using Parsimony) and, for comparison, the model test (Find best Protein/DNA models (ML) implemented in MEGA5 TAMURA et al. (2011) were applied on the data set.

To test whether the data are suitable for estimating neighbor joining trees, NEI et al. (2000) described in their book that the Jukes-Cantor (JC) distance method should be applied on data sets previous to the decision on an appropriate algorithm. The average pairwise distance was calculated to not exaggerate 1.0. Each data resulting in > 1.0 are not suitable for estimating NJ trees. Jukes-Cantor distances were calculated using MEGA5 (TAMURA et al. 2011). This method was applied on the complete data as well as on every partial data set before applying the NJ algorithm.

4.3.2 Phylogenetic analysis

For a start, two algorithms were applied on the data for phylogenetic analyses. The neighbor joining (NJ) method, using Kimura’s two-parameter model from 1980 and a maximum-parsimony (MP) method using PAUP* 4.0b10 (SWOFFORD 2011). A robustness test of phylogenetic hypotheses is not needed (topology test), because no additional marker systems are utilised.

Cleome spinosa was used as outgroup to root the trees, as the Cleomaceae are known to be sister to the Brassicaceae and hence are proven to fulfil the requirements to serve as outgroup.

The complete trees, depicting 624 sequences, can be viewed in the appendix (S15). The NJ algorithm was applied as mentioned above. This amount of data would result in a tree covering several pages. For convenience a sub-data set of 63 sequences was extracted from the original data that is supposed to depict precisely the same. Each tribe is represented by at least one sequence which results in a small-scale copy of the complete data. For further analysis, both data sets are invariably employed, where merely the reduced set is displayed while the entire examinations are attached.

Neighbor joining (NJ) method analyses (SAITOU & IMANISHI 1989) were performed using MEGA5 (TAMURA et al. 2011) to depict evolutionary relationships among the taxa by calculating genetic distance based on Maximum Composite Likelihood of the Tamura-Nei model. This method increases the accuracy of calculating the pairwise distances. Confidence of the clade reconstruction was tested by bootstrapping with 1,000 replicates, which depicts the percentage of replicate trees in which the associated taxa clustered together (FELSENSTEIN 1985). The complete data set was utilised for estimations.

Maximum parsimony algorithm (MP) uses an MP search method to implement parsimony by searching for the minimum number of steps, which equals the minimum change between data. A newer method for larger data sets was applied which is called Parsimony Ratchet implemented with the software PAUP (NIXON 1999, SWOFFORD 2011). After generating an initial tree, the model repeats a certain process (1. Select character of subsets, 2. TBR keeping only one tree, 3. Set character to equal rates, 4. TBR on current tree, 5. Repeat 50-200 times) 200 times (= iterations). Settings were chosen mainly following Nixon's review (NIXON 1999).

4.3.2.1 Divergence time estimates

The dating of the Brassicaceae origin and various other calibration points within this family was previously undertaken with huge effort and have led to highly divergent results, depending on the applied marker systems, substitution rates or calibration points (KOCH 2012). The chalcone synthase has already been successfully utilised for divergence time estimates in previous reviews (BEILSTEIN et al. 2010, COUVREUR et al. 2010, KOCH 2012). To compare those results with newly gathered data at hand, nuclear sequence divergence between groups of taxa were estimated. Because genes accumulate changes over time at a more or less constant rate, the genetic distance between two species, measured by the number of changes accumulated, will be proportional to the time of species divergence. Synonymous substitution rates, also especially for the nuclear chalcone synthase, have been estimated several times, varying among a certain range from 8×10^{-9} to $1{,}67 \times 10^{-8}$ substitutions/site/year (DURBIN et al. 1995, HUANG et al. 2012, KOCH et al. 2001, LAROCHE et al. 1997, WANG et al. 2007), partly based on fossil pollen data. For a first approach, these rates were truncated as minimum and maximum, as secondary calibration dates require boundaries (HO et al. 2010, HO 2007) and will be prospectively abbreviated as *Rate*.

Although it is mostly confuted that a strict molecular clock can be applied on that data, substitution rates are arguable as, firstly, the rate is not tight to a fixed rate and, secondly, groups are allowed to vary among different lineages (DRUMMOND et al. 2006).

An alternative approach to estimate the age of several groups, namely tribes or lineages, was focused on using the Bayesian analyses package. Normally, direct primary calibration via macrofossils is advised to circumvent increasing distortion and noise in the results. But the utilisation of fossils constraints within the Brassicaceae is debated on intensely. On the other hand secondary calibration approaches are often being criticised to produce unreliable results

(SHAUL & GRAUR 2002). Therefore broad prior probability distributions were scheduled on the calibration point.

Secondary calibration was achieved by referring to major minimum angiosperm split ages (BELL et al. 2010, WANG et al. 2009, WIKSTROM et al. 2001). Estimations of absolute divergence times require calibrating the age of at least one node (WANG et al. 2009). The youngest calibration point set the spilt node of Burseraceae (JF728822 *Canarium album*) and Anacardiaceae (KC287084 *Rhus chinensis*), with the most common ancestor *Bursera* and *Schinus*, to 50 mya (CLEAL et al. 2001) the second point to 65.5 mya indicating the split between Malvaceae (EF643507 *Gossypium hirsutum*, EU573212 *Abelmoschus manihot*) and Thymelaceae (EF103197 *Aquilaria sinensis*) and the oldest calibration point was constraint to the split of Hypericaceae (AF461105 *Hypericum perforatum*) and Clusiaceae (FJ197128 *Garcinia mangostana*) dated 89 mya (CREPET & NIXON 1998). All named splits are represented with *chalcone synthase* sequences from NCBI (accession numbers in brackets) which were added to the data set, each with a standard deviation (SD) of 2. The uncorrelated relaxed clock method (UCLN) was used to infer divergence times. This approach will prospectively be abbreviated as *Angio*.

For contrasting reasons, a third approach was tested utilising fossil data (prospectively abbreviated as *Fossil*). The primary calibration was based on two fossils. A normal prior of mean 6 and SD 2 was given to the most recent common ancestor of *Rorippa* and its closest relative in dependence on the *Rorippa* fossil dated to the Pliocene 2 to 5 mya (WALTHER 1995). Another soft calibration point was utilised with a normal prior with a mean 35 and SD 10 was enforced to the node of the crown group of the Brassicaceae family (MANDAKOVA et al. 2010), covering the most frequently estimations from previous calculations, including the oldest putative Brassicaceae fossil from the Oligocene, 22 to 35 mya (CRONQUIST 1981).

The analyses accounted for rate variation using an UCLN drawn from a lognormal distribution and the birth-death speciation model for incomplete samplings and the Hasegawa, Kishino and Yano (HKY) model of nucleotide evolution with four categories and all equal base frequencies. For the calibration, different types of priors were implemented in the settings of BEAUti (Bayesian Evolutionary Analysis Utility), which is the graphical user-interface (GUI) application for generating BEAST XML files (DRUMMOND et al. 2012), to date divergence. Following the standard procedure, four independent runs were conducted. Based on lab experience, 5 x 10^7 generations per analysis from starting trees, with branch lengths satisfying the respective priors on divergence times, sampling every 5,000 generations, were run. This resulted in 10,000 trees each. Four independent runs with each 50 million generations were accomplished.

For each run convergence statistics were analysed with the programme Tracer v. 1.4.1, discarding the first 10% (DRUMMOND & RAMBAUT 2007), using 9,000 post-burn-in generations. Convergence was obtained when reaching stationary phase and a sufficient effective sample size (ESS > 200), each, in the combined file. Using the included programme LogCombiner v.1.7.5. (DRUMMOND & RAMBAUT 2007) to combine both log and tree files, followed by the programme TreeAnnotator v.1.7.5. (DRUMMOND & RAMBAUT 2007) to create maximum clade credibility trees, burning in the first 10% of the trees, what equals 5.000 trees, and, moreover, to determine the 95% posterior density for the trees' nodes. Final tree files were graphically processed with FigTree v1.4.0 (RAMBAUT 2012), a programme belonging to the BEAST group, but distributed separately. This tree viewing programme includes summary information produced by TreeAnnotator.

4.4 Sequence analysis

4.4.1 Alignment analysis of the complete data

All sequence alignments per branch were analysed in different contexts, like divergence calculations within the tribes or sequence statistics of gene elements, to search for abnormalities and peculiarities among the genera. A table was compiled (**Table 7**) depicting each tribe with its basal statistical composition, which together represent the complete data. Within the illustration, divergence data (number of differences) both from the coding and the complete gene were calculated in MEGA5 (TAMURA et al. 2011).

4.4.2 Identifying gene regions

The examination of the complete nuclear gene (promoter, intron and two exons) will help to unravel the differences and similarities among the tribes, genera and species. It, moreover, reveals the discrepancies among the sequences that resulted in unexpected phylogenetic placements. In most studies concerning genes encoding a protein, it is prevailing to only employ the coding region for analysis. Of course, estimations and calculations are just applied on the coding gene and not on the intragenic regions. However, molecular generic elements like the promoter region have been successfully employed to not only compare sequential parts but also to investigate functionality (DE MEAUX et al. 2005). Therefore the complete sequences have been torn apart into their ingredients to apply diverse analysis on.

Therefore different analytical and comparative methods (e.g. motif search, analyses of conserved regions, length comparison) have been applied on the respective gene region.

4.4.2.1 Trinucleotide frequency (k-mers)

DNA sequences display compositional heterogeneity on many scales (KARLIN et al. 1998, KARLIN & LADUNGA 1994, KARLIN et al. 1994). Although DNA composition is proven to be relatively uniform throughout the genome (PAULSEN et al. 2005), k-mer composition is independent and deviations may be an indicator of regions on the DNA that appear as unusual. Previous review has demonstrated that di- and trinucleotide frequencies provide characteristic patterns and are presumably in charge of structural and functional aspects of genome biology (COSTANTINI & BERNARDI 2008). Though illustrations on dinucleotide level tend to result in significant underestimations of several averaged bias as well as in overestimation of others (PORCEDDU & CAMIOLO 2011). In coding sequences, the distribution of the nucleotide bias is expected to be highly dependent on the class of the equivalent trinucleotide. Hence, distribution of all 64 trinucleotides (k-mers) were determined by estimating their frequencies via codon usage bias with MEGA (TAMURA et al. 2013). For illustration a heat map diagram was chosen, beacause this format intuitively distinguishes distinctive characteristics in large-scale data sets. Therefore the software R (R CORE TEAM 2014) was employed, which is an open source statistical environment. Two additional packages namely the ggplot2 package (WICKHAM 2009), available for R, for graphical illustration and a package for transforming input data, namely reshape2 (WICKHAM 2007), converting data for ggplot2, were utilised.

5 Results

5.1 Comparative phylogenetic reconstructions and analyses

5.1.1 Tests applied on data set prior to phylogenetic analysis

Both model tests applied on the complete data set as well as on the reduced set gave similar outcomes.

	Complete dataset	Reduced dataset
PAUP	GTR+G+I	GTR+I+G
MEGA5	GTR+G+(I)	GTR+G+I

Table 3. Results from modeltests on complete and to illustrate representatives from the tribes employed in this study.

After submitting the data sets to different substitution models, the most appropriate one is the General Time Reversible model with γ-distribution and invariant sites with 121 parameters to estimate with the programme MEGA5. Only the complete data set (PAUP) does not display identical results concerning the log likelihood (lnL) for GTR+G and GTR+G+I (therefore I in brackets). All other results suggest that the invariant sites should be added for calculations, therefore the asset for the respective model is defensible. PAUP’s output is in congruence, supported by the highest log likelihood.

The Jukes-Cantor distance method (NEI & KUMURA 2000) resulted in d = 0.160 for the complete coding data set and in d = 0.144 for the reduced data set of 57 sequences depicting a representative excerpt of the complete data. As the d-values are well within the range (d < 1.0), the data, both from the complete as well as from the subset, are appropriate to construct neighbor joining trees.

5.1.2 Comparative phylogenetic reconstructions

The evolutionary history was inferred by the neighbor joining [Maximum Composite Likelihood method (TAMURA et al. 2004)] algorithm which resulted in an optimal tree with a length (L) of 3.25. The evolutionary distances are depicted as base substitutions per site and the confidence probability is shown above branches. Values below 50% are cut off. The resulting gene tree displays 62 *chalcone synthase* genes from 39 tribes of the Brassicaceae plus unassigned genera. Additionally, *Cleome spinosa* is shown as outgroup. This representation exhibits ten groups, from which some contain only one sequence, like *Iberis saxatilis* or *Bivonaea lutea*.

The topology of the NJ tree resembles contrastable estimations highly. Lineage I representing, one of the two crown groups, which are all taxa descended from a major cladogenesis event, is displayed by 19 sequences. This are all tribes assigned to that lineage. The branch of that group is supported by a bootstrap value of 91 percent suggesting high confidence. Two tribes, namely Lepidieae and Cardamineae are positioned outside the remaining sequences from lineage I, what is not in perfect congruence to approved ITS phylogenies (GERMAN et al. 2009, LIHOVA et al. 2006, YUE et al. 2009), where Smelowskieae, Lepidieae and Descurainieae are sister to the rest of lineage I. The Lepidieae, which were already suggested as sister to lineage I are supported by 91 percent reproducibility and the Cardamineae are depicted as sister to lineage I and the Lepidieae. However, Smelowskieae, expected in a sister position to that lineage and here represented by *Smelowskia tibetica*, have swapped placement with Cardamineae. The second crown group assigns a mixture of sequences.

The most diverged part, supported by a bootstrap value of 64 percent depicts all six members from lineage II, supporting previous research results of Eutremeae and Thlaspideae defined as sister group to the remaining lineage II. One Anchonieae sequence (lineage III) is intermingled with that lineage.

Above that group are seven additional sequences from expanded lineage II and 1, as well as below lineage II. Group eight displays the placement of *Cleome spinosa*, which is applied as outgroup, joined by four sequences representing fragments of the Anastaticeae, Arabideae and the Dontostemoneae, from lineage III and expanded lineage III, supported with 99 percent. Another pair of sequences, both assigned to expanded lineage II (*Megacarpaea polyandra* and *Schizopetalon walkeri*), depict a sister-relationship to *Cleome* and its surrounding tribal representatives. The next group among the topology holds six out of seven members from lineage III fostered with 99% bootstrap support. Above, a group of three sequences all from expanded lineage II are arranged.

Below, within group 6, four tribal representatives are gathered, all as well from the expanded lineage. The remaining groups one to five are situated outside the phylogeny suggesting higher diversification rates than the sequences within the phylogeny. *Biscutealla laevigata*, belonging to the tribe Biscutelleae, is not assigned to any lineage yet, due to its clustering outside any of the supported lineages.

This genus builds a group with the unassigned taxon *Ricotia lunaria*. Bivoneae, Cochlearieae and Iberideae demonstrate an even more diverged placement outside the core group of the mustard family. Group one is depicted by Aethionemeae, which was expected to situate outside the core group as it is the oldest tribe of the Brassicaceae. That this tribe arranged with *Physaria pinetorum*, however, is unexpected. But the last-mentioned groups are also supported by appropriate reproducibility values of 80 to 99% and can therefore not be neglected.

This gene tree reconstruction introduced admittedly bears a vast number of inconsistencies in comparison to preceding research, which will be further discussed. For convenience, sequence names are extended to a certain amount to enable affiliations to the respective tribes and genera immediately. Within this illustration of the mustards several tribes are represented by more than one branch. Together there are eight tribes affected within the *chs* data that show this addressed pattern (see **Table 4**).

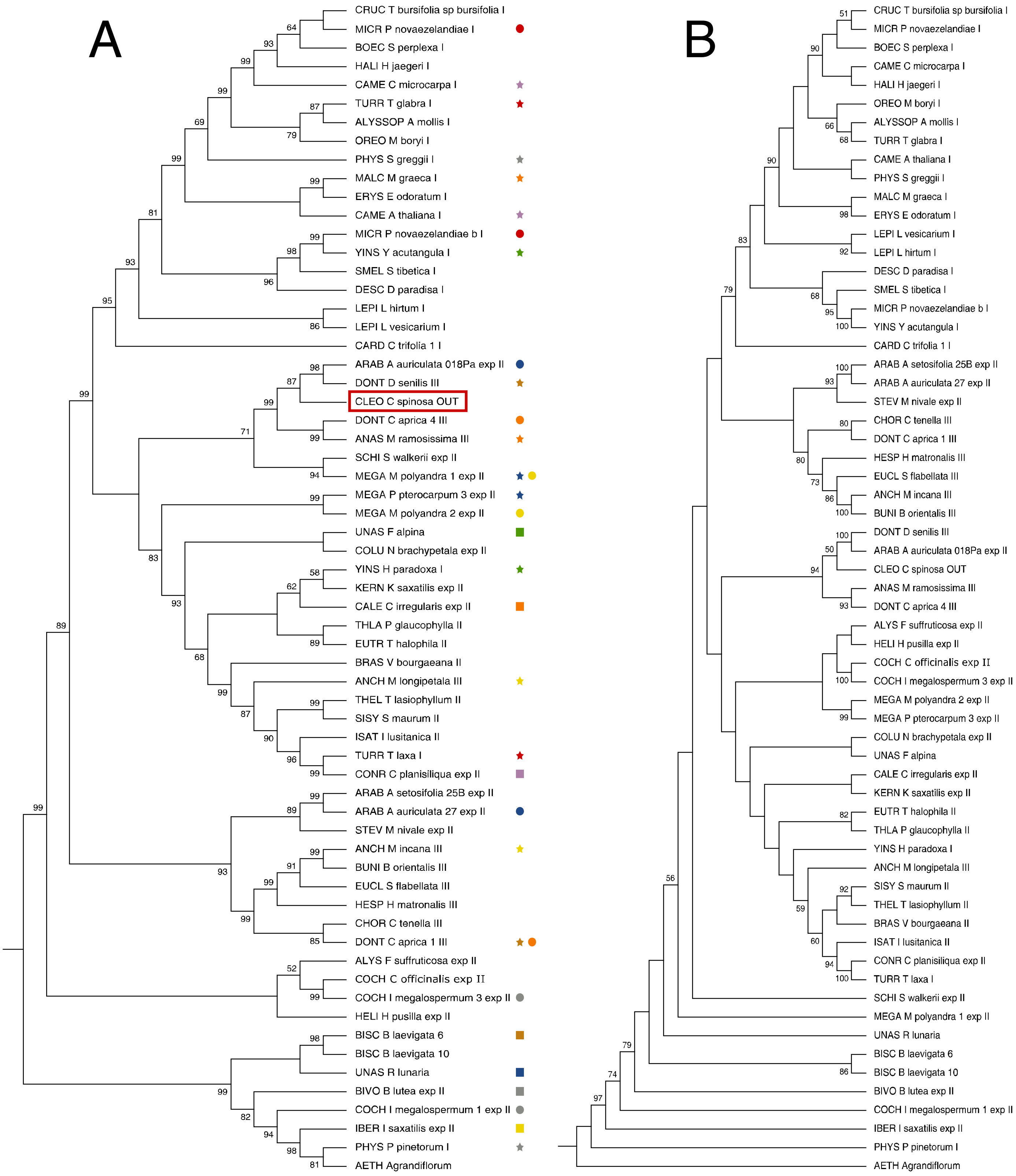


Figure 3. Gene phylogeny of *chs* sequences from all Brassicaceae tribes employed with the putative outgroup *Cleome spinosa* (genomic). Numbers at branches depict bootstrap values based on 1.000 replicates, only percentage above 50% are displayed. Tribal assignment according to AL-SHEHBAZ (2012) is given in front of every sequence, indicated by four capital letters (with exception of ALYSSOP = Alyssopsideae), followed by abbreviated genus and complete species name (written out species name see appendix). Lineage affiliation (BEILSTEIN et al. 2006, FRANZKE et al. 2011) is subsequently indicated by Roman numerals. Colour-coded asterisks, circles and squares highlight sequences to be discussed during the thesis. (A) Midpoint-rooted phylogram of NJ analysis calculating genetic distances based on Kimura's two-parameter model (1980). (B) MP approach as implemented in PAUPRat (NIXON 1999) resulting in a strict consensus tree of 1726 equally parsimonious trees, with a length (L) of 4181 and consistency and retention index of 0.225 (CI) and 0,441 (RI).

The evolutionary history was also inferred using the maximum parsimony method. The most parsimonious tree with length (L) = 4181 is shown. The consistency index (CI) is 0.225, the retention index (RI) is 0.441, and the composite index is 0.116 for all sites and parsimony-informative sites are 0.099. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (NEI & KUMURA 2000). The MP reconstruction depicts eleven groups which are for the most part in good congruence with the NJ tree (see **Figure 3**).

Tribe	Sequence 1	Sequence 2
Anastaticeae/Malcolmieae	*Malcolmia graeca*	*Malcolmia ramosissima*
Anchonieae	*Matthiola incana*	*Matthiola longipetala*
Camelineae	*Arabidopsis thaliana*	*Camelina bursa-pastoris*
Dontostemoneae	*Dontostemon senilis*	*Clausia aprica*
Megacarpaeeae	*Megacarpaea polyandra*	*Pugionium pterocarpum*
Physarieae	*Synthlipsis greggii*	*Physaria pinetorum*
Turritideae	*Turritis glabra*	*Turritis laxa*
Yinshanieae	*Yinshania acutangula*	*Hiliella paradoxa*

Table 4. List of tribes with corresponding genera and species showing signs of a non-monophyletic origin concerning *chs*-phylogeny. Both, sequences 1 and 2, do not represent a single plasmid from the complete data but represent at least two affirmed clones. The sequences listed here are marked within the phylogenetic reconstruction by a colour-coded asterisk and will be discussed in the following chapter.

The evolutionary history was also inferred using the maximum parsimony method. The most parsimonious tree with length (L) = 4181 is shown. The consistency index (CI) is 0.225, the retention index (RI) is 0.441, and the composite index is 0.116 for all sites and parsimony-informative sites are 0.099. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (NEI & KUMURA 2000). The MP reconstruction depicts eleven groups which are for the most part in good congruence with the NJ tree (see **Figure 3**). Lineage I is again monophyletic and supported by a moderate percentage value of 79. The same can be observed concerning lineage III, which clusters with a group of four sequences assigned to expanded lineage II. Differences can be spotted related to the arrangements of expanded lineage II, which, within the NJ estimations, exposes a more dismembered clustering while the parsimonious tree also suggests various groups, but with a more clade-like character. *Cleome* arranges again in the middle of the tree within the same group. The strict consensus tree (not shown) from parsimony analysis was not in congruence with the gene phylogeny computed with the neighbor joining algorithm and the most parsimonious tree. As *Cleome* was defined as outgroup, the estimations resulted in a scarcely resolved tree depicting several polytomies among its backbone.

5.1.3 Divergence time estimates

Divergence time estimates are generally discussed, as they are instantaneously depending on the methods and calibration points as discussed above. Although results from previous research are methodologically thoroughgoing, they present highly divergent estimations of origin and divergence ages. Calculations with the data at hand resulted in three various estimates of divergence times and varied greatly depending on the approaches applied, making it difficult to determine the most reliable methodology. The calibrations based on the range of substitution rates from previous data resulted in a crown age for the Brassicaceae of 25.5 million years. This extrapolation seems to be realistic and within the range of recently suggested evaluations applied on the family (AL-SHEHBAZ et al. 2006, FRANZKE et al. 2009, KOCH & MUMMENHOFF 2006), though the age for the crown group seems relatively young. The calculated mean substitution rate settled at 1.29 x 10^{-8} which leads to this predated approximation. The estimations based on angiosperm split data for the complete data set proposes an age of 34.48 mya, which is nearly 10 million years earlier than calculations based on the synonymous substitution rates. This resembles the outcome of several research results exceedingly (COUVREUR et al. 2010, KOCH et al. 2001, KOCH et al. 2000, SCHRANZ & MITCHELL-OLDS 2006) suggesting ages between 30 and 54 mya. Still, estimations submitted here arrange at the lower age boundaries. On the other hand, estimations from BELL et al. (2010) and WIKSTROM et al. (2001) date the split between Cleomaceae and Brassicaceae to 20 mya respectively 32 mya indicating more recent outcome for the Brassicaceae crown group supporting results exposed here.

The divergence estimations of the listed tribes in **Table 5** are all relatively high which is due to the fact that each of those tribes is divided into two clades. The divergence age indicates the split age of the respective groups, which most likely, is identical to each of the evolutionary events, like polyploidisations. Estimations of the divergence times of the tribes suggest no recent but rather a predating of polyploidisation events. Intra-tribal radiations are only listed for those tribes containing at least two different species (DONT, MICR, ARAB, COCH).

The divergence of the outgroup Cleomaceae from the Brassicaceae is proposed to 42.01 mya (angiosperm split), while the substitution rate range draft argues for this split to occur more recently, namely 28.05 mya. The primary fossil-based calibrations following the approach of MANDAKOVA et al. (2010) seem to have resulted in the highest divergence time values.

Applied Data	**669**		
Constraint	Rate	Angio	Fossil
Runs	4	4	4
Generations	5.00E+07	5,00E+07	5,00E+07
Likelihood	-66623.65	-60367.82	-56494.74
Divergence *C. spinosa*	28.05	42.01	**18.1**
tmrca Brassicaceae	25.5	34.48	39.18
tmrca Lineage I	14.1	21.05	20.92
tmrca Lineage II	11.8	17.99	18.06
tmrca Lineage III	11.5	16.48	17.05
Divergence DONT	23.6	34.48	27.35
Radiation DONT	4.75 – 3.46	11.49 – 11.35	10.95-7.11
Divergence MICR	9.94	14.86	15.26
Radiation MICR	0.59 – 0.56	2.26 – 1.59	1.01 – 1.0
Divergence ARAB	23.6	31.44	28.57
Radiation ARAB	6.98 – 6.56	14.41 – 10.18	16.71 – 14.09
Divergence COCH	21.7	34.48	39.18
Radiation COCH	11.6 – 10.7	20.76 – 18.9	17.37 – 12.65
Divergence MEGA	21.7	31.44	35.52
Divergence YINS	21.7	31.44	27.35
Divergence PHYS	21.7	29.36	39.18
Divergence TURR	21.7	31.44	27.27

Table 5. Parameters and results for the original data set holding 669 sequences of nuclear chalcone synthase genes. Three divergence time estimate approaches are depicted like explained (material and methods) for the putative polyphyletic tribes. Divergence (split age of respective tribe) and radiation values (one for each of the polyphyletic arranged groups) are listed, as well as estimations for the most recent common ancestors (tmrca). Inexplicable results are printed in bold face. Radiation values are only given for those tribes containing at least two different species in each group.

These estimates (39.18 CG mya Brassicaceae with a 95% CI from 2.8 – 12.3) would scale the lineage diversification (lineage I to III) to an age of 20.92, 18.06 and 17.05 mya, which are close to the approach utilising angiosperm split data. Within this estimates lineage I to III are suggested to have diverged 21.05, 17.99 and 16.48 mya. However, the putative outgroup arranges somewhere within the gene tree (compare **Figure 4**) and suggests to have diverged only 18.12 mya from a monophyletic group, containing ANAS (*Malcolmia ramosissima*) DONT (*Clausia aprica, Dontostemon senilis* and *Clausia trichosepala*) and a group of ARAB sequences. Taxonomically and phylogenetically, this clade does not depict expected arrangements. *Cleome spinosa* as outgroup displays a sister-relationship to that peculiar group with a humble divergence time estimate for the split between Cleomaceae and Brassicaceae.

These approaches favour insofar that most of the suggested age spans arrange at accepted estimation spans previously suggested. As fossil calibration is often discussed to distort the calculations to an overly old age, conclusions have to be drawn with cautiousness. However, all three approaches yield reasonable age estimations for the Brassicaceae family, arranging between 39.19 mya and 25.5 mya, and therefore will be all pursued for further divergence time analysis.

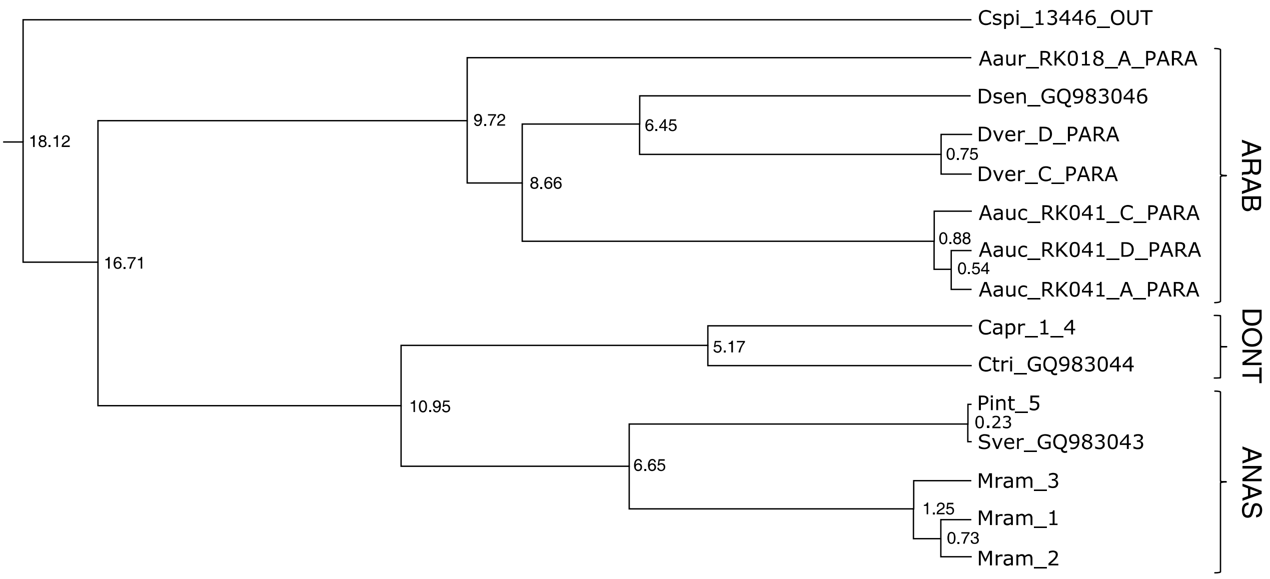


Figure 4. Excerpt from divergence time estimates (Fossil constraint of 669 sequences, see supplementary material S7) calculated with BEAST. The data suggests a postponed divergence of the outgroup, indicating a split from the Brassicaceae after speciation of the family, which is not in congruence with prior research. Values next to nodes are age estimations. *Cleome spinosa* ranks as outgroup of an inner-family disarrangement containing groups of duplicated tribes.

5.2 Sequence analysis

5.2.1 Alignment analysis of complete data

The sequencing procedure resulted in 480 applicable sequences of 236 specimens and 103 genera. All sequences which were completely identical in their composition were removed from the dataset, resulting in a multiple sequence alignment. Sequences from the same species with around 99% identity were kept although considered to result from *Taq* polymerase induced errors, as the preliminary analysis is not sufficient to decide on whether which sequence is the one affected by polymerase lapse. However, there is a tendency towards the most basal clone sequence with the shortest branch, consequently. Differing sequence types were kept as well for further investigations. There was no proof of pseudogenes in the dataset, evidenced by the inspection of the amino acid sequences. As *chs* phylogeny has been the focus of previous studies (JOLY et al. 2009, KOCH et al. 2000, WANG et al. 2007), a thorough sampling of another 144 sequences from the NCBI (National Centre for Biotechnology Information) was available. This material (supplementary material S10) was added to the data set synthesised here to give support to the respective genera and tribes. This resulted in an overall alignment of 624 sequences covering 44 tribes.

		length	C	V	Pi	S	0-fold	2-fold	4-fold
complete	**absolute**	2,343	129	1,979	1,874	94	999	59	52
	in %	100	5.51	84.46	79.98	4.01	42.63	2.51	2.21
coding	**absolute**	1,240	118	1,107	1,007	100	640	44	51
	in %	100	9.51	89.27	81.2	8.06	51.61	3.54	4.11
amino acid	**absolute**	413	26	378	347	31	n/a	n/a	n/a
	in %	100	6.29	91.52	84.01	7.5	n/a	n/a	n/a

Table 6. Statistical attributes of the data set of the complete gene and the coding regions, and the respective values for the amino acid translation, estimated with MEGA5 (Tamura et al., 2011). C is constant sites, V is variable sites, Pi is parsimony-informative sites, S is singleton sites, 0-/2-/4-fold is zero-/two-/four-fold degenerate sites. Each value is shown in absolute numbers and percent.

Though not disposed in phylogenetics, promoter and intron were also investigated. Integration of the non-coding elements of the gene enlarges the alignment close to twice the size. This is due to the high variation within this regions that, consequently, result in the insertions of gaps. The more distantly related the sequences are, the more gaps are present in the alignment, with the result that the overall alignment typically is up to 100% longer than any of the sequences it contains. Although the absolute numbers depicting each statistical attribute are significantly higher in the complete gene, the relative amount depicted in percent demonstrates the difference between the complete and the coding gene. Nearly 10% of the coding regions are constant all over the complete Brassicaceae family, which means that 129 nucleotide sites are conserved for all 624 sequences. Within the complete gene only additional eleven constant sites can be found, which result in about only the half amount depicted in percent. A site that is not variable is referred to as a constant site. The variable sites contain at least two types of nucleotides. Some variable sites can be singleton, containing at least two types of nucleotides, of which maximal one occurs multiple times, or parsimony-informative, where two or more nucleotide types occur and at least two of them with a minimum frequency of two. The amount of parsimony-informative aligned characters is nearly twice the size of cpDNA reviewed by (LEE et al. 2002) and even eight times more than in ITS (BOWMAN et al. 1999). The degeneracy state of the sequences should be highlighted as more than half of the sites of the amino acid encoding gene are zero-fold degenerated meaning that every second site contains nonsynonymous changes, nearly 10% more than could be found in the completely sequenced regions. The two-fold degenerate sites present those sites of which one out of three changes is synonymous. Consequently, the four-fold degenerate sites, where every change is synonymous, are represented by 2% respectively 4% of all sequences.

5.2.2 Alignment analysis of complete data set with focus on tribes

The average alignment accuracy of the coding sequences resulted in a p-distance of 0.381 which is < 0.8 and therewith far beyond the minimum value for reliability. This 0.381 correspond to 62% identity which is well within the acceptable range. The alignment accuracy of the reduced dataset is calculated with p = 0.157 which displays even more precision than among the complete data and, respectively, results in circa 84% identity between the sequences. The results for the procedure conducted for each tribe resulted in a range from d = 0 to d = 0.31, which implies an identity of the alignments between 69% (Cochlearieae) and 100% (Alysseae, Buniadeae, Crucihimalayeae, Oreophytoneae), which clearly is given by the number of species the alignments contain. Therewith every alignment fulfils the requirements of reliability.

In the majority of further estimations and investigations, only the coding sequence of the gene will be applied to the bioinformatics tools, as the promoter and the intronic region show adverse impact on the output. To avoid such contort, non-coding regions are neglected as they, additionally, do not directly account for crucial information of the gene’s evolutionary journey.

To give an overview over the sequence identities, bundled in tribes, a diagram was calculated. All coding sequences were included, split into the regarding tribal arrangement, with average values describing the nucleotide and translated amino acid identity for each tribe. Therefore all constant sites, calculated with MEGA5 (Tamura et al., 2011) were transformed into percentage values.

The diagram depicts two values for each tribe, namely DNA identity on the x-axis and amino acid identity on the y-axis, both in percent for each tribe. This estimations roughly display a line through the origin, with most of the points well above 75% identity on both axis demonstrating a much higher identity within the tribes than among the complete family (~10%), which was to be expected. First, this underpins the monophyly of the tribes and secondly supports the recent phylogenetic re-arrangements that lead to a tribal system reflecting natural relationships. Another peculiarity is that most of the points can be spotted slightly above that imaginary line and due to the fact that the results on the y-axis mostly represent a commensurately higher value, which is of course due to the degeneracy of the genetic code. However, eight of the points, representing tribes Bivoneae, Buniadeae, Calepineae, Erysimeae, Heliophileae, Kernereae, Oreophytoneae and Schizopetaleae can be spotted under the line resulting from a higher DNA than amino acid identity. In most of these cases only a fractional difference of less than 1% is responsible for the result. Only within the Erysimeae a difference between DNA and amino acid translation of 6.38% can be observed. This means that the genera within that tribe show an untypical pattern suggesting that most, or at least a multiple from the other tribes, show nonsynonymous changes at most sites (zero-fold degenerate sites). The one spot representing a quite low DNA identity in comparison to the others and the amino acid identity represents the tribe Malcolmieae. Within this tribe the genera show a low nucleotide identity (45%) which all seem to be third codon positions and, therefore, synonymous changes.

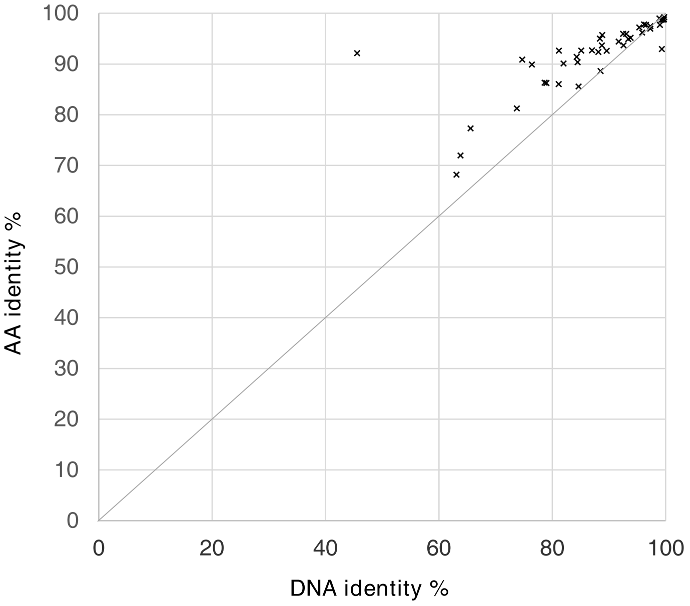


Figure 5*.* Nucleotide (x-axis) and amino acid (y-axis) identity for all 44 tribes are depicted. Bisecting line for optical support.

The display of the statistical attributes of solely the coding region of the data, assigned to the respective tribes (**Figure 6**), immediately shows that the data received from experimental assignment hints to some inconsistencies among the sequence constitutions which could be of crucial concern for further investigations. The conserved sites among the coding region are in the majority of cases well above 900, with most of them between 1.000 and 1.200 residues, which indicates that a huge amount of the sequence is of conserved origin and does not describe deviations from this expected stable trend. Tribes ARAB, CAME, CARD, MICR, PHYS, BRAS, ANAS, ANCH, DONT with conserved residues below 1.000 hint to disunity within the tribe, as they consequently depict more variable sites. A steep drop of conserved sites can be observed within tribes COCH (nearly no conserved sites) and MALC suggesting further examinations. Variable and parsimony informative sites, as well as singletons, obey a more or less moderate description, mostly not surpassing the 400 residue mark. A steep increase can of course be viewed in the two named tribes, as counterpart to the dropped values of conserved sites.

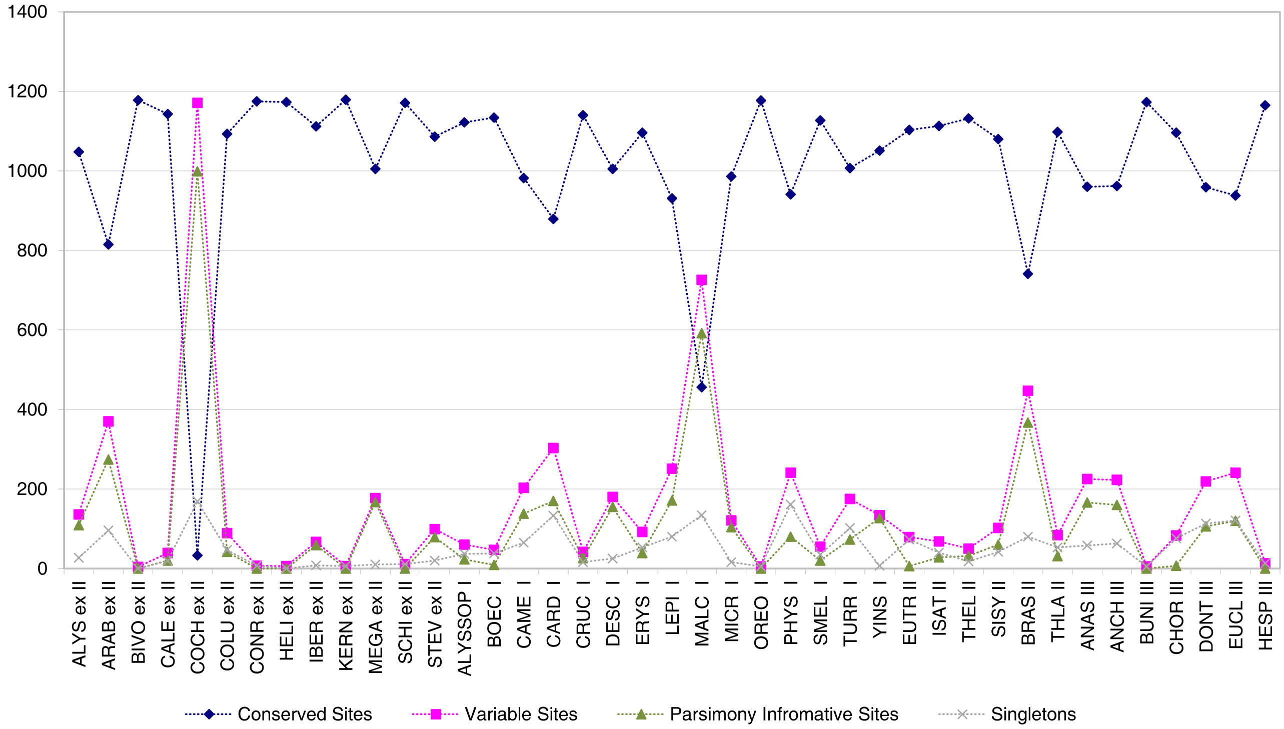


Figure 6. Basic sequence statistics (conserved sites, variable sites, parsimony informative sites and singeltons) of employed tribes. X-axis images tribes and lineage, y-axis number of nucleotides.

5.2.3 Alignment analysis of complete data set with focus on sequences

The analysis of the tribes, lineages and genera respectively results in huge amounts of data necessary to retrace output gained by phylogenetic analysis.

Table 7 shows the results from distance calculations within the respective tribes. The highlighted rows show divergences within the tribes that are significantly higher ($p \leq 0.05$) than within the other tribes. Most of the values around 50 distances per tribe and above can be spotted in those tribes already mentioned above. However, tribes Cardamineae, Microlepidieae, Brassiceae and Biscutelleae are also above 50 digits of differences. Biscutelleae and Microlepidieae will be discussed later in this investigations. The appearance of the Brassiceae is not unexpected here, because the tribe is often described as problematic concerning its arrangement and systematics which is due to its tribal specific triplication event (LYSAK et al. 2005), although the tribe is one of the most morphological distinct among the family. This eponymous tribe belongs to the major lineages among the mustards, containing 49-54 genera and 240 species (LYSAK et al. 2005). This tribe is also holding the most samples within the dataset presented here, which automatically leads to a wider range of divergence between the respective specimens. The higher the amount of data from diverse species added, the higher the number of differences calculated. The commensurate consequences, but vice versa, can be observed in case of tribes represented by a small amount of genera, like the Oreophytoneae.

They are illustrated by one species and therefore show only very little (2) differences between the sequences.

Note that the tribes Halimolobeae, Heliophileae and Bivonaeeae are represented by only one to two sequences from the same specimen (see **Table 1**), therefore no average, minimum and maximum divergence can be depicted. The average number of differences transferred to percent reveals a divergence rate within the tribes of less than 1% up to nearly 9%. The maximum number of differences can be found within the Physarieae which is close to 200 changes within the tribe which equals 7,5% intra-tribal distinction and therefore argues for significant distinguishable DNA sequences. Another eleven tribes, marked with asterisk, are conjectured to hold sequences which significantly differ from each other. As the tribal system meanwhile reportedly describes actual kinship these results are unexpected to a certain extend.

The outgroup, *Cleome spinosa,* was also tested for divergence. The three sequences available also suggest significant differences among the gene. Therefore the blast function for DNA samples from NCBI was employed to search for resemblance with other genes. This resulted in two different output files.

Lineage	**Tribe**	**Average (d)**	**Max.**	**Min.**	**length (bp)**	**Average(d%)**
expanded II	Alysseae	12.860	27	0	1,185	1.085
expanded II	Arabideae	65.090	117	0	1,185	5.648*
expanded II	Bivoneae	n/a	4	4	1,182	n/a
expanded II	Calepineae	16.500	28	5	1,185	3.924
expanded II	Cochlearieae	51.320	105	0	1,203	5.099*
expanded II	Coluteocarpeae	17.990	52	0	1,182	1.521
expanded II	Conringieae	3.500	5	2	1,182	0.296
expanded II	Heliophileae	n/a	6	6	1,179	n/a
expanded II	Iberideae	42.830	64	1	1,179	3.632
expanded II	Kernereae	3.000	4	2	1,185	0.253
expanded II	Megacarpaeeae	84.430	137	2	1,182	7.142*
expanded II	Schizopetaleae	3.670	6	1	1,183	0.310
expanded II	Stevenieae	32.290	65	0	1,185	2.724
lineage I	Alyssopsideae	16.250	27	2	1,182	1.374
lineage I	Boechereae	11.200	26	1	1,182	0.947
lineage I	Camelineae	60.01	102	0	1,185	5.064*
lineage I	Cardamineae	53.160	128	0	1,182	4.497
lineage I	Crucihimalayeae	12.380	19	1	1,182	1.047

Lineage	Tribe	Average (d)	Max.	Min.	length (bp)	Average(d%)
lineage I	Descurainieae	49.480	90	0	1,185	4.175
lineage I	Erysimeae	10.990	32	0	1,188	0.925
lineage I	Halimolobeae	n/a	n/a	n/a	1,179	n/a
lineage I	Lepidieae	68.900	126	0	1,185	5.814*
lineage I	Malcolmieae	6.980	14	0	1,182	0.590
lineage I	Microlepidieae	52.210	99	0	1,182	4.417
lineage I	Oreophytoneae	2.000	4	0	1,182	0.169
lineage I	Physarieae	89.670	193	0	1,194	7.510*
lineage I	Smelowskieae	22.470	43	2	1,182	1.901
lineage I	Turritideae	50.240	118	0	1,185	4.239
lineage I	Yinshanieae	67.670	113	1	1,185	5.710*
lineage II	Eutremeae	12.310	44	1	1,182	1.041
lineage II	Thelypodieae	12.530	34	0	1,182	1.060
lineage II	Isatieae	22.820	33	3	1,182	1.930
lineage II	Sisymbrieae	17.670	32	0	1,182	1.494
lineage II	Thlaspideae	40.600	60	0	1,185	3.426
lineage III	Anastaticeae	97.830	169	0	1,185	8.255*
lineage III	Anchonieae	103.670	178	1	1,185	8.748*
lineage III	Brassiceae	77.420	128	0	1,188	6.516*
lineage III	Buniadeae	2.500	4	1	1,179	0.212
lineage III	Chorisporeae	11.780	47	0	1,179	0.999
lineage III	Dontostemoneae	92.870	115	11	1,179	7.877*
lineage III	Euclidieae	46.940	89	2	1,179	3.981
lineage III	Hesperideae	8.670	12	4	1,179	0.616
	Biscutelleae	64.440	122	0	1,188	5.424*
	Aethionemeae	41.800	118	3	1,179	3.545
Cleomaceae		54.900	71.3	23	1,187	4.625*

Table 7. Divergence within the tribes and outgroup. Data depicts the length and number of differences, d, (in absolute numbers and percentage) of the coding gene (for information concerning the complete gene and additional percentage values see appendix.) within the respective tribes. Minimum and maximum values are calculated showing the range of variability. Grey colored rows mark variance within the tribe above 50 distance steps. Significant differences within the tribes are marked with an asterik (Max. = maximum, Min. = minimum, n/a = not available).

The first result suggested 85% identity with a query coverage of 85% and a total score of 507 that the blasted sequence equals *Phoenix dactylifera* (Arecaceae) for scaffold 39:383485:384744 and 39:379191:380480, while the second output file for scaffold

229:1056422:1057707 showed a total score of 996, a query coverage of 97% and an identity of 83% matching with *Anthirium andraeanum* (Araceae). All three sequences, also they could not directly be connected to the Brassicaceae, display *chalcone synthase* genes.

5.2.4 Identifying gene regions

5.2.4.1 Promoter

The promoter region (primer start within ACE-MRE region, seven base pairs before the A-box) of the complete data set was analysed concerning length variability and (highly) conserved regions. The shortest promoter, found in *Iberis semperflorens* sequence 2 and 3 (Isem 2 and 3; list of lab IDs see supplementary material S10), counted 116 base pairs, while the longest counted 186 (Acor 1 and 2, Cmon 5, Crup 2) nucleotides. The promoter identity among the complete data was, with 88.2% on nucleotide and 83.8% on amino acid level, unexpectedly high.

The identities within the tribes were between 67.3% and 100% owing to the fact that some tribes, like the Kernereae, are only represented by one genus or even species. In principle, it is self-evident that the divergence between the promoter sequences rises the more different species are involved in the analysis. But when distances within the promoter sequences of *Biscutella laevigata* for example are investigated, an intra-species identity of only 76.4% can be observed, suggesting a certain evolutionary distance between the sequences.

Although there is no defined threshold clearly separating an expected non-coding intra-species divergence from increased divergence, suggesting evolutionary distance, variability around a quarter of the promoter region indicates disproportionally difference among the non-coding region at hand. In other words: around 75% identity of the promoter region within the same species is too much to talk about allelic variation. Two different types of promoter sequences could be identified, differing significantly in length. One set of promoters is 167 long, while the shorter only shows 132 nucleotides. The first 38 base pairs do not display any distinction. After a deletion of 19 nucleotides, the sequences exhibit only short identical parts, with exception of the promoter end, where 6 bases are identical. The regulatory signals mentioned in the next passage could also be detected.

Therefore it cannot be excluded that the functionality of the region is preserved. As a part of the promoter is missing, further indications are simply not given, but the sequence divergence can denote a change in functionality of the gene.

Tribe	Intron Range	Exon 1 Length	Exon 2 Length	∑ Length (coding)
AETH	83	184	995	1179
ALYS	81-92	184-190	995	1179,1185
ALYSSOP	81-91	187	995	1182
ANAS	92-125	181-190	995	1176,1185
ANCH	75-85	184,187	995	1179,1185
ARAB	52-131	187-193	995	1182,1185
BISC	67-99	184	995	1176,1179,1188
BIVO	70	187	995	1182
BOEC	82-83	185	995	1182
BRAS	71-257	178, 187, 190, 193	995	1185,1179,1173,1188,1191
BUNI	71	184	995	1179
CALE	81-96	187	995	1182
CAME	82-96	187, 190	995	1182,1185
CARD	53-85	187	995	1104,1182,1200
CHOR	72-88	184	995	1179
COCH	72-110	184,187,189,190, 194, 199	995	1179,1182,1185,1191, 1194
COLU	84-105	187	995	1182
CONR	96	187	995	1182
CRUC	81	187	995	1182
DESC	77-98	187, 190	995	1182
DONT	83-189	181	995	1176,1179
ERYS	61-92	190,193	995	1185, 1188
EUCL	64-104	184	995	1179
EUTR	104-133	187	995	1182
HALI	n/a	187	n/a	n/a
HELI	80	184	995	1179
HESP	68	184	995	1179
IBER	74-87	184	995	1179
ISAT	80-98	187	995	1182
KERN	83	190	995	1185
LEPI	78-92	187, 190	995	1182
MALC	67-70	187	995	1182
MEGA	84-116	184, 187	995	1179, 1182
MICR	77-81	187	995	1182
OREO	71	187	995	1182
PHYS	77-146	178, 187	995	1173, 1182
SCHI	98	187	995	1182
SISY	81-96	187	995	1182
SMEL	80-83	187	995	1182
STEV	87-96	187	995	1179
THEL	69-93	184, 187	995	1182
THLA	77-91	187	995	1182
TURR	77-92	181, 187	995	1170, 1182
UNAS	66, 90	187	995	1182, 1185
YINS	76-108	187, 190	995	1182, 1185, 1188

Table 8. Overview of all employed tribes within this study. Exonic (exon I and exon II), intronic, as well as the corresponding length values of the complete coding region of the *chalcone synthase* gene are given. N/a = not available.

The putative regulatory signals, like the A (CCGTCC) -and TATA-box (TATA) or the MRE (Myb recognition element) could be detected in almost every gene. The A-box was incomplete in clones from *Arabis soyeri* (Asoy 6), *Coluteocarpus vesicaria* (Cves 8), *Pseudocamelina* *glaucophylla* (Pgla 3) and *Turritis laxa* (Tlax 1-1), whereas the TATA-box was damaged in an *Arabis soyeri* (Asoy 5) and *Pseudocamelina glaucophylla* (Pgla 3) clone. The MR element (ACCTAC) was only once incorrect (Pgla 3). As nearly all remaining promoter sequences (the complete data set does not provide a promoter region) share these active sites without any evidence for mutation, and therewith exhibit a high degree of uniformity, it can be concluded that catalytic conservation among *chs* respectively within the promoter region is not only common, but plays an essential role for the functionality of the gene. It further can be confirmed that the A-box and MR element are a highly conserved region in all sequences.

Only the Euclidieae show a consequent transversion at position 17, which actually is situated between these two elements. Some other unspecific point mutations could be detected which could also derive from sequencing errors. A grouping of the region to the respective lineage eased the aligning tremendously and revealed some specific sequence patterns. Each tribe displays a specific arrangement of indels and mutational changes which look quite distinctive.

5.2.4.2 Intron

The intronic region is available for 543 sequences and depicts a range from 52 nucleotides (Amon CK4_A and Amon CK4_uA) to 257 nucleotides (Cann 1). Most plant *chs* superfamily genes have one intron that splits the Cystein (Cys) in the consensus sequence (KODURI et al. 2010), which also is applicable to the data at hand. Within the whole family, chalcone synthase does always display only one intronic region, which is a phase-1 type (T/GC). They also stick to the GT-AG rule, with exception of Pgla 3, stating that all eukaryotic nuclear introns start with GT and end with AG, serving as splice donor and acceptor sites.

Although this part of the gene is too variable to align it properly among the whole family, there are at least identifiable tendencies, due to the fact that the intron belongs to the conserved non-coding sequences (CNS) bearing the potential to regulate gene expression. In *Pachycladon* e.g. are two different patterns among the eight species, pinpointing the divergence of the respective allopolyploid group. One intron is 77, while the other is 88 base pairs in length, both with only four point mutations among the diverse sequences but with an inter-intronic p-distance of 0.187. This suggests that the divergence of both groups happened chronologically far enough, – 15.06 ± 6.38 mya are suggested (JOLY et al. 2009) according to the CHS data – to give rise to the accumulation of the respective mutational events resulting in two kinds of intronic regions with an overall identity of around 80%. The inter-intronic sequence identity of each group of ~89% furthermore indicates two parallel radiation events which end up in two sets of sequence species differing only to a very small amount.

Another interesting observation could be detected in *Pseudocamelina glaucophylla* (Pgla 3), which already was discussed above. The intronic sequence of this clone is, like Pgla 1 and Pgla 2, 97 base pairs in length but shows a distance of ~50%. Moreover, the Pgla 3 intron displays no thymine, but more than 77% adenine bases resulting in long poly-adenine stretches (up to twelve) and repetitive elements. Interestingly, the coding parts of that genes share nearly identical sequence content.

5.2.4.3 Exon 1

The exonic region is divided into two parts. Exon 1 exhibits an overall mean distance of p = 0.116, an average length of 187 and a range of 21 base pairs (**Table 8**). Most tribes are defined by one typical length from exon 1, while two tribes show a high heterogeneity in the length depiction, indicating high diversity for these tribes (COCH and BRAS). Most of those tribes which show two different kinds of the first exonic region are again those which are nested within different positions in the phylogeny. Among the tribes an even higher conservation, described via identity, could be observed. Hence, identities between 88.8% (Physarieae) and 100% (Kernereae) were computed, with an average value well above 90%.

5.2.4.4 Exon 2

Exon 2, where complete, shows a thoroughgoing length of 995 base pairs among the Brassicaceae family, regardless of tribe, duplication or other evolutionary influences. An insertion of one codon in exon 2, in comparison to other angiosperms, can be observed, which seems to be exclusively found in the Brassicaceae. The observed overall identity of exon 2 among the Cruciferae is 86.7%, illustrating a relatively low and slightly higher level of divergence compared to exon 2. It was expected that the average identity should be higher among the second exon, but this can be due to the fact that several sequences do have an incomplete exonic region.

The highly conserved cystein residue at amino acid 169 (WANG et al. 2007), hence in exon 2, is thought to be part of the 4-coumaroyl-CoA binding site, required for enzymatic activity, and therefore essential for the functionality of the enzyme. This residue could be detected in all chalcone synthase sequences employed here, although the position slightly varies among the respective genera and tribes. In most cases it could be screened between amino acid position 167 and 170, and often at the expected 169th residue. This shift in position is supposedly owing the fact that brisk small scale rearrangements, implying insertions and deletions, repeatedly restructure this gene. This can be viewed in **Table 9** demonstrating the variability in the respective coding region. Although exon 2 is less affected by this, length variations in the first exonic region of course subsequently influence positioning in the second part of the exon.

Several more additional conserved signatures were reported (JEZ & NOEL 2000) like His 307, Asn 340 and Phe 219, all from the catalytic centre of the chalcone synthase. Supposedly these residues are also conserved in all chs-like enzymes. As there is an extremely huge amount of sequence identity, even between distantly related genera from different families, there consequently are plenty of more conserved residues among that gene, which have not been reported yet.

5.2.5 Conserved residues of the complete gene

Nucleotide sequences showed high levels of identity among nucleotide and amino acid level. However, this does not guarantee that the sequences depict that enzyme which is in charge of the catalysation of the production of the chalcones. Model organisms, like *Humulus lupulus*, from other families (Cannabaceae) showed sequence variants which displayed more than 98% nucleotide and amino acid identity with the true *chalcone synthase* but were still identified as oligofamily members (MATOUSEK et al. 2006). Therefore, all sequence variations have to be checked for certain conserved residues, which are in charge for the functional *chalcone synthase*. Those residues applied have been previously been reported as distinct and influential positions among the gene. Amino acid changes within these highly conserved and existential residues immediately have control on the function (FUSSY et al. 2013, MATOUSEK et al. 2006, OKADA et al. 2003, OKADA et al. 2004).

conserved residue	amino acid	position	changed to	sequence
active centre	Cystein (C)	164	L	Cpyr s17 D; Iabu B
	Histidine (H)	303	N	Tgla AF112091
	Nsparagine (N)	336	nv	nv
coumaroyl binding site	Serine (S)	133	F	Imeg 6
	Glutamate (E)	192	nv	nv
	Threonine (T)	194	I	Tlax 1-3; Bcre 1; Bcre 2
	Threonine (T)	197	M/I	Acan RK214A; Agra 5-2; Dver C; Dver D
	Serine (S)	338	T/L/P	Cexc B; Cexc D; Cgro B; Coff B1; Cpyr s16 B; Cpyr s17 B2; Iaca B; Aalp CK1 H; Aver CK7 C

conserved residue	amino acid	position	changed to	sequence
cyclisation pocket	Threonine (T)	132	A	Cexc B; Cexc B2; Coff B2; Cpyr s16 B2; Cpyr s17 B2; Iaca B2
	Methionine (M)	137	nv	nv
	Isoleucine (I)	254	nv	nv
	Glycine (G)	256	R/E/L	Caes B1; Cpyr B2; Iaca B; Iaca B2; Rcan GQ983020; Bori 1-3; Spub 1;Spub 3; Smat GQ983041; Dver C, D
	Phenylalanin (F)	265	L	Elit 1
cyclisation pocket/ active centre	Phenylalanin (F)	215	L	Dsop2; Dsop 4; Chir 1; Egal 1; Egal 2; Egal 3; Egal 4; Amon CK4
geometry of active site	Proline (P)	138	nv	nv
	Glycine (G)	163	I/D/R/V/S/-	Mmar 1; Hmat 2-2; Coff B, B2; Iabu B;Boxy 2; DverC, D; Aauc RK018A; Aauc RK041A;C,D; Anov RK127 D
	Glycine (G)	167	C/-	Mniv 3; Lala KE156373; Aauc RK041 3
	Leucine (L)	214	P	Lcam 1
	Aspartate (D)	217	nv	nv
	Glycine (G)	262	E	Smat GQ983041
	Proline (P)	304	S/R	Cmol FJ645084; Smau 3; Cmic GQ983008; Lhir 2
	Glycine (G)	305	E	Alyr AF112104
	Glycine (G)	306	S	Imeg 7
	Glycine (G)	335	D	Mniv 3
	Glycine (G)	374	R	Fsuf 1
geometry of active site/ cyclisation pocket	Proline (P)	375	S/Q/L	Lhir 2; Lhir 3; Lhir 4; Lhir 5; Cexc B2; Cexc B; Isav 1; Isav 2; Isav 3

Table 9. Amino acid changes within conserved residues (FUSSY et al. 2013, MATOUSEK et al. 2006, OKADA et al. 2003, OKADA et al. 2004). Substitutions are indicated by the amino acid site and its change to the respective amino acid. Sequences are abbreviated, first letter is initial genus name, while the species name is reduced to three letters, followed by the individual’s clone number. E.g. Mniv 3 = Macropodium nivale clone number 3. Nv = no value, meaning that no sequence depicts an aa change here; - = aa change to delition.

Amino acid changes occurred, but mostly outside the conserved residues, (see **Table 9**), depicted by MATOUSEK et al. (2006). These are characteristic for the functional *chalcone synthase* gene, like its formation of the active site, the active centre, the cyclisation pocket, and the coumaroyl binding site (MATOUSEK et al. 2006). In the majority of cases only one change from one amino acid to another can be observed. As the original sequence, the functional sites were taken from, derives from hop (*Humulus lupulus* L.), some of the changes, maybe especially those which depict a multiple amino acid switch, could derive from sequences divergence. But, as the residues discussed here are conserved due to functionality of the gene, it is more likely that the changes derive from interfamilial r sources. A majority of deficiencies cumulate clones that are assigned to genera or tribes bearing problems with phylogenetic arrangements. Especially the sequences from the tribe Cochlearieae cover every category in the residue column, with exception of the combined residue "cyclisation pocket and active centre". Together 29 sequences represented here derive from this tribe, which, however, is represented with the most sequences in the data set, as well. Other tribes gathered here are Turritideae, Brassiceae, Arabideae, Descurainieae, Buniadeae, Cardamineae, Lepidieae and Alysseae, but most of them provide only one sequence. Six alignment positions (nv) representing conserved sites, did not show even one mutational hint in the amino acid alignment. In respect to the size and divergence of the specimen, these sites are highly conserved within the whole Brassicaceae and most likely are of ample functional importance.

5.2.6 Trinucleotide frequency (k-mers)

DNA sequences display compositional heterogeneity on many scales, therefore trinucleotide frequencies potentially deliver information on sequence constitution. Codon NCN is preferred in any genome, while this in plant genomes corresponds the codon GCG. So, C is highly preferential as second codon letter, which was already suggested previously by NIIMURA et al. (2003).

No common behaviour of trinucleotides can be spotted, although some groups depict analogical appearance of the trinucleotide distribution. CALE and COCH display a similar amount of the respective trinucleotides, which tends to behave in an anti-parallel nucleotide usage. While most of the tribes show an intensive occurrenc of the codons GAG, GAC, AAG and CUC, which confirm the observation that G is highly preferred as first and third letter (KOZAK 1999, NIIMURA et al. 2003), these are only used to a maximum value of 9.3 in those two tribes. Heliophileae, Iberideae, Physarieae and Aethionemeae show a pattern that looks shifted in comparison to the remaining tribes. These results, with exception of Calepineae, were expected as prior outcomes already foreshadowed irregularities among the outliers. A bias change among the codon third position could be detected in the alignment, close to the beginning and end of the gene, which create a species specific pattern.

These changes are mostly not detectable in the amino acid sequences because the codon third position hardly affects the aa composition. Biases in base appearance close to the termination or initiation codon are possibly signals to control the initiation or termination of a translated region (NIIMURA et al. 2003) and determine translation efficiency.

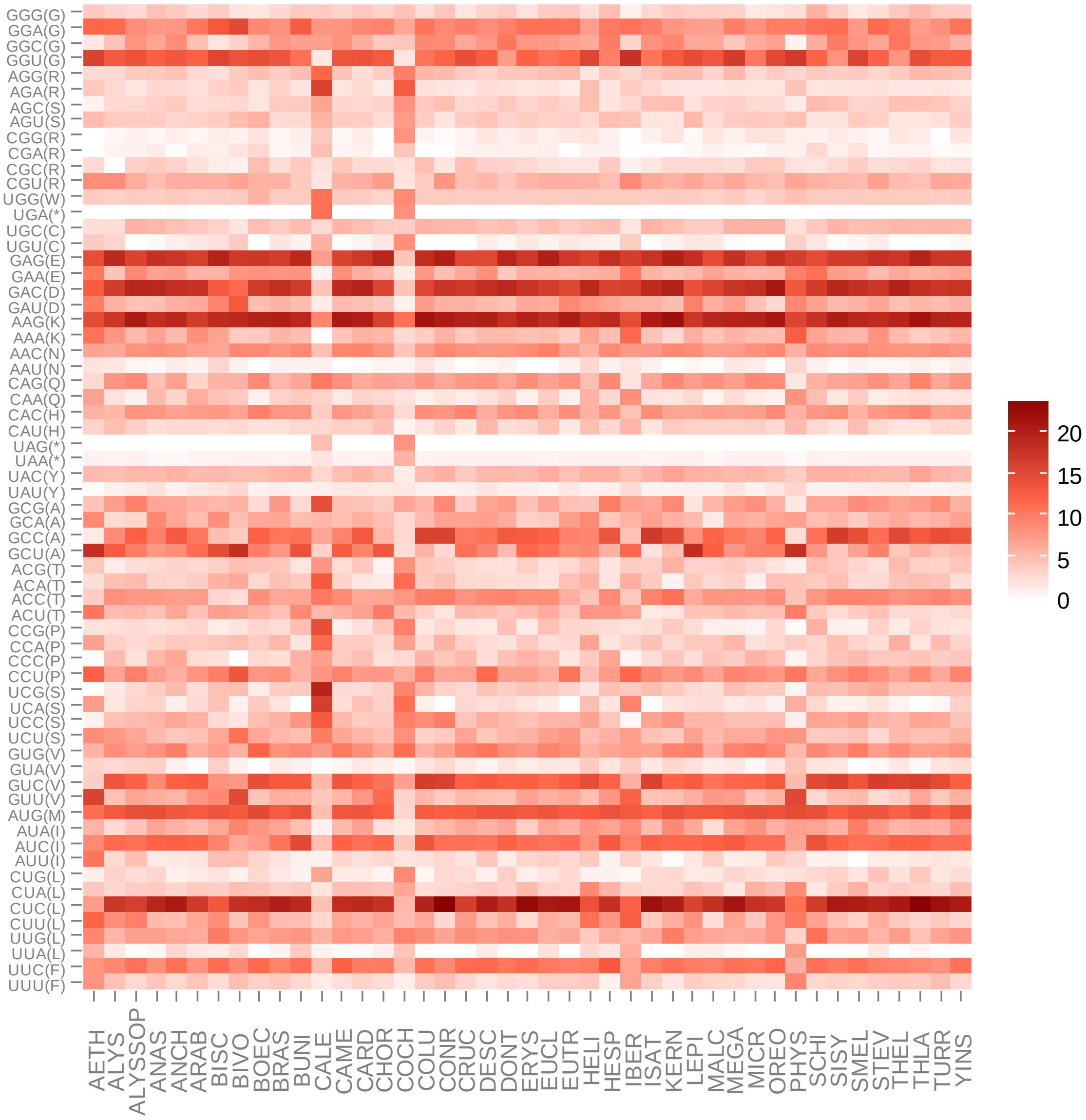


Figure 7. Signature of trinucleotides in the chalcone synthase gene in Brassicaceae tribes depicted as heat map. Colours correlate with the codon usage bias indicated by scheme to the right, which shows amount of trinucleotide use with average number in the plot.

6 Discussion

The phylogram of *chs* depicts several incongruences compared to expected as well as previously demonstrated results (GERMAN et al. 2009, WARWICK et al. 2010, WARWICK et al. 2007, WARWICK et al. 2009). These estimations show that the corresponding *chs*-based phylogenetic hypothesis is frequently not in congruence with known phylogenetic reconstructions (BAILEY et al. 2006, BEILSTEIN et al. 2006, KOCH et al. 2001, KOCH 2000).

These incongruences are not supported by low bootstrap values on the majority (see **Figure 3** and S16), which would indicate a lack of consistent signal across sequences sampled and would deliver a straight forward explanation. Most nodes are well above 50% support. Therefore, noise ratio as well as an oversized amount of variable or informative sites can be excluded. Although a considerable argument concerns the fact that a single-gene alignment can comprise the challenge that it might not depict too much diverse information, but, the other way round, sequences are too identical to result in well resolved and supported branches.

Evolutionary induced factors like lineage sorting, convergent evolution, or ancient intergenic recombination events (SYVANEN et al. 1994) can only be considered to a certain amount, as bootstrap support is too high.

It has also to be taken into account that the employed data set is relatively large concerning number of sequences as well as the coverage of tribes, compared to other studies (CONNER et al. 2009, JOLY et al. 2009, KOCH et al. 2001, O'KANE 2003, ZHAO et al. 2010) displaying high bootstrap values. The number of operational taxonomic units (OUT) immediately influences estimations in so far as the number of possible trees increases exponentially (FELSENSTEIN 1978). Thus distances of capacious trees display a tendency to depict lower bootstrap support, as some nodes are not frequently considered by the applied algorithm in the sampled data.

The considerations listed above could all be taken into account for low bootstrap support and poorly resolved gene tree reconstructions which could not be evidenced here. Hence, this moreover supports the quality of the alignment, the calculations are based on and excludes several options to be in charge.

Consequently, the gene tree depicted in **Figure 3** is authentic but discloses incongruences which have to be dissolved.

A very demonstrative difference, compared to affirmed Brassicaceae phylogenies is the location of the outgroup, *Cleome spinosa*. This sequence is marked by a frame in the NJ tree in **Figure 3**. The family of the Cleomaceae is applied within several studies to serve as reliable outside information to root the mustard family (BAILEY et al. 2006, GERMAN et al. 2009, JOHNSTON et al. 2005, LIU et al. 2012). According to the definition of an outgroup, *Cleome spinosa* is supposed to be more distantly related to the ingroup sequences than the ingroup sequences are to each other. The Cleomaceae diverged from their common ancestor between 42.01 and 28.95 mya, thus supporting a legitimate outgroup status.

The reconstructions suggest that *Cleome* is not appropriate to anchor the gene tree at hand. The *C. spinosa* sequence clusters with two clones from lineage II, namely *Clausia aprica* and *Malcolmia ramosissima* (see **Figure 3**). The branch length of the putative outgroup suggests a more recent divergence from the Brassicaceae close to the origin of lineage II, which definitely does not depict the real evolution of the sister family. The reason for the unexpected clustering behaviour could be due to its difference viewed in the genomic sequence. The sequences derive from the complete genomic data DQ415920 to DQ415922 and result in three *chs* loci within the whole genome, due to a triplication event within the family (JIAO et al. 2012, SCHRANZ & MITCHELL-OLDS 2006). Neither of the loci results in an appropriate amino acid sequence which could be aligned to the Brassicaceae correctly. One copy depicts a insertion of eighteen nucleotides (scaffold 229:1056422:1057707) at the end of exon 2, which on the one hand enlarges the coding region to a relatively huge extend of six codons and, on the other hand, implies a shift in the reading frame, leading to a postponed termination codon at the end of exon 2 (alignment upon request). The remaining two sequences (scaffold 39:383485:384744 and scaffold 39:379191:380480) both show an insert of twelve nucleotides, resulting in four additional codons at the end of the second exon, while the first named displays an additional deletion of nine nucleotides in the first exon. All three copies have changed their appearance but are still in frame. No premature termination codon implies non-functionality but it is quite likely that this gene encodes for a protein with a function at least slightly different from that of *chs*. All three sequences, although they could not directly be connected to the Brassicaceae, display *chalcone synthase* genes.

The increased length of the genes is somehow unexpected as SCHRANZ & MITCHELL-OLDS (2006) argue that the gene loss among the compact *Cleome* genome is greater compared to the *Arabidopsis* genome. Therefore it can be reasoned that the *chs* loci among the *Cleome* genome are not (yet) affected by genome size reduction but by other evolutionary processes.

These indels either display an older duplication a polyploidisation event followed by functional divergence after polyploidisation or a chromosome rearrangement. The multiple homeologous small scale copies were rearranged by inversions and translocations within the diploidised genomes. These events could have led to the present-day gene number and length variation within the Cleomaceae.

In case of functional divergence this sequence depicts the fate of a copy on its way to gene loss. After an individual gene duplication, the redundant copy, which is not under selective pressure, gathers deleterious mutations, leading to non-functionality and finally results in an exclusion from the DNA. Most of the dispensable genes, resulting from the last polyploidy event have already been lost from the genome, only around 27% of duplicates (BLANC et al. 2003) retain in the genome (reference from *Arabidopsis thaliana*). So, there obviously is a trend towards reduction by massive gene loss. As the three sequences at hand derive from genomic DNA and no other putative *chs* sequences could be spotted this means that at least one out of these three *chs*-like sequences must be functional as plants are not capable of living without these genes, as chs catalysis are responsible for the initial reaction within flavonoid biosynthesis (CAIN et al. 1997). But this implies that at least some additional small-scale evolutionary process must have worked on this nuclear gene.

Moreover, divergence time stimates argue that Cleomaceae split from their common ancestor between 42.01 and 28.95 mya (see **Table 22**) what is in absolute congruence with ages estimated by BLANC et al. (2003), proposing 40 to 24 mya for the same event. Thus *Cleome* is supporting a legitimate outgroup status. However, the third estimated divergence time, 18.1 mya does not seem to fit into that draft.

It is suggested that *Cleome* evolved independently from the Brassicaceae and underwent a triplication event more recently than the Brassicaceae's duplication event. This results in different ages for ancient polyploidy events within the two sister lineages Brassicaceae and Cleomaceae (HALL et al. 2002, SCHRANZ & MITCHELL-OLDS 2006). Therefore it can be reasoned that the *Cleome spinosa chs* sequence applied here depicts one copy deriving from the triplication event which still maintained within the genome. This is assisted by the gathering of sequences for which *Cleome* provides the outgroup position (see **Figure 4**) within divergence time estimates. This clade contains data from tribes DONT, ARAB and ANAS. Genera within that last mentioned tribe additionally show a low nucleotide identity (45%) which all seem to be third codon positions and, therefore, synonymous changes (**Figure 6**). Those tribes have to be further investigated, like already suggested in **Table 4** (with exception of ARAB, which will be discussed lateron in this thesis) and **Table 7**, indicating high divergence within the several tribes. This table will be analysed within the following chapters.

As a consequence, the Cleomaceae as outgroup was removed from the data set. This resulted in an alignment length of 1200 bp and 48.8% identity in comparison to 1218 bp and 45.8% identity with *Cleome* included.

For further investigations, tribe Aethionemeae, as the most basal tribe of the mustards, replaces *Cleome* and functions as outgroup. Moreover, the tribes listed in **Table 4** will be highlighted and surveyed for potential conspicuities causing the arrangements discussed above.

The overall appearance of the gene tree is self-evident for the fact that the *chalcone synthase* gene does not end up in a thoroughgoing re-drawing of a backbone phylogeny for the Brassicaceae. Although there are only nine nodes from more than one hundred within the cladogram that show a bootstrap value less than 50 (between eleven and 49) which suggest that the evolutionary history resembles the representation of the gene quite a lot, several questions remain open concerning the evolution of the gene that led to such a complicated image.

Part 2: Trouble with the Tribal Arrangement

The amount of data and already sustained results suggest to undertake several approaches to receive insight into the diverse levels of DNA quality and constitution with a focus on the tribes which already emerged distinctive features listed in **Table 4**. As one group of *chs* sequences (sequences in first column) are those which arrange at the expected position within phylogenetic analyses, while the second column depicts the counterparts arranging at unexpected positions, those groups will be further referred to as “expected” and “unexpected”.

To identify putative irregularities and maybe evidence or explanations for their origins, the analyses are conducted in the surrounding of the complete family to warrant commensurability. A cursorily view on the data is definitely not sufficient to reveal differences and key aspects which are in charge for the performance of DNA sequence data. Therefore, diverse methods were applied on, for a start, the complete data, to approximate meaningful outcomes step by step.

7 Material and methods

7.1 Comparative phylogenetic reconstructions and analyses

The NJ algorithm was repeatedly applied on the modified dataset. *Cleome spinosa* was excluded, resulting in a representative dataset of 62 sequences. However, the settings are completely identical to those described in part 1 (see 4.3.2).

The same holds true for the MP search method. Minimum change estimations are based on the assumption that the tree, which is most likely, requires the fewest number of changes to appropriately explain the data (HALL 2011). Parsimony methods do not utilise specific evolutionary nucleotide substitution models for the estimations of phylogenetic reconstructions. Hence, a search method has to be applied to find the minimum number of steps, which was explained in part 1.

For this chapter an additional algorithm was applied on the dataset, namely the maximum likelihood method (ML). This is a powerful statistical method that seeks for the tree that makes the data most likely, by applying the log-likelihood, an explicit criterion, on the tree to compare the diverse substitution models. ML analysis were also conducted in Mega5 (TAMURA et al. 2011). A new modeltest was previously conducted resulting in the same output like in part 1 where the best fitting substitution model is the General Time Reversible Model (GTR) with γ distributions and plus Invariant Sites (rates among sites). The Maximum Likelihood Heuristic Method was set to Nearest-Neighbor-Interchange (NNI).

7.1.1 Test for recombination

Within literature, one of the two most cited divergence-based approaches utilise the GENECONV software package v 1.81 (SAWYER 1989) to test for incongruities between a given species phylogeny and a gene tree that has been estimated from DNA sequences (INNAN 2011). GENECONV was originally designed to detect allelic conversion, but has subsequently been used to detect ectopic gene conversion (EGC), as well. The software searches for stretches of sequence identity between sequences that extend further than would be expected by chance. A given model of independent evolution between the loci and permutation tests (here 10,000) which are used to establish statistical significance. The complete data file was applied on the programme, fragment limits were set to minlength = 1, minnpolys = 2 and minscore = 2. GENECONV detects gene conversion by looking for sufficiently similarly aligned segments between a pair of sequences.

7.1.2 Divergence time estimates

Like in chapter 1 (5.1.3), three approaches were applied on the dataset where *Cleome* was excluded. With exception of the outgroup file, no changes in the setting of BEAUti (DRUMMOND et al. 2012) were deployed in order to both guarantee the comparability of the output and to define the impact of the inappropriate outgroup.

7.1.3 Lineage through time plots (LTT)

The genetic behaviour of the sequences argue for estimations linked to divergence times of these species plotted against the birth and maybe death of the gene in the respective group. LTT daigramms plot the number of lineages in a clade that have any living descendants from the respective data against time (RABOSKY & LOVETTE 2008).

LTT plots were constructed with the single MCC trees, using the ape package (PARADIS et al. 2004) of the R software environment (R CORE TEAM 2014). These plots illustrate an overall pattern of diversification. LTT were created for the tribes causing confusion among the constructed phylogenies and are suggested to be putatively of non-monophyletic origin concerning the evolution of the *chalcone synthase*.

7.2 Sequence analysis

7.2.1 Alignment analysis of the complete data

For an overview the complete data was analysed, mostly with models and tools implemented in MEGA5 (TAMURA et al. 2011) or MEGA6 (TAMURA et al. 2013), to receive basic statistical values and first hints pointing to further analysis methods and grouping strategies. Therefore the data set was divided into the single tribes, coding and complete DNA sequences and nucleotide versus amino acid compositions were analysed. It is not feasible to depict any chunk of outcome for every lineage, tribe, genus or even species. Hence, achievements are displayed or discussed to a) give an overview and basis for comparison and b) depict significant peculiarities.

7.2.2 Identifying gene regions

The examination of the complete nuclear gene will help to unravel the differences and similarities among the tribes, genera and species. It, moreover reveals the discrepancies among the sequences that resulted in unexpected phylogenetic placements. In most studies concerning genes encoding a protein, it is prevailing to only employ the coding region for analysis. Of course, estimations and calculations are just applied on the gene's part of interest and not on the intragenic regions. But molecular generic elements like the promoter region have been successfully employed to not only compare sequential parts but also to investigate functionality (DE MEAUX et al. 2005). Therefore the data has been analysed not only as entire coding or complete gene, but also separated into their respective structural parts.

7.2.3 DNA motif search among gene

For further analysis of the coding and non-coding parts of the chalcone synthase gene, MEME version 4.9.1 (BAILEY et al. 2009) was employed, which is a motif-based sequence analysis tool. The statistical significance of each motif is given in E-values, starting with the motif of highest significance, which means a low E-value, here set to a threshold of 0.05. The E-value is based on the lnL ratio, width, sites, background letter frequencies and the size of the applied data.

The chosen database for the motif search is JASPAR CORE (2014) for plants. Further settings allowed a search for maximum 100 motifs with a minimum length of 6 and a maximum of 30 nucleotides.

7.2.4 Transition/transversion rate bias

An additional factor influencing an accurately phylogeny depends on the pattern and amount of homoplasy present in a dataset. To reduce that effect character-state weighting, based on a model of DNA evolution (BROUGHTON et al. 2000), was employed. The transition/transversion bias is known to be a general possession of DNA sequence evolution. In virtually all DNA sequences transitions (T←→C, A←→G) have been noted to occur at higher frequencies than transversions (T←→A, T←→G, C←→A, C←→G) and gather multiple substitutions at their fast-evolving sites. This results in the accumulation of a stochastic signal in the sequences, demonstrating homoplasies (GOJOBORI 1983, WAKELEY 1993, YANG & YODER 1999).

It has been demonstrated in several reviews that low levels of genetic divergence results in an increased transition/transversion (ti/tv) ratio and, vice versa, at high levels of divergence, transition/transversion appears to be low. At levels around 20% divergence or more, both substitution types were demonstrated to show equal frequencies (YANG & YODER 1999). Therefore the transition/transversion bias was calculated using DAMBE's 5.3.115 (XIA 2013) tool for sequence analysis estimating nucleotide substitution patterns, depicting a detailed output. This beholds generalised assumptions about different types of character changes (BROUGHTON et al. 2000). Genetic distance analyses were based on a highly hierarchical hypothesis test of alternative models implemented in Modeltest 3.7 (POSADA 2003).

7.3 Compositional heterogeneity among DNA

7.3.1 GC content

Among species within a phylogenetic group, genomic GC% values can cover a wide range that is particularly evident at third codon positions. Previous research has demonstrated that individual genes depict a homostabilising propensity to adopt a comparatively uniform GC%, defined as (micro)isochores that fill a certain niche and depict an explicit characteristic pattern among that respective gene, which are said to strongly associate with the genome organisation (EYRE-WALKER & HURST 2001). In the majority of cases the nucleotide composition is most diverse in the second codon position, which is of high interest concerning amino acid determination (FORSDYKE 2004). Therefore nucleotide compositions were calculated with MEGA5 (TAMURA et al. 2011) and checked for identity and similarity. All codon positions were considered.

7.3.2 Base composition

Base composition analysis suggest that the synonymous codon usage bias is significant and influenced by various factors as mutational bias, gene function or translational selection (XU et al. 2008), although Chargaff's rule (CHARGAFF et al. 1952, ELSON & CHARGAFF 1952) states that any cell from any organism display an 1:1 ratio of purine (A and G) and pyrimidine (T and C) bases. This specifically means that both the amount of guanine equals that of cytosine, while the same holds true for adenine and thymine. In other words, G = C and A = T, concerning base composition.

Another measure to study the codon usage biases in genes (and genomes) is the effective number of codons (ENC or Nc). It has been proven that this measures are among the most reliable to show codon usage bias (COMERON & AGUADE 1998).

Therefore, CodonW v. 1.4.2 (SHARP et al. 2005) was employed to estimate the codon usage indices of an overall GC, as well as an GC3 (third codon position) content and, additionally, the ENC.

7.3.3 Trinucleotide frequency

The exertion and the motivation of the employment of trinucleotide frequencies has already been explained in the previous chapter. The disparities are within the utilised data set. The plot estimated here contains only those eight tribes which are argued to be not of monophyletic origin. Therefore all sequences assigned to those tribes were gathered in one record to facilitate data comparison on a small scale level comparing the usage of every single codon within the groups.

For illustration a heat map diagram was chosen, beacause this format intuitively distinguishes distinctive characteristics in large-scale data sets. Therefore the software R (R CORE TEAM 2014) was employed, which is an open source statistical environment. Two additional packages namely the ggplot2 package (WICKHAM 2009), available for R, for graphical illustration and a package for transforming input data, namely reshape2 (WICKHAM 2007), converting data for ggplot2, were utilised.

7.4 Adaptive evolution

When homologous DNA sequences are compared, almost always do silent or (nearly) neutral mutations outnumber the amount of replacement mutations, which is owing to the fact that most purifying selections are eliminated. To identify evidence of either positive or negative selection, the number of replacement and silent mutations have to be normalised to the number of silent and replacement sites in the respective gene.

The ω ratio (dN/dS) is used as measure of the operation and pressure of selection applied on the protein level, which is demanded here.

The corrected proportions of nonsynonymous substitution per nonsynonymous site (dN) and synonymous substitutions per synonymous site were estimated from the sequences based on maximum likelihood methodology of YANG (1997) by using the CODEML which is incorporated in the software package PAML (YANG & YODER 1999).

To test the statistical significance of the dN/dS ratio, a likelihood ratio test (LRT for hierarchically nested models) has to be applied on the data. The test verifies the goodness-of-fit between two models and depicts whether individual ratios or only one ratio are valid for the tree. Therefore the log likelihood for both models has to be compared. Twice the result from the difference between the likelihoods is used as the score for the chi-square value, as the LRT statistic approximately follows a chi-square distribution. The degree of freedom needs to be individually considered for each dataset to find the respective critical chi-square value with a probability of 5% ($p \leq 0.05$) from standard statistical tables.

Selection can operate at miscellaneous levels and therefore different questions can be posed. An evolutionary pathway method, the Nei-Gojobori method, in comparison to the ML method mentioned above, can be tested with MEGA5 (TAMURA et al. 2011). Both analyses are based on the comparison of codons. This method intends to test whether positive or negative selection was operating as these sequences diverged. Therefore each tribe has to be tested individually with MEGA’s tool *codon based Z-test for selection*, where an overall average with variance estimations of 1,000 bootstrap replications was applied. The substitution type has to be set to *Syn-Nonsynonymous* while the Nei-Gojobori method (proportion) has to be chosen.

7.4.1 Origin of purifying selection

To answer the consequently rising question, where in the gene the selection occurred, dN/dS values have to be estimated among each codon. A ML reconstruction of ancestral states under a Muse-Gaut model (MUSE & GAUT 1994) of codon substitution and General Time Reversable model of nucleotide substitution was estimated. This model is implemented in MEGA6 (TAMURA et al. 2013), originating from the HyPhy software package (POND et al. 2005), and estimates selection among each codon. This option calculates the strength of selection (positive or negative) operating upon each individual codon in an alignment and provides statistical support measures of each estimate.

Fisher's Exact Test of Neutrality was additionally applied on the data to estimate the number of synonymous and nonsynonymous differences among the sequences, using the Nei-Gojobori method (NEI & GOJOBORI 1986). This test is utilised tests of selection can be conducted to examine the null hypothesis of the neutral evolution.

7.4.2 Synonymous substitution rate

As functional loci, genes of the *chs* family are likely to be shaped by natural selection (WANG et al. 2007). Only synonymous sites tend to be neutral and are capable to provide information for dating split events. The number of nucleotide substitutions at synonymous sites (Ks) were calculated for the coding region of every tribe, and, moreover, an overall Ks was estimated, as well. Calculated data can easily be applied on the formula for synonymous substitution rates to obtain the remaining values by transposition of the formula, r (sometimes also found as μ) = dS/2T. Like already discussed, an influence on the rate of substitution among the respective tribes, due to varying functional constraint, is expected.

7.4.3 Ancestral sequence reconstruction

This method could deliver a projection by which estimated ancestral states resolve some relatedness within the phylogenetic reconstructions (OMLAND 1999). The idea is that those reconstructions may offer hints for the gene evolution among the mustards by revealing some evolutionary structure within the ancient sequences, as those are variations of the recent ones. It moreover may indicate variations among the sequences that could have arisen (BROOKS 1999). So, already available genetic information can be employed in order to determine evolutionary routes and timing (RONQUIST 2004). MEGA5 (TAMURA et al. 2011) offers a maximum likelihood method to estimate those sequences, including ancestral gaps. Reconstructions were executed mainly following the directions of the manual by HALL (2006).

8 Results

8.1 Comparative phylogenetic reconstructions and analyses

The evolutionary relationship of all three algorithms applied on the dataset without *Cleome spinosa* resulted in a nearly absolute congruence concerning the topology displayed by

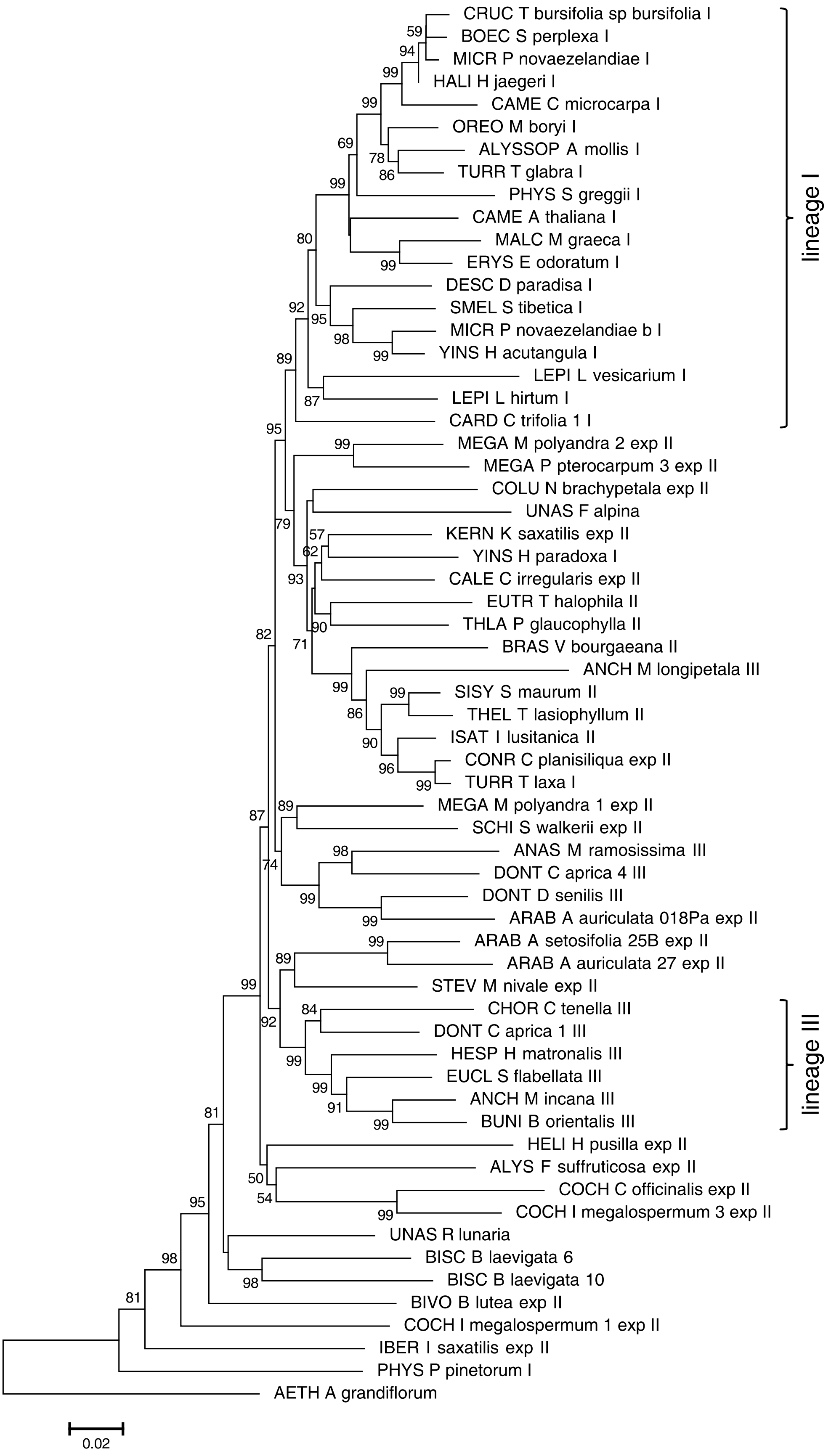


Figure 8. Neighbor joining tree of the reduced data set without *Cleome spinosa* as outgroup. Bootstrap values above 50% are plotted next to the respective node. Lineage I and lineage III are displayed as monophyletic clades. While lineage II, as well as expanded lineage II contain additional *chs* sequences from other lineages. MP and ML analyses were also conducted and can be viewed in the appendix (supplementary material S21 and S22).

the neighbor joining tree in **Figure 8**. The NJ method lead to an optimal tree with the sum of branch length = 3.142. The confidence probability (multiplied by 100) that the interior branch length is greater than zero, as estimated using the bootstrap test (1,000 replicates) is shown next to the branches (DOPAZO 1994, RZHETSKY & NEI 1992). Moreover, the branch lengths are depicted in the same unit as those of evolutionary distances used to infer the respective phylogenetic tree.

What is striking in these gene tree phylogenies is that the exclusion of *Cleome* immediately leads to, firstly, nearly congruent trees implying different algorithms and, secondly, to a gene tree phylogeny which looks straightforward, as AETH, as the most basal tribe, arranges at the uttermost position within the arrangements. However, the ML and the MP phylogenies do not depict such extraordinary high bootstrap values like it can be viewed in the NJ tree (not shown, compare supplementary material S21 and S22). Concerning the maximum parsimony analysis of taxa, the most parsimonious tree (not shown) with the length = 4008 is displayed in the appendix, with a consistency index (CI) of 0.22788, a retention index (RI) of 0.44853 and a composite index of 0.118 for all sites. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches, but only values above 50% are displayed (FELSENSTEIN 1985). Only the more recent nodes and the nodes which mark the split into the respective lineages are supported with values above 50%. Lineage I for example shows a bootstrap support of 68%, while lineage III, at least, is supported with 83%, while the remaining data is only moderately backed.

The molecular phylogenetic analysis via ML method resulted in a tree with the highest lnL of -21.9243 and a topology which are superimposable with tree displayed above. The initial tree for the heuristic search was obtained by applying the NJ method to a matrix of pairwise distances estimated using the MCL (Maximum Composite Likelihood) approach. The percentage with which taxa cluster together is again much lower than in the NJ tree and the values above 50% are also more rarely distributed.

Although bootstrap values do not support every node consummately, identical behaviour of the associated taxa within diverse reconstruction methods suggest an entity among the data. In all employed analysis methods, lineage I was well facilitated with moderate to high bootstrap values, as well as lineage III which is monophyletic in all representations. Lineage II members do cluster within one group which seems to be polyphyletic as additional sequences are added to the group and seem to share the recent common ancestor. Several samples from expanded lineage II (KERN, CALE, COLU, CONR, YINS and MEGA) are associated with lineage II. The *chs* reconstruction at hand suggests that *Conringia planisiliqua*, as well as

Turritis laxa are both assigned to lineage II, while EUTR and THLA, which are in a sister position, are arranged closer to expanded lineage II. The remaining members of that expanded lineage are still allotted in smaller groups among the tree. One well supported (89%) small clade of three sequences from that lineage, namely two ARAB sequences and one STEV, behaves as a sister group to lineage III and, with 92% support suggests that those two groups share a common ancestor.

Moreover, the tribes which already became apparent to hold severe inconsistency during analysis in chapter one, are highlighted with colour-coded asterisks **Figure 3** and will be intensely investigated within that chapter as they seem to completely disarrange the phylogenetic relationships within the mustard family.

The remaining arrangements resemble those from part 1 keenly and are therefore not again discussed in detail.

Hence, the gene tree reconstructions do not perfectly maintain the expected phylogeny of the *chalcone synthase* gene experienced by other genes like ITS. The results at hand recommend further investigations.

8.1.1 Test for recombination

GENECONV (SAWYER 1989) results in a sum file (output format), were 103 polymorphisms were detected to be permuted (10,000 permutations) and the maximum BLAST-like scores can be seen in **Table 10**.

	Max Score	**Sim p-value**	**S.D.s above Sim Mean**	**S. D. of Sims**
Inner Frags Score	8.229	0.0560	1.75	1.0829
OuterSeq Frags Score	2.467	0.5048	0.37	1.3097

Table 10. Test for recombination. GENECONV (SAWYER 1989) tests for gene conversion by scanning for identical gene fragments between pairs of sequences from a DNA alignment. P-values ($p \leq 0.05$) are calculated to assess statistical significance of observed fragment lengths.

The global value of $p \leq 0.05$ is used as indicator for further analysis of potential conversion tracts detected by the programme GENECONV (SAWYER 1989). Given that the programme cannot distinguish between a gene conversion event and unequal crossing over, significant output alludes to conversion events or tracts (MONDRAGON-PALOMINO & GAUT 2005).

None of the p-values showed a significant output, as both are above the critical value. This suggests that gene conversion could be excluded as potential declaration of approach. However, it is important to consider that most gene conversion events may not be detected by this programme, if sequence identity levels are above 70%, what can be observed mostly among coding *chs* sequences. It, additionally, is conditioned by the fact that GENECONV is biased towards detecting most recently converted regions that have not yet accumulated mutations (MONDRAGON-PALOMINO & GAUT 2005).

8.1.2 Divergence time estimates

The comparison of the split data sets is supposed to show drifting results. This hypothesis is valid for the divergence time estimates calculated in BEAST, as well. Direct and secondary calibration approaches most likely lead to incongruent estimations.

Applied Data		**668**	
Constraint	Rate	Angio	Fossil
Runs	4	4	4
Generations	5,00E+07	5,00E+07	5,00E+07
Likelihood	-56134.85	-59942.44	-55872.19
tmrca Brassicaceae	24.9	29.66	39.3
tmrca Lineage I	14.7	17.6	20.48
tmrca Lineage II	8.64	10.64	13.99
tmrca Lineage III	10.1	15.93	16.46
Divergence DONT	13.3	15.93	22.83
Radiation DONT	8.29 – 6.48	9.94 – 0.71	5.14 – 0.91
Divergence MICR	11.5	12	14.91
Radiation MICR	0.59 – 0.56	0.68 – 0.65	0.87 – 0.85
Divergence ARAB	18.5	22.38	29.69
Radiation ARAB	7.1 – 5.77	11.51 – 6.89	10.73 – 9.29
Divergence COCH	24.9	26.16	39.3
Radiation COCH	12.2 – 8.42	14.2 – 12.52	16.57 – 13.08
Divergence MEGA	16.8	20.98	39.3
Divergence YINS	18.5	22.38	25.6
Divergence PHYS	24.9	23.6	28.13
Divergence TURR	11.3	23.6	28.13

Table 11. Parameters and results for the original data set holding 668 sequences of nuclear *chalcone synthase* genes, *Cleome spinosa* was removed. Three divergence time estimate approaches are depicted like explained (material and methods) for the putative polyphyletic tribes. Divergence (split age of respective tribe) and radiation values (one for each of the polyphyletic arranged groups) are listed, as well as estimations for the most recent common ancestors (tmrca). Radiation values are only given for those tribes containing at least two different species in each group.

When the complete data was applied, relatively strong variations, due to the approaches and the inappropriate *chs* sequence from *C. spinosa*, occurred. The estimates via range of synonymous subsition rates again resulted in the youngest outcome and assesses the crown age of the Brassicaceae family, with 24.9 mya, into the early Oligocene. This estimation is well within the range of previous research (COUVREUR et al. 2010). The mean rate settled at 1.29 x 10^{-8} (95% HPD 7.08^{-9} and 1.1^{-8}) which is a bit faster than expected. This might be due to the fact that the polyphyletic data included displays increased divergence. The output received via dating with released angiosperm split calibrations resulted in older estimations for the crown group, namely 29.66 mya. While the third approach, the fossil calibration, resulted in an estimation of 39.3 mya for the crown age of the family, dating its origin back to the Eocene (54-35 mya). Without the outgroup, a trend towards younger estimations can be scheduled, especially in the Angio approach, which displays a crown age which is antedated 5 million years. The estimated lineage ages are in relative congruence among all three approaches, arguing for lineage I to be the oldest (between 20.49 and 14.7 mya) and lineage II as the most recently derived group with estimations between 13.99 and 8.64 mya. The age estimations for the tribal divergences still settles at relatively high rates. Therefore, the excluded outgroup did not affect all estimations listed.

8.1.3 Lineage through time plots

Changes in diversification effect the delineation of all organisms among the data and also in single clades. Therefore the expectation of those estimates is exponential growth in case the rates of speciation are constant over time. Significant deviations indicate that diversification is changing as a function of time. This expected results can be viewed in the plot received from each tribe suggesting a polyphyletic presentation. Each tribe affected was plotted separately (not shown) depicting a single progress which is due to the fact that the number of sequences for the single tribes are not that high (between six and 19). Thus, the development of species among one tribe would result in a meaningful output and the division into the two groups within each tribe would result in even less administrable data. Therefore all of those (complete) tribes were gathered in one plot, delivering valuable information of their origin and evolution.

For each tribe a plot was estimated predicting diverse modes of evolution or a parallel development of the clades among these tribes.

Therefore each curve in the plot consists of two diagrams depicting the particular group of sequences following the gene tree reconstructions.

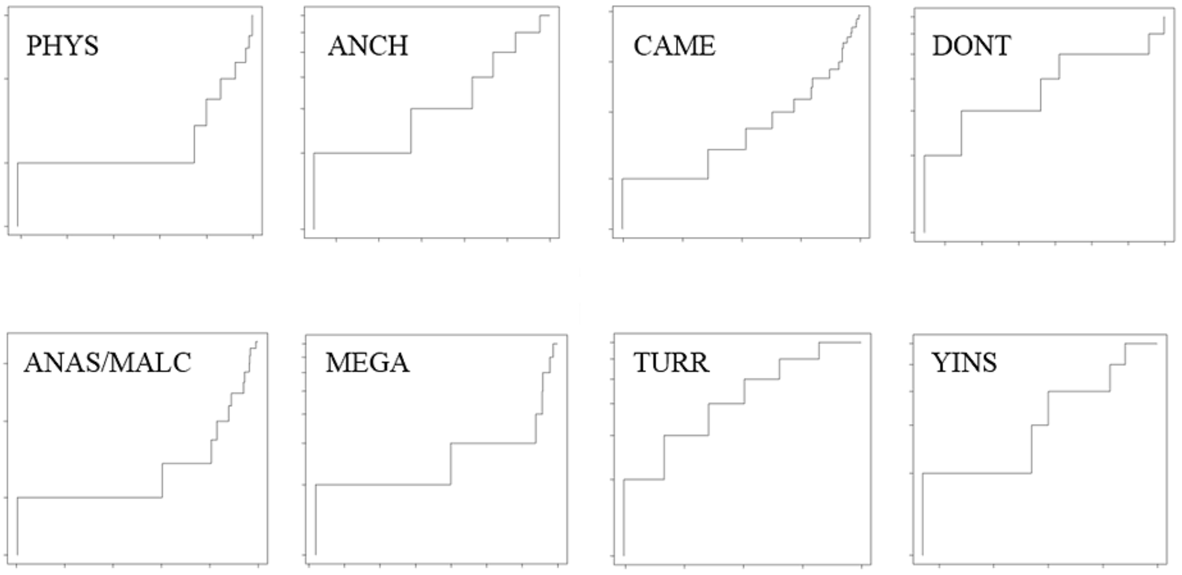


Figure 9. Schemes of lineage through time plots for eight feigned polyphyletic tribes, although proven to be monophyletic. Number of sequences (ln) on y-axis are plotted against the age on the x-axis (cut off for abridgement). Sequences are put into one estimation (expected and unexpected) for each tribe.

The illustration describes the anticipated origin of the species annotated to the respective tribe. A vertical line indicates the erratic increase of species, while a horizontal line without rise suggests that for that time span no speciation has taken place with reference to the data employed. A relatively long plateau, like it can be observed in the PHYS or MALC/ANAS suggests that the origin of the respective tribe is marked by the start of the curve, while the speciation, the burst of the species, followed only after a longer period of time. In this context, it can be reasoned that the second rise marks the effective date for an evolutionary process like a duplication. The opposite process is shown in tribes DONT or TURR where the tribe immediately starts with an early burst of cladogenesis and increase in species numbers suggesting an early polyploidisation event. The plots turn out more significant the more data it is added.

8.2Identifying gene regions

8.2.1 Promoter

The promoter region of the eight tribes were surveyed and visualised via Multiple EM for Motif Elicitation (MEME), a motif-based sequence analysis tool, discovering de-novo motifs (BAILEY et al. 2009). The output files partly revealed no motif aberration among the expected and the unexpected group and variability could only be detected due to different species and genera employed. Especially within those tribes with a huge amount of data, motifs vary significantly. The most promising tribes for representative comparison are therefore those which hold sequences from either the same genus (close kinship) or, what is even of more interest, sequences from the same species indicating mutational variation. Hence, tribes ANCH (*Matthiola incana* and *M. longipetala*), MEGA (*M. polyandra*) and DONT *(Clausia aprica*) were investigated. Concerning the Megacarpaeeae, *Pugionium pterocarpum* was excluded and only *M. polyandra* data was compared. First indication for variable promoter sequences here is the length. While the expected data shows a length of 152 base pairs, the unexpected sequences are all longer (170 bp). Concerning the sequence pattern, an additional motif is present in the longer sequences (five motifs), while the shorter ones do only depict four motifs, where three of those can be found in both groups. Nearly the same pattern is perceptible within the two groups of the ANCH. The unexpected promoter, again, is longer with 171/172 bp compared to 156 bp in the expected ANCH data. Six or rather seven motifs could be identified of which four are shared.

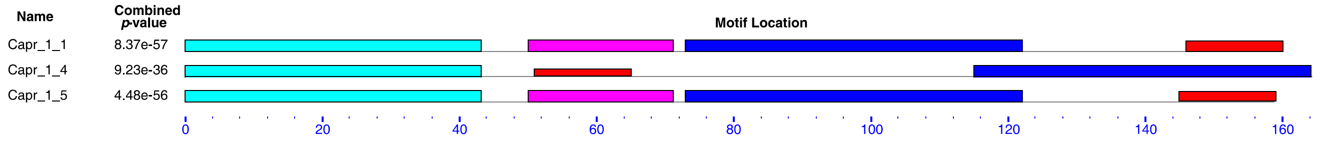


Figure 10. Illustration of promoter region with p-values from *Clausia aprica* (DONT). DNA sequence motifs are calculated by MEME (BAILEY et al. 2009) and indicated by blocks in various colours and height. Identical colour symbolise identical motifs, while the height of the respective motif block is proportional to -log (p-value), truncated at the height for a motif with a p-value of 1e^{-10}.

Clausia aprica, like *M. polyandra*, shows up with two sequence collocations among the phylogenetic trees. This hints not only to a polyphyletic origin of the respective tribes but also to distinct duplication events, which will be discussed later on (see 9.4). As it can effortlessly be recognised in the figure above, there are two varying types of promoter regions within that species recognisable. The first and the third are identical, while the second varies in length and in the amount of motifs estimated. The sequences with four motifs are effectively shorter counted in base pairs (160 bp) than the middle one (166 bp), which only depicts two distinct sequences. Those variations within DNA regions, although belonging to the non-coding part, are meaningful while this pictographic self-explanatory method depicts the results in an intriguingly elementary way.

8.2.2 Intron

All introns with noticeable intra-tribal ranges were compared. The partly striking differences in the length of that non-coding region was further examined via t-test, to find out whether there is a significant difference between that two groups and which group is affected by that phenomena. The suggested tribal splitting, which is dealt with within this chapter was also factored, cognisable by its bold digits.

The reason for the tribe Camelineae to depict two distinct groups will be discussed later (see 9.3).

Obviously, there are some tribes left in the table bare of discussion, which do not belong to the group of tribes reviewed in this chapter. Those remaining intronic regions will be discussed later on in the following chapter 11.3.2.

The results show that most of the tested introns bear (highly) significant difference in length. With tribe Physarieae, no statement can be made, as no intronic region for group 1 is available. Only within the Anastaticeae/Malcolmieae, the Camelineae and the Arabideae the results were not significant. The latter result can be explained by the two shorter sequences from Aver C and Dver D (each 73 base pairs). If these are ignored, the two groups will also differ significantly (asterisks in brackets). The ANAS/MALC group, quite expectedly, shows no significant t-test for $p \leq 0.05$, as this group recently depicts two tribes (AL-SHEHBAZ et al. 2014), instead of only ANAS, which does not show any hint for unexpected phylogenetic behaviour. Therefore the first row of the table displays brackets enclosing the output for these re-arranged phylogenetic situation. This moreover demonstrates the adequate employment of chalcone synthase for phylogenetic reconstructions, resolving unclear situations.

The intronic region clearly depicts relatedness and deliver hints concerning the level of divergence. In some tribes, like the Smelowskieae, the intron varies only among three sites even between different genera, resulting in a p-distance of 0.025. Most of the tribes employed here show a distance between zero (tribes with minimum one species) and 0.366. But there is a cleavage within the distance values dividing the tribes into two distinct groups. The first group gathers tribes with distances up to 0.2 whereas values above that build the second group. These all belong to either that tribes that are situated at a basal position (e.g. Cardamineae, Biscutelleae) or that are not monophyletic within the phylogenetic representation (Megacarpaeeae, Microlepidieae, Cochlearieae, Yinshaniea) predating the divergence of the respective tribe. This leads to a rule of thumb that predicts that the less heterogeneous an intron appears within a tribe the younger the divergence of the species. A value roughly around 0.2 or higher induces an older divergence or a duplication event. Therefore, with every taxonomic rank intronic identity decreases.

tribe	Group 1	Group 2	p-value
Anastaticeae/Malcolmiaeae	92, 125	67, 70	0.13714)
Anchonieae	85	75	0.00014**
Arabideae	52-91	73, 115, 131	0.1985 (**)
Biscutelleae	67	99	0.0001**
Camelineae	86, 87, 89, 91	82, 83, 96	0.20656
Dontostemoneae	83	136, 138, 189	0.01405*
Megacarpaeeae	84, 86	101, 116	0.0087**
Microlepidieae	77	88	0.00014**
Physarieae	n/a	77, 78,83, 146	n/a
Turritideae	77	92	0.00895**
Yinshanieae	76, 85	108	0.00064**

Table 12. Significant variability of intronic length within tribes. The column titled group 1 exhibits the intronic length of sequences which resulted in a phylogentically expected pattern, while those of group 2 depict sequences from a rather unexpected position, with exception of tribes Biscutelleae, Camelineae and Microlepidieae, where it cannot be scheduled, which group holds the expected sequences. Digits in bold mark the tribes of putative polyphyletic origin. Significant differences between the group's intronic length are marked with asterisk (* = significant, ** = highly significant).

8.2.3 Exon 1

The initial part of the exon, after the start codon, is more diverse, while the remaining first exon shows a notable identity. A tendency could be observed that all clones, assigned to lineage I depict a constant second base triplet, coding for alanine (A). Two exceptions could be found, namely three species that display an alanine although they belong to other lineages (Isav 1-3 and Dver RK239 B to expanded lineage II, and Ebou 1 to lineage II). Vice versa, sequences that are assigned to lineage I and do not depict an A as second codon, namely Ypar 10-2 to 10-4, Tlaxa 1-1 to 1-5, Dvir 1 and 2 and Mlon 1-3. With exclusion of Dvir 1 and 2, the remaining sequences belong to these tribes listed in **Table 12** and **Table 13** depicting tribes that externalise some sort of internal issue, e.g. *Yinshania acutangula* exposes an alanine as second codon, while *Hilliella paradoxa* does not, although both are assigned to lineage I. This result perfectly underlines the outstanding position of *H. paradoxa*, debated later on in the discussion. With the two species left it can be concluded that the difference in the respective beginning of the coding region is due to PCR or sequencing error and therefore not reflecting the actual sequence part and therefore can be ignored.

It consequently can be subsumed that, in case the second codon of exon 1 is alanine, there is a nearly 100% chance that the following sequence belongs to lineage I. However, the most frequent second codon is glycine (G), viewed in each remaining lineage with exception of lineage I. No further interrelation can be detected.

8.2.4 Exon 2

Within the second exon a 12 residue chalcone- and stilbene synthase signature motif, (W)GVLFGFGPGLT, is known, close to the carboxyl-terminus (MARTIN 1993). The conserved amino acid sequence is present in the CHS/STS (TROPF et al. 1994, WANG et al. 2007) family among all angiosperms.

Motif sequence changes in the CHS protein imply functional divergence, although these functions are not known, yet (WANG et al. 2007). The table depicts the amino acid replacements in the reviewed tribes reviewed in this chapter, which can be categorised as conservative or radical changes (HUGHES et al. 2000). No other part of the data was affected.

Sequence	W	G	V	L	F	G	F	G	P	G	L	T	charge	polarity
Polyphyletic Tribe														
Yacu 1-3, 1-5, 1-8												S	neutral	polar
Alyr AF112103												I	neutral	polar/nonpolar
Cbur GQ983009				S									neutral	nonpolar/polar
Cbur_AY612785				S									neutral	nonpolar/polar
Mlon 1, 2, 3				S									neutral	nonpolar/polar

Table 13 A conserved motif at the carboxyl-terminus of the chalcone synthase. Each amino acid sequence was compared to the conserved.

Any change of category is counted as radical difference while a change within the category is evaluated as conservative. Concerning the charge of the amino acids, no radical categorical change could be observed, the neutral amino acids are changed to another aa neutral in its charge, while there were several changes in the polarity. Only one change was conservative, **Table 13** [change from (non)polar to (non)polar] while the remaining four changes mostly depict a switch in the category from a nonpolar residue to a polar one and only once vice versa. These amino acid substitutions may most likely result in a dramatically change of structure and chemical properties of the respective proteins (WANG et al. 2007). This can also result in the pseudogenisation of the gene within the named sequences. The nine sequences of four species affected do belong to the tribes Yinshanieae, Camelineae and Anastaticeae, which are three out of eight relevant tribes discussed. This strengthens the hypotheses of evolutionary divergent events within these tribes or of taxonomic inconsistencies not itemised yet.

8.2.5 DNA motif search among gene

Again, a motif search was applied on the reduced data, containing eight tribes each divided into two groups, of one which depicts the tribal data of the expected group, while the second shows the unexpected data. For evidently review of the output from MEME (BAILEY et al. 2009), the expected and unexpected data sets for each tribe were put into one group. The height of the resulting motif blocks is proportional to - log (p-value), truncated at the height for a motif with a p-value of $1e^{-10}$. Within the coding region a pattern could be observed, which points to the beginning of the first exonic region as proprietary of the pivotal DNA difference between the expected and unexpected sequences. This trend could be observed in six out of the eight tribes investigated here. The not affected tribes are CAME and TURR, while the latter depicts a similar small-scale addition of eight nucleotides within the coding region but within the second exon (bp 943-950). The tribe Camelineae display complete identical motifs among the coding region, indicating no severe difference among the genes, suggesting that no real unexpected sequences are investigated here (see discussion 9.3). There are also affected regions in the second exons depicted in the tribes MEGA and YINS of which the latter is depicted in the figure as representative delineation. In MEGA the second deviation in exon 2 affects eight base pairs (bp 1082-1089) which build an additional motif (which does not automatically equal with additional nucleotides, as not each displayed motif is completely identical in length) results from a previous motif-shift.

The YINS, which are shown in the **Figure 11**, display that additional motif at exon 1 (bp 1-11) and an accessorily motif difference in the second exon (second last motif, dark blue and brown). The expected three sequences (*H. acutangula*) hold this at location 1,114-1,151, while the lower three sequences (*H. paradoxa*) demonstrate this eight base pair variation between base 1,147 and 1,154 with an overall gene length of 1,184 to 1,181 in *H. acutangula*. To put this in a nutshell, the beginning of the first exon already delivers evidence for the difference of gene types resulting in relatively extreme consequences (see gene tree). These motif searches are additionally facilitated via the highly significant p-values left to the motif blocks. MEME (BAILEY et al. 2009) only displays motif blocks which represent non-overlapping sites and p-values better than 0.0001.

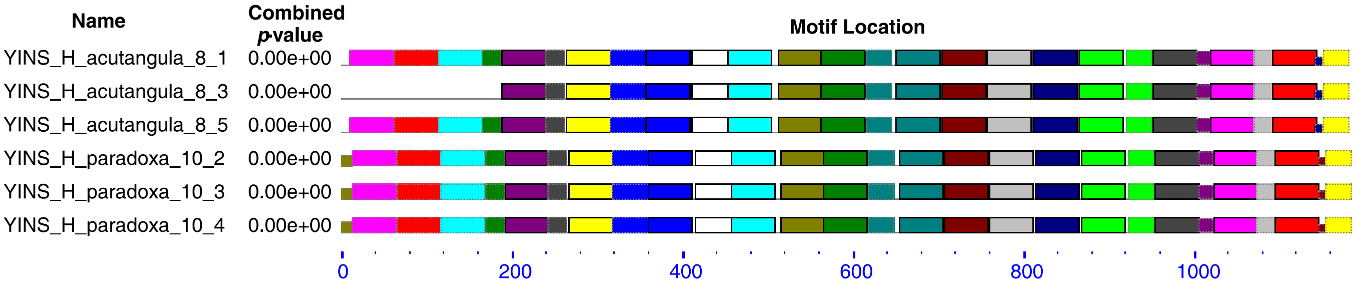


Figure 11. Motif search with MEME (BAILEY et al. 2009) among the eight feigned polyphyletic tribes. Tribe Yinshanieae is depicted as example. Both groups (expected upper three and unexpected lower three sequences) are displayed with p-values. Note that the second sequence (YINS_H_acutangula_8_3) is not completely sequenced.

8.3 Compositional heterogeneity among DNA

8.3.1 GC content

The mean GC content in the complete mustards utilised here is distributed between 42.5% and 62.8%, where the most probable content is 53.8% (see S30). Previous review suggested that plants show a relatively small distribution of GC content in comparison to prokaryotes (range ~20) or algae (range 13%), which cannot be confirmed here. (JEZ & NOEL 2000) described an even higher content for the ORF in *chs* of 59% on average (no range given). Among the third codon position the range is even close to 30%, starting with a minimum GC content of 37.7%, ranging up to 65.7%.

High GC-rich stretches are linked with coding regions. This is especially seen in eukaryotic exons and introns, which show significant differences in base-composition. The average GC% content, mentioned above, within the coding region is nearly twice the size of the GC content of the intronic region, which shows an average of 27.3%. That the length of coding regions is related to GC content (POZZOLI et al. 2008) could not be verified or falsified here, as the *chalcone synthase* does only vary slightly concerning its length and therewith no significant statements are feasible. SUEOKA (1961) argued that the variation in GC content is driven by neutral mutational biases and is most evident in those sites under the least selective constraint.

The stronger the constraint the slower its evolutionary rate of substitutions and higher the chance for maintenance within the genome. It was further ascertained that mutational patterns in *Arabidopsis thaliana* are also AT rich and show evidence (SUEOKA 1961) of correlation. This directly leads to the inspection of the base compositions for the listed tribes presumably arguing for mutational appearance among some sequences or even tribes. Concerning the impact on the substitution rate, a relatively slow rate would be expected for tribes with low variation in the GC content, while, the converse argumentation is, that tribes with a faster rate display a higher AT content and stronger variations in the GC content.

8.3.2 Base composition

Figure 12 depicts the base composition in the *chalcone synthase* gene among all employed tribes. It can be noticed that there is no regular or even parallel application of the bases. In most tribes the GC content is higher than the AT content, although varying among codon positions.

Analysis showed, that the average percentage of GC content was generally higher at the first (average GC1 = 58.3%) than at the second (average GC2 = 41.7%) codon position. It was observed that the GC1 content was always (with exception of the Cochlearieae) higher than the GC2 content. This coincides with base composition analysis (Xu 2008). Thus the variations of the synonymous first and second codon positions might be caused by translational selection utilising G or C at the synonymous second position.

XU et al. (2008) stated, that species with a close genetic relationship always present a similar codon usage pattern. In case this holds true it can be reasoned that the groups depicted in the diagram above do not witness close relatedness. The average GC3 content of all eight tribes employed here, differs 5.8%, with 62.8% for the tribes with expected phylogenetic arrangement and 57% for the tribes of not expected placement. This may not seem to be an exceedingly ample difference, but with a closer look on the bias it becomes peculiar. The C3 content (not shown) of the expected groups depicts an average value of 21.8% and a G3 average of 35.2%, while the values shift significantly concerning the unexpected-placed group. Here values of 36.4% for C3 and 26.4 for G3 hint to a huge range within the sequences expected to be of close genetic origin. Both values within the two groups differ significantly, while the C3 values are higher and the G3 values are lower. This can be interpreted as evidence for the heterogeneity within those tribes. It is most likely that the results of the ENC plot and base composition analysis (**Figure 12**) indicate that the codon usage pattern was influenced by mutational bias as well as other factors such as translational selection. As mentioned above, this bias of different codons immediately affects the synonymous to nonsynonymous rate ratio, as mutation bias results in an accelerated replacement rate of amino acids, especially in that regions with less functional constraint. Therefore an increased mutational rate is expected for that groups of the tribes which resulted in unexpected placements.

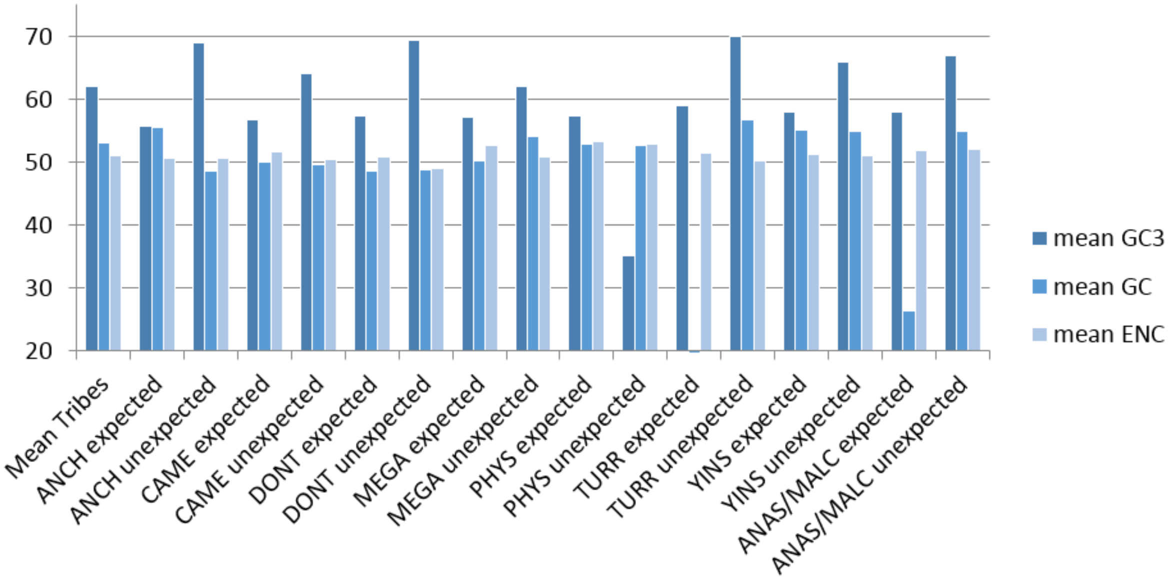
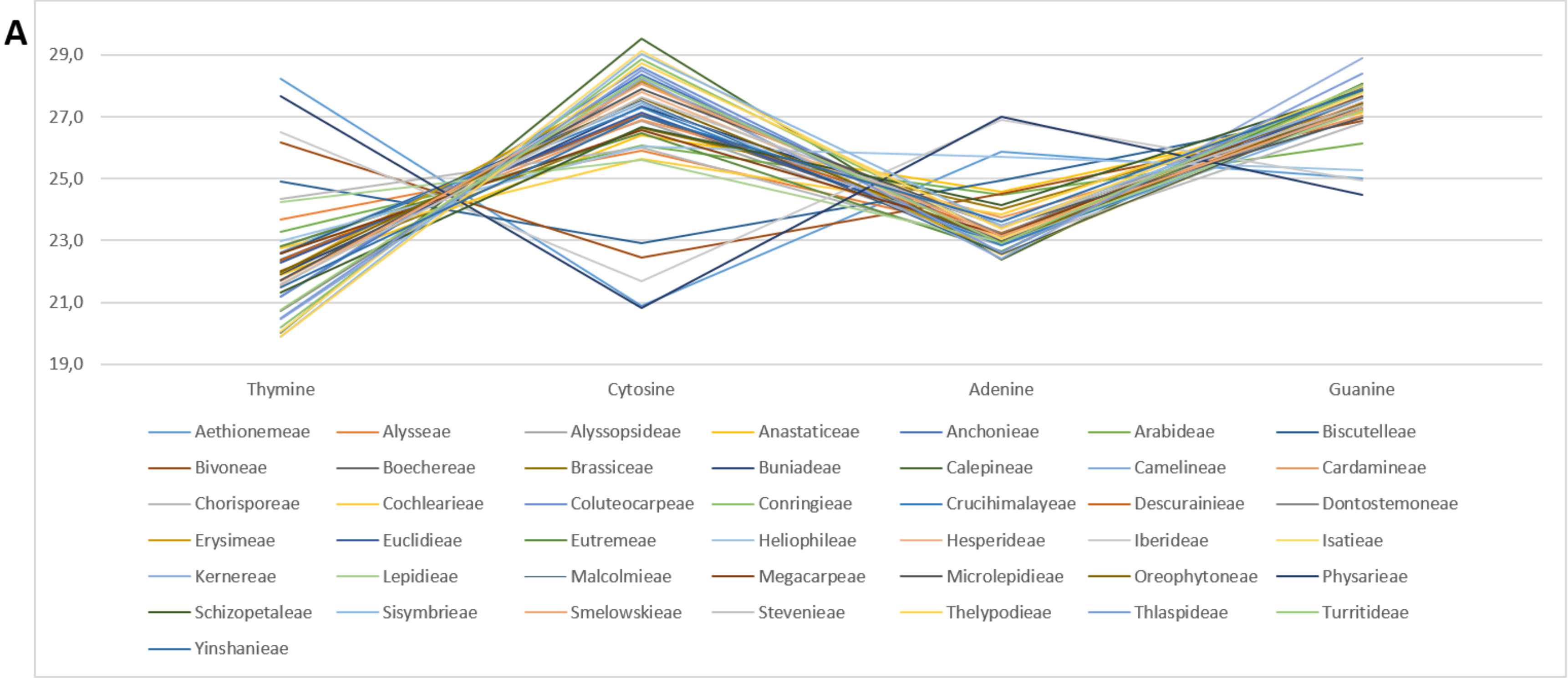


Figure 12. Mean GC3, mean GC content and effective number of codons (ENC) as a measure of overall average codon usage bias in all tribes averaged and in tribes depicting a polyphyletic origin within the *chalcone synthase* gene. Evolutionary analyses were conducted in DAMBE v.5.3.105 (SUN et al. 2013, XIA 2013). Note that for Turritideae no mean GC value could be calculated due to low number of complete sequences.

However, the base composition of the complete codons of the averaged tribes resulted in an unequal output. In comparison to the bar chart depicting selectively GC focused data, the plot in **Figure 12** employs all tribes, also holding the arguable sequences recently discussed, each with its entire amount of sequences, while the bar plot depicts each tribe with polyphyletic delineation in two categories. While the first triplet of the bar chart ("Mean Tribes", which is the averaged value of all tribes with exclusion of theses tribes examined aside) shows the expected values, namely a mean GC at third codon position well above 60%, a mean GC around, but above 50% and an effective number of codons lodging roughly at 50%, as well. This impressively demonstrates the range between the expected and the unexpected values. For the additional eight groups, with Anchonieae (ANCH) for example, the group with the sequences clustering at the expected placement, show a slightly to low GC3 value, but still in an acceptable range, while the unexpected group has an GC3 which is exorbitant reaching a value close to 70%. A similar observation can be done among all but one (PHYS) unexpected groups. Note that there are groups with an extremely low GC3 value, which are the PHYS (unexpected) and the ANAS/MALC (expected). With the last, the explanation is very obvious: The sequences gathered here were very recently (AL-SHEHBAZ et al. 2014) divided into two tribes, which can be underpinned with these results. ANAS/MALC expected are the Anastaticeae, with exclusion of some *Malcolmia* species, which are now arranged within the revitalised Malcolmieae (*Malcolmia graeca* for example, here the unexpected group).



B

Tribe	Base composition Thymine	Cytosine	Adenine	Guanine
Aethionemeae	28.2	20.9	25.9	25.0
Biscutelleae	24.9	22.9	24.9	27.2
Bivoneae	26.2	22.5	24.5	26.9
Iberideae	26.5	21.7	26.9	24.9
Physarieae	27.7	20.8	27.0	24.5

Figure 13. A) Base composition of *chalcone synthase* gene among all tribes. X-axis displays the bases, while the y-axis indicates the respective amount in percent. B) List of base composition for tribes with anti-parallel appearance, resulting from A. Base compositions are listed in percent.

Therefore their output, with exception of the GC value in the expected group, are very close to the average values of all remaining tribes.

Tribes alluded in **Figure 13** B) show a GC content around 50% or below, which is not in congruence with reported values and results gained from the data at hand. The polyphyletic tribes, with exception of PHYS, seem to even out the diverse values when packed together. Ergo, lukewarm inspections of the DNA sequences would not have revealed the discrepancies among some of the tribes.

The five addressed tribes here (B) hold an outstanding position within the phylogenetic arrangements, see **Figure 3**. They are all nested outside the gene tree lineages and are close to Aethionemeae. These results suggest that a low GC, or vice versa, a high AT content can be found in sequences depicting a basal position. This, in turn, facilitates the thesis that the mentioned tribes, concerning their evolutionary journey, have to be told apart from the sequences creating a core phylogeny.

8.3.3 Transition/transversion rate bias

The saturation plot displays the coding sequences of the complete dataset minus *Cleome spinosa*. Nucleotide saturation was analysed by plotting the number of observed transitions (Ti/s) relative to that of transversions (Tv/v) against genetic distance values.

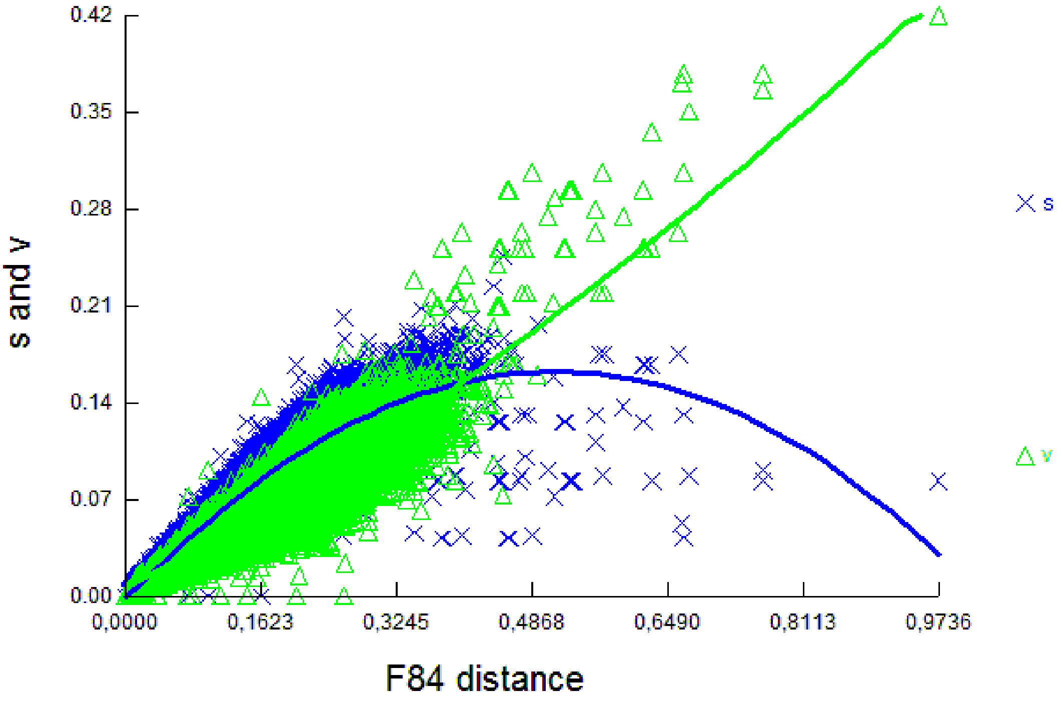


Figure 14. DAMBE (Xia 2013) substitution saturation plot of codon third position for the *chalcone synthase* gene among the complete dataset. The number of transitions (s) and transversions (t) is plotted against the F84 distance.

Increased nucleotide transitions (s) mean that multiple changes per site are more likely. The plot clearly shows that both substitution types accumulate slowly and almost linearly until a certain point (around 0.4), indicated by the majority of symbols for transitions and transversions. This results from the fact that both quantities (s and v and F84 distance) increase directly proportional to the point where saturation occurs. The curves clearly denote an antiparallel development, especially foreshadowed by a bundle of transition-outliers, arranging in a cloud more parallel to the x-axis. The transitions (blue crosses) precisely show a fast increase in divergence, while the Ts/Tv ratio drops steeply. This demonstrates the high genetic divergence among the data utilised here. Consequently, the alignment underlying this analysis is saturated concerning the transitions shown by the curve (exceeding transversions partly), which arises from the fact that uncorrected distances among third codon position are underestimated.

8.3.4 Trinucleotide frequency

The heat map estimated from the doubled (expected and unexpected group) tribes resulted in a demonstrative confirmation of the expectations following from previous data analysis. The tribes are arranged in pairs so that each group containing the expected and unexpected data can be immediately investigated. A counter-rotating pattern is very obvious in each of the tribes.

Starting with the double-tribe ANAS and MALC, the map underpins commandingly the differences concerning codon bias use. The unexpected groups depict an extensive use of the codons CUC, AAG, GAC and GAG coding for the amino acids leucine (L), lysine (K), aspartate (D) and glutamate (E), which, unexpectedly, are exactly the same codons highlighted in the previous chapter, although all complete tribes were employed.

This suggests that the unexpected sequences represent the overall trends concerning codon usage bias more precisely than the expected. Besides, this clarifies the already mentioned effect of the acquaintance with averaged data and generalised conclusions drawn from those. The heat map presented in the last chapter therefore contorts the data to the disadvantage of the sequences with an expected behaviour in estimations. In summary, the base composition data advocates for the evolutionary distances gathered within the sequences which are supposedly of close kinship, although some data even derives from identical individuals. Three approaches are conceivable: either this results confute the thesis that closely related species display a similar codon usage bias, or the data compared via this method does not show homologous genetic sequences or the sequences illustrate data which was coined by diverse developmental and mutational processes. The second bullet point can be ruled out, as homology has been checked. Most likely the remaining argumentations can be put together, as firstly, the species *are* related and, secondly, hold a genetic mixture of acquainted influences imaged via diversifying sequence composition. There is clear evidence that the evolutionary history of *chalcone synthase* tells a fascinating story of gene development. As a consequence, these affected tribes have to be revised for further analysis.

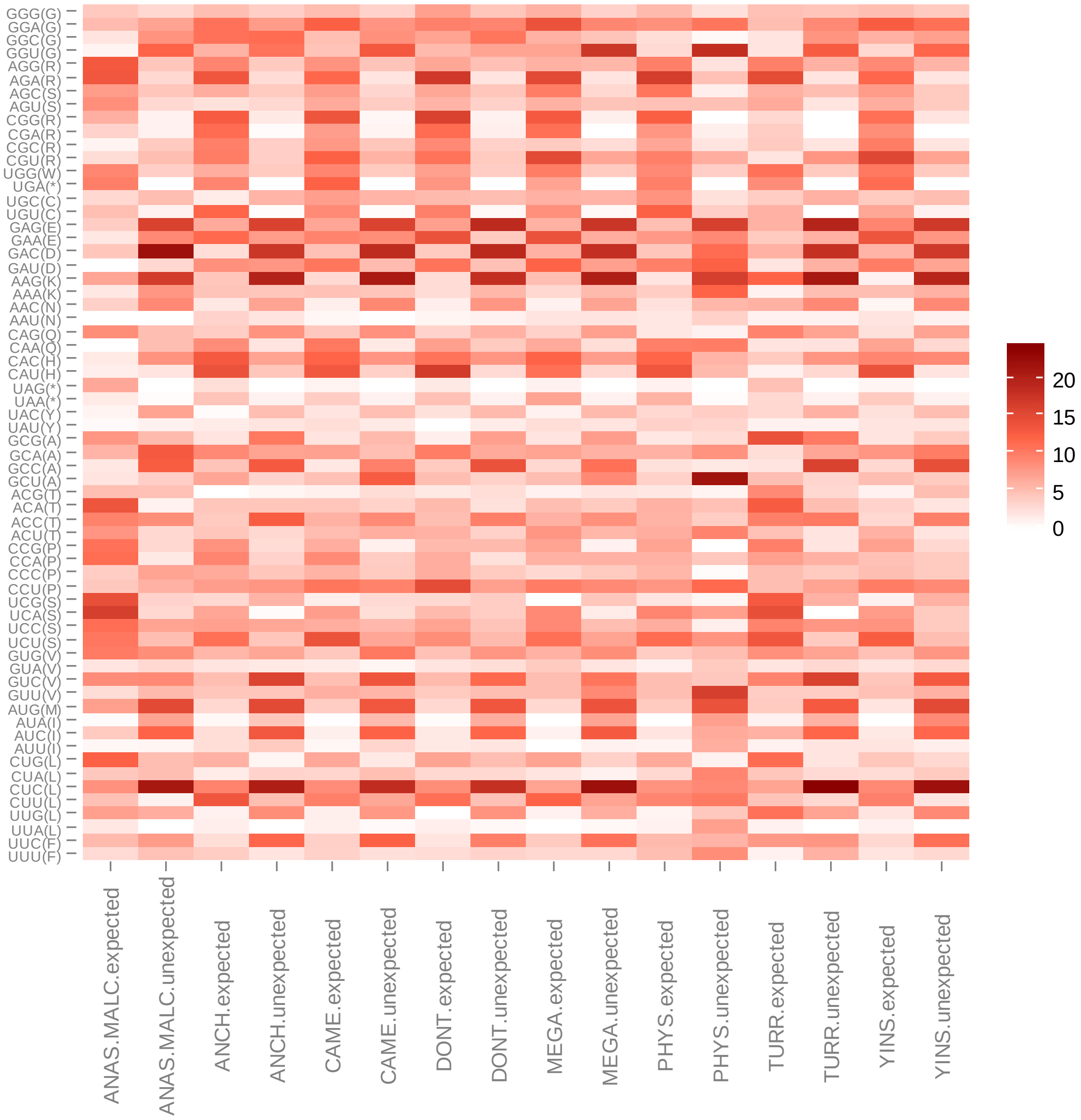


Figure 15. K-mers frequencies depicted as heat map. Colours correlate with the codon usage bias. Split tribes (expected and unexpected) are illustrated in separate columns on the x-axis, base triplets (codons) with amino acids in brackets are show on the y-axis.

8.4 Adaptive evolution

The investigation of adaptive evolution is an appropriate tool to closely analyse the information gained from recent outcome. The table below (**Table 14**) contains information on the selective mode of the chalcone synthase gene among all tribes from this study. The dN/dS ratio (here ω) is used as measure of the operation of selection. For each tribe separate CODEML files were run, comparing each sequence to every other sequence from the same tribe. A dN/dS ratio < 1.0 is probative of purifying (negative) selection, while a ratio > 1.0 is taken as evidence for diversifying (positive Darwinian) selection and a ratio = 0 depicts generally neutral selection. The products of pseudogenes normally do not contribute anything to the fitness. Therefore does an ω very close to 1 often hint to pseudogenes, as the intensity of selection is mirrored in the degree of that ratio. There are no signals for pseudogenes detectable. The dS value in some tribes displays relatively high numbers (see Arabideae, Brassiceae or Cochlearieae), which is a sign for substitution saturation among that branches. The affected tribes are those with the highest data sampling and consequently the most different species, which automatically results in higher, as more diverse, substitution rates. In parallel, the respective dN values are also around one order of magnitude higher than the remaining values. The overall dN/dS values observed here imply that the *chs* gene is under purifying selection among all employed tribes since each diverged from a common ancestor, with exception of *Murbeckiella boryi***,** the only species from the tribe Oreophytoneae, which dispalys an ω value above 1.

Employing the Z-selection among the tribes, the overall probability that dS exceeds dN within the Aethionemeae is p = 0.01 and therefore highly significant. Hence, the hypothesis of strictly neutral evolution (dN = dS) has to be rejected in favour of the alternate hypothesis of purifying evolution. Comparable results (p values all between 0.0 and 0.03) could be observed for all tribes with exception of the Oreophytoneae. They showed neither significant results for purifying (p = 0.29) nor positive selection (p = 0.98). This means that not any significant conclusion about averaged selection can be drawn.

However, MEGA5 (TAMURA et al. 2011) offers another application to test selection additionally on pairwise comparison. This resulted in no significant outcome as well, which is obvious since all sequences are from one species and one individual. This would only result in significant and reasonable outcome if a comparison between different loci would be estimated.

The pairwise selection tool was hence only applied on tribes with more than one species or with a tendency for more than one *chalcone synthase* locus. As expected, most of the tribes show a trend towards purifying selection. If negative selection occurred within a tribe, at least

Tribe	dS	dN	ω	r = dS/2T
Aethionemeae	0.435	0.038	0.089	2.41x10^{-8}
Alysseae	0.379	0.013	0.035	**1.28x10^{-8}**
Alyssopsideae	0.197	0.015	0.078	4.75 x 10^{-9}
Anastaticeae	1.187	0.040	0.034	2.48 x 10^{-8}
Anchonieae	0.913	0.066	0.072	2.24 x 10^{-8}
Arabideae	2.300	0.127	0.055	5.35 x 10^{-8}
Biscutelleae	0.539	0.030	0.056	**1.17x10^{-8}**
Boechereae	0.122	0.016	0.131	3.05x10^{-9}
Brassiceae	4.973	0.178	0.035	**9.11x10^{-8}**
Buniadeae	0.005	0.003	0.649	(1.36x10$^{-10)}$
Calepineae	0.095	0.015	0.159	(2.28x10$^{-9)}$
Camelineae	0.844	0.054	0.064	1.94x10^{-8}
Cardamineae	1.424	0.097	0.068	2.78x10^{-8}
Chorisporeae	0.294	0.019	0.064	(7.07x10$^{-9)}$
Cochlearieae	5.149	0.169	0.032	**1.19x10^{-7}**
Coluteocarpeae	0.286	0.033	0.117	6.75x10^{-9}
Conringieae	0.0069	0.006	0.869	(1.63x10$^{-9)}$
Crucihimalayeae	0.139	0.005	0.041	3.26x10^{-9}
Descurainieae	0.588	0.082	0.140	1.33x10^{-8}
Dontostemoneae	1.362	0.041	0.029	2.77x10^{-8}
Erysimeae	0.267	0.021	0.081	6.4x10^{-9}
Euclidieae	1.281	0.068	0.534	2.81x10^{-8}
Eutremeae	0.264	0.017	0.065	5.91x10^{-9}
Hesperideae	0.038	0.003	0.087	(7.54x10$^{-10)}$
Iberideae	0.191	0.015	0.082	4.77x10^{-9}
Isatieae	0.209	0.023	0.111	4.78x10^{-9}
Kernereae	0.006	0.005	0.709	(1.31x10$^{-10)}$
Lepidieae	1.094	0.061	0.056	1.99x10^{-8}
Malcolmieae	0.283	0.042	0.148	6.84x10^{-9}
Megacarpaeeae	0.702	0.032	0.045	**1.24x10^{-8}**
Microlepidieae	0.477	0.062	0.055	**1.14x10^{-8}**
Oreophytoneae	0.003	0.005	1.667	(6.39x10$^{-11)}$
Physarieae	1.538	0.035	0.022	3.08x10^{-8}
Schizopetaleae	0.011	0.009	0.818	(1.53x10$^{-10)}$
Sisymbrieae	0.306	0.024	0.081	7.08x10^{-9}
Smelowskieae	0.191	0.011	0.061	4.41x10^{-9}
Stevenieae	0.404	0.025	0.062	1.12x10^{-8}
Thelypodieae	0.162	0.026	0.161	3.93x10^{-9}
Thlaspideae	0.315	0.021	0.067	7.0x10^{-9}
Turritideae	0.569	0.096	0.168	**1.44x10^{-8}**
Yinshanieae	0.617	0.017	0.028	**1.57x10^{-8}**

Table 14. Synonymous (dS) and non-synonymous (dN) substitutions as well as ω values (dN/dS) for each tribe are listed. The row to the right is the calculated synonymous substitution rate (r = dS/2T) averaged among each tribe. Bold rates suggest an accelerated synonymous substitution rate (above expected values). Rates in brackets result from one-species calculations.

30% were effected. In most of the tribes between two third and 95% of the compared species depict purifying selection among the tested gene since they diverged. Only tribes Biscutelleae, Brassiceae, Isatideae, Bivoneae and Cochlearieae show some additional tendencies (maximum of 20% of species) for positive Darwinian selection. This is no opposition, as, firstly, the species and rather *chs* may have evolved differently after the divergence from one and another and, secondly, the evolutionary journey of a gene may vary due to other influences, as well. From these results it can be concluded that the evolution of these genes among most species tested here has been under strong purifying selection during some, but not all, of its history.

Concerning the tribes named above, it can be also subsumed that those, additionally, were under positive selection during a certain time. Some ω values seem to be disproportional high which is due to the fact that these tribes (Buniadeae, Conringieae, Kernereae, Oreophytoneae, Schizopetaleae) are all represented by only one species which consequently results in small amounts of substitutions.

Exactly those values result, while estimating the synonymous substitution rate (r), logically in higher exponents than expected, illustrating a lower rate.

So, all rates with e^{-10} or even e^{-11} (displayed in brackets in the table) can be neglected for further calculations. The bold numbers depict increased substitution rates putatively suggesting that *chs* among those tribes evolves a magnitude faster than among the rest of the family. This is due to the fact that the data sample assigns sequences that are relatively diverged from their counterparts, which could result in misinterpretation of the results.

8.5 Origin of purifying selection

As there is a further interest of finding the origin that has been subject to negative selection, as well as its strength, HyPhy was applied on each tribe to test selection among each codon. Within every tribe no significant value for any codon could be detected (see supplementary material S13), neither under positive nor under negative selection. This is most likely due to the fact that the values calculated are averaged among the entire tree (HyPhy’s estimations are based on an implemented tree algorithm) and codons under selection, only over some branches, are not reflected with this test.

None of the tribes showed significant results (see supplementary material S14). This indicates that the null hypothesis (no significant difference between the groups) cannot be rejected. Hence, suggested tendencies for selection cannot be accepted.

8.6 Synonymous substitution rate

8.6.1 Estimation of divergence ages utilising synonymous substitution rates and dS values

The synonymous substitution rate was calculated for each tribe, as well, utilising the calculated dS values and six different values for r taken from previous reviews (DURBIN et al. 1995, HUANG et al. 2012, KOCH et al. 2001, LAROCHE et al. 1997, WANG et al. 2007, WOLFE et al. 1989).

Given the fact that the sampling is not complete, estimated tribal ages are approximated values, especially those tribes represented by one species.

With the calculated values for dS for every tribe, age calculations have been made. Tribes with only one species result in extreme low calculations, mostly below one million years, suggesting that those tribes diverged very recently.

This obviously is due to the fact that the dS values are one to two magnitudes smaller compared to other samples, as it is calculated among sequences of one species. The age estimations shown here are only valid for the divergence age of the respective species, like *Kernera saxatilis* (origin between 0.75 and 0.18 mya).

Tribe/Rate	$T_{(r=1,5x10^{-8})}$	$T_{(r=4x10^{-9})}$	$T_{(r=8x10^{-9})}$	$T_{(r=1,67x10^{-8})}$	$T_{(r=5x10^{-9})}$	$T_{(r=1x10^{-8})}$
Author	Koch et al.	Wolfe et al.	Durbin et al.	Wang et al.	Li et al.	Huang et al.
Aethionemeae	14.51	54.38	27.19	13.02	43.5	21.75
Alysseae	12.63	47.38	23.69	11.35	37.9	18.95
Alyssopsideae	6.57	24.63	12.31	5.9	19.7	9.85
Anastaticeae	39.57	148.38	74.19	35.54	118.7	59.35
Anchonieae	30.43	114.13	57.06	27.34	91.3	45.65
Arabideae	76.67	287.5	143.75	68.86	230	115
Biscutelleae	17.97	67.38	33.69	16.14	53.9	26.95
Boechereae	4.07	15.25	7.63	3.65	12.2	6.1
Brassiceae	165.77	621.63	310.81	148.89	497.3	248.65
Buniadeae	0.17	0.63	0.31	0.15	0.5	0.25
Calepineae	3.17	11.88	5.94	2.84	9.5	4.75
Camelineae	28.13	105.5	52.75	25.27	84.4	42.2
Cardamineae	47.47	178	89	42.63	142.4	71.2
Chorisporeae	9.8	36.75	18.38	8.8	29.4	14.7
Cochlearieae	171.63	643.63	321.81	154.16	514.9	257.45
Coluteocarpeae	9.53	35.75	17.88	8.56	28.6	14.3
Conringieae	0.23	0.86	0.43	0.21	0.69	0.35
Crucihimalayeae	4.63	17.38	8.69	4.16	13.9	6.95
Descurainieae	19.6	73.5	36.75	17.6	58.8	29.4
Dontostemoneae	45.4	170.25	85.13	40.78	136.2	68.1
Erysimeae	8.9	33.38	16.69	7.99	26.7	13.35
Euclidieae	42.7	160.13	80.06	38.35	128.1	64.05

Tribe/Rate	**$T_{(r=1,5x10^{-8})}$**	**$T_{(r=4x10^{-9})}$**	**$T_{(r=8x10^{-9})}$**	**$T_{(r=1,67x10^{-8})}$**	**$T_{(r=5x10^{-9})}$**	**$T_{(r=1x10^{-8})}$**
Author	Koch et al.	Wolfe et al.	Durbin et al.	Wang et al.	Li et al.	Huang et al.
Eutremeae	8.8	33	16.5	7.9	26.4	13.2
Hesperideae	1.27	4.75	2.38	1.14	3.8	1.9
Iberideae	6.37	23.88	11.94	5.72	19.1	9.55
Isatieae	6.97	26.13	13.06	6.26	20.9	10.45
Kernereae	0.2	0.75	0.38	0.18	0.6	0.3
Lepidieae	36.47	136.75	68.38	32.75	109.4	54.7
Malcolmieae	9.43	35.38	17.69	8.47	28.3	14.15
Megacarpaeeae	23.4	87.75	43.88	21.02	70.2	35.1
Microlepidieae	15.9	59.63	29.81	14.28	47.7	23.85
Oreophytoneae	0.1	0.38	0.19	0.09	0.3	0.15
Physarieae	51.27	192.25	96.13	46.05	153.8	76.9
Schizopetaleae	0.37	1.38	0.69	0.33	1.1	0.55
Sisymbrieae	10.2	38.25	19.13	9.16	30.6	15.3
Smelowskieae	6.37	23.88	11.94	5.72	19.1	9.55
Stevenieae	13.47	50.5	25.25	12.1	40.4	20.2
Thelypodieae	5.4	20.25	10.13	4.85	16.2	8.1
Thlaspideae	10.5	39.38	19.69	9.43	31.5	15.75
Turritideae	18.97	71.13	35.56	17.04	56.9	28.45
Yinshanieae	20.57	77.13	38.56	18.47	61.7	30.85

Table 15 Tribal ages [T = (dS/r)/2E^{-6}] estimated with synonymous substitution rates (synonymous substitutions per site per generation) from previous reviews. Coloured rows highlight under (light grey) - or overestimation (grey) of calculations.

Tribes marked in darker grey are massive overestimations of divergence age, resulting from high dS values, which firstly proves that species assigned to those tribes hold numerous substitutions, suggesting high divergence among that groups and secondly, that the placing within the gene tree reconstructions exactly reproduces the difference among sequences.

If all marked tribes are omitted, plausible values for T, ranging roughly within results from prior studies, can be observed. Especially results between the fourth (WANG et al. 2007) and sixth synonymous substitution rate (HUANG et al. 2012) match the BEAST calculations (S4 and S5) rather well. These calculations still include the tribes with the inapplicable dS values.

8.6.2 Estimations of synonymous substitution rates utilising dS values and divergence ages

Table 14 suggests synonymous substitution rates (r) extrapolated using dS values and estimated divergence times from the BEAST analyses (see supplementary material S9) as established rates cited above.

Lineage-wise estimations of synonymous substitutions among the complete data sample surprisingly point to average values close to those reported previously (see **Table 15**). For lineage I an average of r = 1.02x10^{-8} was computed, while lineage II diverged at an even higher rate with r = 2.67x10^{-8} and within lineage III the synonymous substitution rate arranged well between the previous with r = 1.58x10^{-8}.

Subsuming the results, they are suggestive of a *chs* gene evolving at a more or less expected rate with a tendency to increase its amount of synonymous substitutions per site per year. But it has to be kept in mind that outliers, with well divergent sequence assembly, expeditiously influence the pace of such rates. Hence, the dataset has to be purged.

8.7 Synonymous substitution rates for outliers

Summarised results for the synonymous substitution rates (r) among tribes resulted in overestimations of some rates, meaning that a not representative accelerated rate was assumed. The most striking values were corrected by evaluation of the respective tribal datasets. The unexpected sequence species were detached as they are in authority for the deviating dS values. This immediately led to decelerated rates shown in table below. Regarding the Brassiceae, no re-calculations could be conducted as the tribe does not show any sign for duplications. Unfortunately the tribe is not well resolved and inscrutable concerning *chalcone synthase*. Hence, the original values were kept.

Tribe	**r=dS/2T**	**r=dS/2T rev**
Anchonieae	2.24x10^{-8}	6.43x10^{-9}
Arabideae	5.35x10^{-8}	4.68x10^{-8}
Brassiceae	9.11x10^{-8}	n/a
Cochlearieae	1.19x10^{-7}	3.58x10^{-8}
Dontostemoneae	2.77x10^{-8}	6.75x10^{-9}
Physarieae	3.08x10^{-8}	1.58x10^{-9}

Table 16. Excerpt of ***Table 14***. Original estimated synonymous substitution rates compared to revised (r = ds/2T rev) rates. Unexpected sequences for each tribe were removed, resulting in decreased accumulation of mutations.

After interchange of the synonymous substitution rates, an overall rate for *chalcone synthase* among the Brassicaceae was calculated, yielding to two results. The first rate is calculated with all tribes listed above and depicts a value of r = 1.26 x 10^{-8} while for estimations of the second rate the one-species tribes were eliminated. This led to a slightly accelerated rate of 1.53 x 10^{-8}. Both rates are well within previous suggested rates. Additionally values for r for lineage I, II and III were extrapolated and resulted in the following rates: r (lineage I) = 1.04 x 10^{-8}, while r (lineage II) = 1.99 x 10^{-8} and r (lineage III) = 9.9 x 10^{-9}.

As expected, lineage II displays the fastest rate. This is most likely due to the fact that this lineage, with six tribes, is the smallest and statistical outliers, like the Brassiceae, carry weight. Hence, the average value is shifted, suggesting that lineage II accumulated more mutations per site per year which, as a logical consequence, leads to the youngest estimations of the lineages. It was reasoned that the lineage diverged between 20.6 to 15 mya, due to given estimates.

8.8 Ruling out non-conformance

The (W)GVLFGFGPGLT *chalcone*- and *stilbene synthase* signature motif within the second exon has already been successfully applied on the dataset in order to detect peculiarities indicating sequence divergence. As this motif is highly conserved and present among all angiosperms, it is appropriate to scan reference data for that sequence.

Additional Sequences	**W**	**G**	**V**	**L**	**F**	**G**	**F**	**G**	**P**	**G**	**L**	**T**	**charge**	**polarity**
Bra017147			L	G	L	A					I		neutral	nonpol/pol
Bra000559			L	G	L	A					I		neutral	nonpol/pol
Bra011566			L	I	L	A					V		neutral	nonpol
Bra034658			L	I	L	A					V		neutral	nonpol
Aly_470071			L	G	L	A					I		neutral	nonpol/pol
Aly_490563			L	G	L	A					I		neutral	nonpol/pol
Aly_491172			L	I	L	A					V		neutral	nonpol
AT1G02050			L	G	L	A					I		neutral	nonpol/pol
AT4G00040			L	G	L	A					I		neutral	nonpol/pol
AT4G34850			L	I	L	A					V		neutral	nonpol
Thhalv10007831			L	G	L	A					I		neutral	nonpol/pol
Thhalv10028565			L	G	L	A					I		neutral	nonpol/pol
Thhalv10025417			L	I	L	A					V		neutral	nonpol
Carubv10003743			L	G	L	A					I		neutral	nonpol/pol
Carubv10007475			L	I	L	A					V		neutral	nonpol
EF643507_Gossypium hirsutum													neutral	nonpol
EU573212_Abelmosch. manihot													neutral	nonpol
FJ197128_Garcinia mangostana													neutral	nonpol
AF461105_Hyper. perforatum													neutral	nonpol
FJ887898_Citrus unshiu													neutral	nonpol
KC287084_Rhus_chinensis													neutral	nonpol
JF728822_Canarium_album													neutral	nonpol
EF103196_Aquilaria sinensis					L	S					F		neutral	nonpol/pol
NM_116221_Atha_STS			L	G	L	A					I		neutral	nonpol/pol
GU948866_Cgrandiflorum_STS			L	G	L	A					I		neutral	nonpol/pol
DQ518912_Lunularia_cruciata													neutral	nonpol

Table 17. A conserved motif at the carboxyl-terminus of the *chalcone synthase*. Each amino acid sequence from additional reference material was compared to the conserved pattern in the second exon. Sequences with GeneBank accessions were taken from NCBI, while the remaining data was generated via Phytozome or CLC workbench and, moreover, transcriptome data was added (nonpol = nonpolar, pol = polar).

To prove and compare the data, additional reference material was collected and constructed. As it has be proven that stilbene developed out of *chalcone synthase* several times (TROPF et al. 1995, TROPF et al. 1994) and that both enzymes differs only in two amino acid changes, an intensified search for similarities of STS (stilbene synthase) among the data seems advisable. As the resemblance is that high, it is suggested that erroneously stilbene or other closely related enzymes like *chalcone isomerase* (CHI) or other polyketide synthase enzymes (PKS) which are also involved in the flavonoid biosynthesis have been unwittingly been sequenced during lab work. In addition, the CHS/STS gene family among the angiosperms partly holds high numbers of family members (DURBIN et al. 1995, FLAVELL et al. 1998, VAN DER KROL et al. 1990), although this is not known for the Brassicaceae. Unknown gene family members, which derive from various duplication events, could easily disarrange the phylogenetic reconstructions and therefore any other analysis as well, especially if it is not assumed. In the majority of cases the genes of such families keep the same or a closely related biochemical function (DAUGHERTY et al. 2012).

Therefore two stilbene synthase enzyme sequences from *Arabidopsis thaliana* and *Capsella rubella* were taken from GeneBank (BENSON et al. 2009, SAYERS et al. 2009) (accession numbers are listed in the table). Phytozome v.9.1. (GOODSTEIN et al. 2012) is a comparative online platform holding a genome browser and tools that, like blast search or keyword search, deliver manifold options to compare and analyse sequence data. Sequences from this research with uncertain background or origin were blasted against available whole genome data to verify whether these genomes hold similar episodes within their DNA. 78 and 96% identity for the first and between 72% and 96% for the latter. *Thellungiella halophila* and *Capsella rubella* resulted in three and two sequences, with identities between 71% and 87% and 82% to 94%, which can all be viewed in table **Table 17**. All sequences were defined as *chs*-like. The *Sisymbrium irio* genome was downloaded from the BioProject PRJNA202979 available on NCBI (HAUDRY et al. 2013) and was loaded as blast databank, further procedure was the same like explained for phytozome.

Motif sequence changes in the CHS protein imply functional divergence, although these functions are not known, yet (WANG et al. 2007). The table depicts the amino acid replacements in the tribes reviewed in this chapter, which can be categorised as conservative or radical changes (HUGHES et al. 2000). Any change of category is counted as radical difference while a change within the category is evaluated as conservative. Concerning the charge of the amino acids, no radical categorical change could be observed, the neutral amino acids are changed to another aa neutral in its charge, while there were several changes in the polarity.

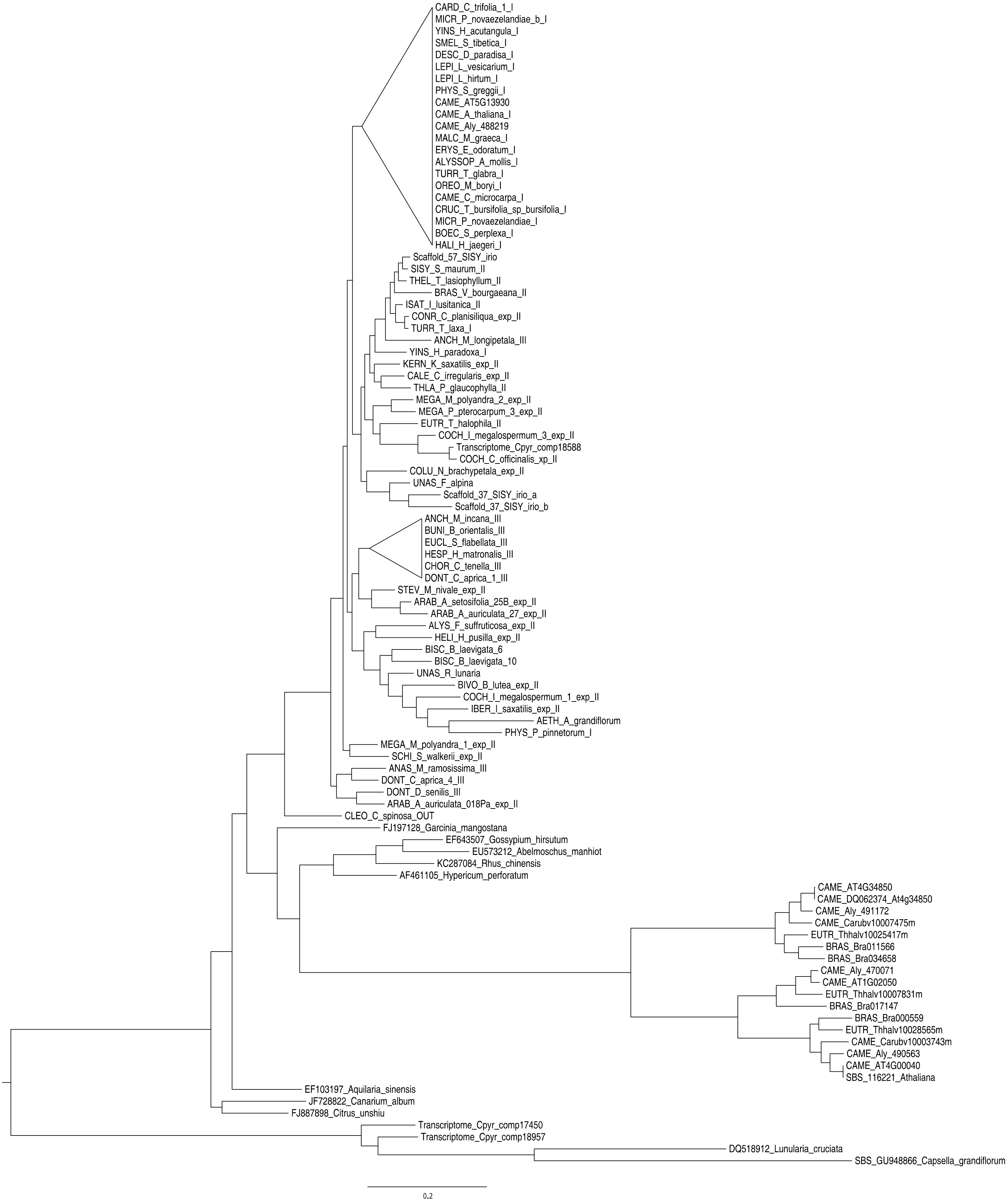


Figure 16. Phylogenetic reconstruction (ML) of the representative dataset with sequences from NCBI, CLC and Phytozome v.9.1., as well as transcriptome data form *Cochlearia pyrenaica*. Monophyletic lineages 1 (triangle top) and 3 (triangle below) are collapsed.

All changes were radical and only from nonpolar to polar. Most likely, an alteration in those amino acids results in a change of structure and therefore function. The affected sequences are all gathered in one group among that represented tree, most likely showing family members adequate to chromosome 1 in *Arabidopsis thaliana* as well as the *stilbene synthase* gene from the same species, which also shows a radical change.

The sister group to chromosome 1 is a group holding sequences from chromosome 4, does not exhibit any change, indicating that both groups (chromosome 1 and 4) carry different functions. It can be argued that the copies, which developed in parallel to those on chromosome 1 on *A. thaliana*, are more diverged than those on chromosome 4. The cladogram of the diverse sequences hints to the fact that chalcone synthase could either also be a multigene family in the Brassicaceae or the extra members are on their pass through to expulsion from the genome. The sequence material from diverse other angiosperms (used in BEAST estimations as well) and even one *Bryophytes* example from *Lunularia cruciate* also helped to arrange.

The back-cloth of that excursion to multigene families and other sequence analogue data could thoroughgoing illustrate that the DNA data which is investigated here, does supposedly not include any closely related genes as stilbene synthase or, moreover, resemble other *chs*-like DNA episodes from other chromosomes. Besides, it should be noted that chalcone synthase sequences, even from relatively far related organisms (*Bryophyta*) were still akin enough to unresistingly align and analyse. This attests how highly conserved this gene is.

8.9 Ancestral sequence reconstruction

The character mapping lead to 54 ancestral sequences estimated from the respective nodes (57-110) from a reduced file (see **Figure 16**) calculated by ML algorithm. This resulted in an alignment with sequence lengths between 1176 and 1185 base pairs, resembling the recent alignment lengths with a range from 1176 to 1188. The ancestral alignment depicts some gaps at the beginning of exon 1 analogue the modern exonic region. The more distantly related the sequences are, the more gaps within the constructed sequences are expected and vice versa.

The accuracy score for the DNA sequences, which is the mean of the probabilities of all the bases, displayed a minimum value of 0.9497 and delivers a good reflection of the overall accuracy of estimated ancestral sequences (HALL 2006). This means that every base has a chance of minimum 94.97% of being correct. The probability of the entire sequence correctness is the product of the most probable base (MPB) probabilities. The log of each probability is a relatively small negative value, higher than that of the complete tree. As the resulting sequences do display a minor amount of gaps resulting in an alignment of approximately the same length

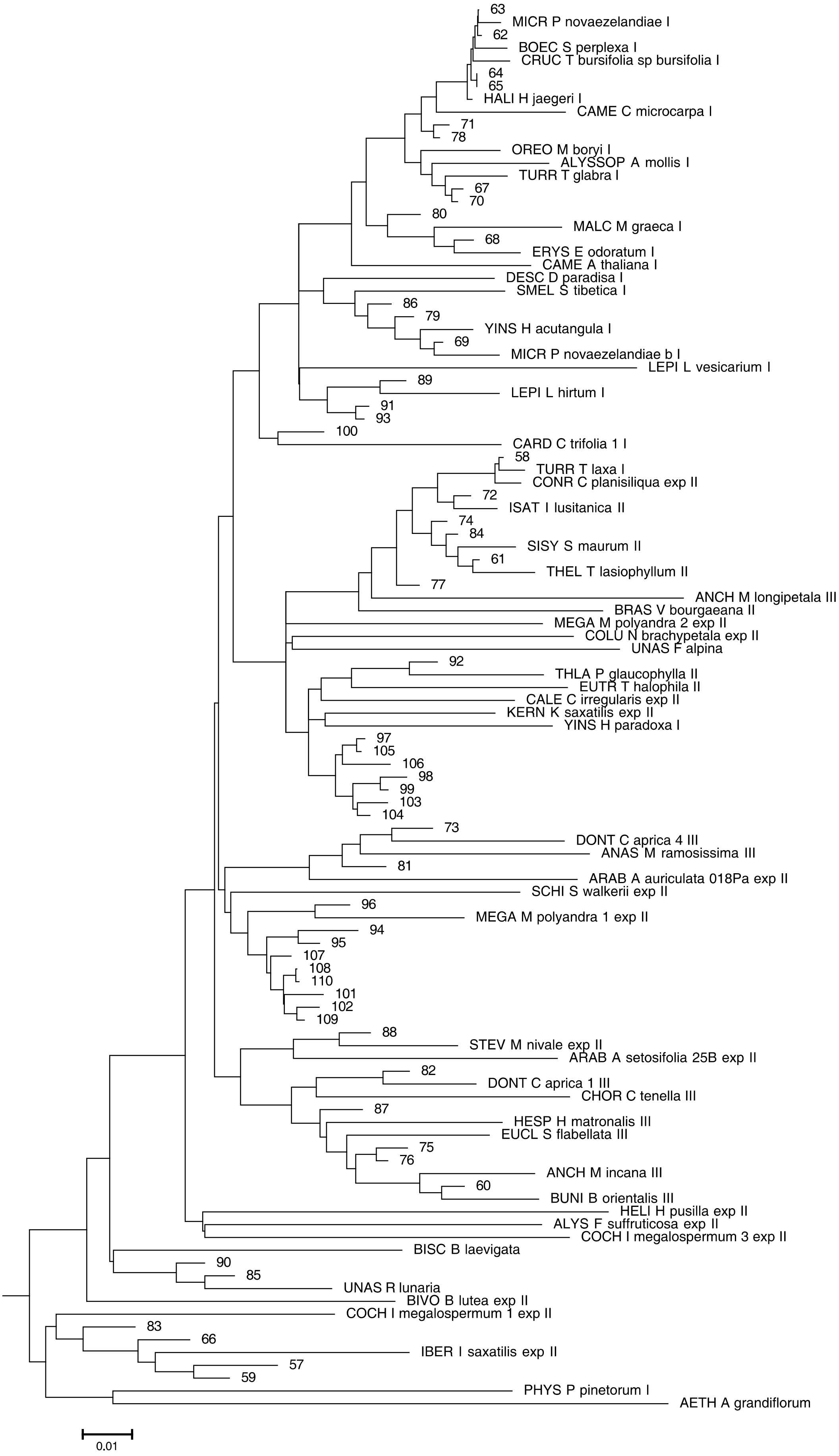


Figure 17. Estimated ancestral sequence reconstruction of *chalcone synthase* gene sequences conducted in MEGA5 (Tamura et al. 2011). 54 estimated ancestral sequences are depicted with the recent *chs* data employed in this study.

as the recent sequences it can be concluded that the gene is either highly conserved and/or the sequences diverged relatively recently. The resulting phylogenetic calculations furnished each dichotom node with an estimated ancestral sequence suggesting miscellaneous evolutionary trends.

In most cases it can be concluded, that the ancestor of two recent sequences is closer to one modern species sequence than to the other, indicating that either intermediate sequences, bridging the evolutionary distance, are not sampled or that one of the recent sequences accumulated more mutational steps, since they diverged, than the other. A peculiar result concerns *Aethionema grandiflorum* and the sequences clustering close. Their ancestral sequences seems to display a younger age than the respective modern sequence. This could underpin the suggestion of duplication events resulting in two gene copies, each. The data on hand indicates that in these examples both copies are maintained, at least in this temporal snapshot. These basal sequences seem to have heaped mutations, resulting in a still functional sequence with an evolutionary distance higher than that of the estimated ancestral sequence.

9 Discussion

The tribes of the mustard family are meanwhile all monophyletic, forming well supported clades, thanks to through research, especially during the last two decades e.g. (AL-SHEHBAZ et al. 2006, COUVREUR et al. 2010, KOCH et al. 2001, WARWICK et al. 2010). The tribal nomenclature therefore displays no longer an artificial system, but describe natural relationships between genera and species.

As the monophyly of tribes and even lineages has been proven e.g. (AL-SHEHBAZ et al. 2006, BAILEY et al. 2006, BEILSTEIN et al. 2008, KARL & KOCH 2013, KOCH et al. 2005, LIU et al. 2012, RESETNIK et al. 2013) via distinct approaches, the performance of *chs* has to be scrutinised. As indicated by KOCH et al. (2007), phylogenetic constructions based on a one-marker-sytems are limited in their value to establish robust phylogenies. However, *chs* was not employed to unravel phylogenies. As far as known, no comprehensive delineation of this nuclear encoded marker gene, comprising the majority of the accepted tribes, is available. Hence, eight tribes recognised within this study, which demonstrate feigned polyphyly, were investigated. Those divided systematic units do *not* question confirmed cladistic relationships, but rather pinpoint evolutionary events, gathered in the genomes of the taxa. This relatively short DNA unit is capable to hint at several divers occasions which will be discussed individually for the respective tribe.

9.1 Anastaticeae/Malcolmieae

This tribal is intricate due to the actuality that rearrangements concerning taxonomy and phylogeny are going on continuously. Based on the fact that the complete genus *Malcolmia* was assigned to the ANAS until this year and is now divided into four already existing or newly re-established tribes, and besides, the data at hand encloses three *Malcolmia* genera, assertions have to be done. *Malcolmia ramossisima* was kept within the ANAS but the genus name was replaced by Marcus-Kochia [(AL-SHEHBAZ et al. 2014) and D. German (pers. comm.)]. *Malcolmia maritima* and *Malcolmia graeca* (s.str. *Malcolmia*), together with other species, were excluded from the ANAS as they share a vast number of morphological and molecular data with the ERYS. Re-established MALC are a sister tribe to ERYS, which is in absolute congruence to the data introduced here (**Figure 8**) and moreover supported by a bootstrap value of 99%. Therefore the previous putative polyphyletic arrangement of ANAS within members of lineage I and III is hence futile and underpins the capability of the nuclear *chalcone synthase* to unravel phylogenetic challenges. From here on, Anastaticeae and Malcolmieae will be treated as two distinct tribes.

But there are still open issues concerning the tribe ANAS in the data at hand. *Cithareloma vernum* GQ983043 and *Malcolmia ramosissima* should be situated close to each other, as both are assigned to the tribe ANAS of lineage III. The former condition is fulfilled while the ANAS are the only tribe from that respective lineage that is not arranged among the group of lineage III, but only with one species from the tribe DONT (see discussion Dontostemoneae). So, both groupings are proven as existent and it is therefore suggested that the tribe ANAS sensu AL-SHEHBAZ et al. (2014), underwent at least a tribal-wise duplication event, if not even lineage-wise or even remaining from the alpha WGD, which resulted in a two times over appearance of the chalcone synthase gene among the genomes.

What is even more striking is the fact that within *M. ramosissima* sequences variability in the coding region settles between 92.48% and 96.03%, while the complete gene shows a similar overall identity of 93.86% to 96.76%. To make meaningful argumentation from that result, it is suggested that the tribe Anastaticeae underwent a separate polyploidisation event after the last WGD. The "wrong", meaning unexpected, gene tree arrangement of the lineage III assigned tribe is supposed to be the outcome from *At*-α while the intra-species (*M. ramosissima*) disparity is generated by a recent gene duplication event resulting in two paralogous copies of *chalcone synthase*. This incidence must have happened currently (less than 2 mya), as the amount of paralog-identity is not advanced enough to illustrate the loci at separated locations within gene tree arrangement. On the other hand, the coding region is diverse enough to indicate an even older duplication event. This puzzling outcome (coding and complete gene identity close to similar) maybe hints to concerted evolution, as the two paralogous copies are more closely related (compare gene tree arrangemenet) than to other orthologs from related species.

As a tendency to diploidisation is reported among the Brassicaceae (KASHKUSH et al. 2002, LYNCH & CONERY 2000, MA & GUSTAFSON 2005, WOLFE 2001), functional divergence is another manifest approach to explain the fate of the duplicated genes. Here it seems like the sets of duplicated genes underwent different processing concerning members of lineage III. Six (ANCH, EUCL, DONT, CHOR, HESP, BUNI) out of seven tribes (ANAS) kept supposedly only one of the *chs* copies on the same locus. This definitely advocates for orthologs of *chs* among those different species. Orthology, as well as paralogy are key concepts in evolutionary genomics. Orthologs are genes that derive from a vertical descent as they are related via speciation (FITCH 1970). Orthologs are homologs that derive from a single ancestral gene present in the last common ancestor of the compared species (KOONIN 2005).

Concerning the outlying tribe ANAS two scenarios can be imagined. Either representatives for tribe ANAS maintained both copies were the second could not be sequenced by mischance. Consequently, these sequences, resulting from a duplication event, would be defined as paralogs, displaying an additional locus, most likely even on another chromosome. The missing representative would consequently be the ortholog to the other lineage III species, as they are homologous sequences placed on the same locus complying the same function. The alternative hypothesis is, that the tribe ANAS already underwent the gene loss of the additional redundant copy, maybe even directly after polyploidisation, and resulted in the phylogeny at hand. A tangible reason for the maintenance of the paralog instead of the ortholog is no obvious fact and can hardly be explained without further investigations. As ANAS is arranged with different DONT species it is suggested that the first scenario is conceivable.

9.2 Anchonieae

The tribe ANCH is treated as inconspicuous and, to the best of knowledge, nothing concerning phylogenetic abnormalities has been discussed previously. *Matthiola incana*, is arranged within lineage III, were it was expected. It depicts a close relatedness to the tribe BUNI and illustrates the deepest node within the lineage. The more peculiar is the arrangement of *Matthiola longipetala*, which is nested in the middle of lineage II, separated by a comparatively long branch. A thoroughgoing explanation for the positioning is long-branch-attraction of the gene due to shared homoplasies. It can be eliminated that this arrangement is due to experimental or taxonomical lapses as both was double-checked. The branches within this clade are also supported with very high bootstrap values between 86 and 99 percent which support the reproducibility of that clustering. Moreover, lineage II is entirely present with six tribal representatives. Also within that group another sequence pair (*T. laxa* and *C. planisiliqua*) can be observed at the deepest node, suggesting that lineage II within *chs* phylogeny is not monophyletic.

An arguable hypothesis for the polyphyletic appearance of ANCH, although speculative, is, that this tribe as well, kept both *chs* sequences since the last WGD. As this *Matthiola* sequence is relatively diverged from the surrounding sequences, meaning that it has accumulated several mutations since the duplication, it can be proposed that *M. longipetala* indeed is a relict sequence carried within the genome since the duplication. Immediately after a polyploidisation event, both sequences are redundant meaning that they are identical and no preference can be predicted. As this appearance in the ANCH could only be witnessed within one species, this presumably is a still working gene on its way to non-functionalisation ending long-term in gene loss. However, it is striking that the mutational accumulation is obviously in progress, while the gene still keeps its function.

9.3 Camelineae

The tribe was found to be polyphyletic within *chs* illustrations, with one subclade comprising *Capsella bursa-pastoris*, *C. rubella* and *Camelina microcarpa*, while the other included all *Arabidopsis* species (*A. lyrata*, *A. thaliana*, *A. halleri*) employed within this study. The *Arabidopsis* clade seems to be more basal and depicts a group that acts like a sister tribe to tribes Turritideae, Alyssopsideae, Oreophytoneae, Camelineae (with exception of genus *Arabidopsis*), Halimolobeae, Boechereae, Microlepidieae A-copy sensu JOLY et al. (2009) and Crucihimalayeae, all members of lineage I. A similar placement and division of the Camelineae was observed by studies using the chloroplast gene *matK* (LIU et al. 2013), the phytochrome A phylogeny by BEILSTEIN et al. (2008) and another nuclear encoded marker, ITS (GERMAN et al. 2009). In a recent review by COUVREUR et al. (2010) employed a supermatrix analysis, resulting in similar findings concerning the split within the tribal phylogeny. The cleavage between both Camelineae groups is even wider within this *chs* study, which is moreover also underpinned by another chs study focusing on the tribe Cochlearieae (KOCH 2012). It is not only the tribes Boechereae and Halimolobeae which are nested within, but also Microlepidieae (A-copy) and Crucihimalayeae. The disagreement of this tribe suggests further investigations. It, moreover, can be suggested, based on several identical results listed above, that the Camelineae need supplemental revision or tribal affiliations and should be subdivided into at least two separate tribes.

9.4 Dontostemoneae

The tribe Dontostemoneae, rather of small dimensions, is restricted to central and eastern Africa and was only established in 2007 (AL-SHEHBAZ & WARWICK 2007). *Chalcone synthase* data from Dontostemoneae has not been employed very often until now. Previous research analysis from ZHAO et al. (2010) suggest that the tribe is arranged close to Hesperideae and Chorisporeae, supporting the findings at hand. As all of the named tribes belong to lineage III, the expected arrangement of *Clausia aprica* within that group seems not be surprising. Astonishingly, sequences from the review named above are nested within a second position among phylogenetic reconstructions, both arrangements supported with a 99 % bootstrap value. The question rises whether one group of sequences, containing *Dontostemon senilis*, *D. elegans*, *Clausia trichosepala* and *C. aprica* depicts the genuine placement or the group holding a second copy of *C. aprica* and all the other members from lineage III. On the other hand, the former group is also surrounded by another lineage III member, but only one (see **Figure 8**). More conspicuous is the fact that both placements are arranged within the close neighbourhood of ARAB species, which belong to the indistinct expanded lineage II. But, like mentioned above, the tribe ARAB is stated to have undergone an additional duplication event. Hence it is proposed that the DONT are affected by the same or a parallel incidence, indicating that either the duplicated sequences derive from the alpha whole genome duplication in the course of the rapid radiation event, as this historically seems to be the nearest encounter of both tribes concerning time flow. Or an independent duplication event, most likely close to the divergence of the DONT and lineage III, which is often discussed to be the oldest of age, having diverged between 35 and 28 mya (BEILSTEIN et al. 2010, COUVREUR et al. 2010). Independent from the exact duplication occurrence, it is proposed that the discussed tribe underwent an arbitrarily duplication event resulting in paralogous gene copies on two loci among the nuclear core genome which are still present and functional momentarily.

9.5 Megacarpaeeae

This Brassicaceae tribe is neither very widespread nor very well known. To the best of knowledge no chromosome counts of the species on hand, phylogenies or further thoroughgoing information are available. The two genera employed here depict no monophyletic resolution. *Megacarpaea polyandra* and *Pugionium pterocarpum* clusters at two positions within the *chs* reconstruction. The tribe is assigned to expanded lineage II and is therefore supposed to arrange among other genera and tribes from that lineage, potentially in a polytomic manner with a lack of resolution. Both species partly fulfil that expectations, although it is not explicitly obvious whether one of them is an outlier due to the fact that both arrange among other sequences from the same lineage. It is most likely that the *P. pterocarpum* position represents the actual placement, as two additional sequences of *M. polyandra* are arranged in a neighbouring position and depict a recent divergence from each other. Moreover, group eight, which also contains the other *M. polyandra* sequence, seems to be a receptacle for incongruous and debatable arrangements. Concerning the tribe's monophyly, it is suggested that a duplication event took place, most likely before the diversification of the lineages, what makes it verisimilar that these sequences even descend from the last whole genome duplication event. Further discussions regarding the double appearance of *M. polyandra* are postponed to the following chapter (see 12.4).

9.6 Physarieae

This tribe was also found to depict a polyphyletic arrangement concerning *chs* phylogeny, although it was several times depicted as monophyletic (BAILEY et al. 2006, FUENTES-SORIANO & AL-SHEHBAZ 2013). The tribe belongs to lineage I, where one of the genera employed, arranged. *Synthlipsis greggii*, recently defined as part of the DDNLS clade by (FUENTES-SORIANO & AL-SHEHBAZ 2013) nested, as expected, within lineage I, with a bootstrap support of 69%, in a more basal position with a suggested close relationship to the tribe Descurainieae, one node more outside, and sister to Malcolmieae and Erysimeae. This placement has often been verified (BAILEY et al. 2006, FUENTES-SORIANO & AL-SHEHBAZ 2013, KOCH et al. 2007) by several marker systems. But a second grouping of members of that tribe can be found at a position not only outside the lineage but also outside every defined lineage for the Brassicaceae at a very basal situation. The clones discussed here are both from the genus *Physaria* (*P. fendleri and P. pinetorum*), which are depicted as members of the PP (*Paysonia* and *Physaria*) clade (FUENTES-SORIANO & AL-SHEHBAZ 2013) and are known, especially *Physaria*, for their large C-values (up to C = 2.34) in combination with a relatively low chromosome number (up to 2n = 8). In *Physaria bellii* for example, a 2C content of 4.7 and a chromosome number of n = 8 was detected by LYSAK & LEXER (2006). It was therefore reasoned that the taxa showing this pattern underwent a polyploidisation event followed by subsequent downsizing of the chromosome number by fractionation which is accompanied by chromosome rearrangements. Hence, at least the genus *Physaria* is suggested to be of paleopolyploid origin (LIHOVA et al. 2006, LYSAK et al. 2009, LYSAK et al. 2005). But, expansion of the chromosome size and the genome size joined by decreasing chromosome numbers are characteristic for the whole tribe of the Physarieae which supports the suggestion of a paleopolyploid origin of the complete tribe. This finding should help explaining the hypothesis for the pattern observed of the genus *Physaria* within the *chs* phylogeny consequently. Both the split of the tribe and the very basal arrangement of the two species, also supported by JOLY et al. (2009), indicates that the Physarieae underwent a past polyploidisation event resulting in duplicated genomes, each carrying a copy of the chalcone synthase. This, most likely, was followed by loss of one gene, as there is only one copy for each species available, gradually transforming the polyploid into a quasi-diploid genome (MANDAKOVA et al. 2010). It can be ruled out that the additional copies are indeed present and could simply not be sampled by the cloning procedure as several reviews depict the exactly identical situation concerning the tribe Physarieae, especially the genus *Physaria* (JOLY et al. 2009, MANDAKOVA et al. 2010). This approves the argumentation that, after the last whole genome duplication event, the PP clade, or at least the genus *Physaria* kept one of the duplicated *chs* copies while the DDNLS group maintained the other copy and silenced the redundant gene. It is well supported via whole genome information that large proportions of duplicated genes are eliminated from the genome over time (BLANC & WOLFE 2004).

9.7 Turritideae

Within this tribe another polyphyletic arrangement could be observed. Between the two species, *Turritis laxa* and *Turritis glabra*, an identity among the coding regions of only 55 respectively 82 percent could be observed. It is thus not astonishing that the species arrange at more distant positions to each other, what actually does not purge the reason for that distinction. Especially the positioning of both species is exceptional. *T. glabra* arranges, as anticipated, among highly supported lineage I, while *T. laxa*, supported by 99 percent bootstrap value, both in the NJ as well as in the MP reconstruction, echoes with CONR as deep node within lineage II. Blast results also support the concordance of the sequences from *T. laxa* and *C. planisiliqua*, although a close relatedness can be expulsed, as both are even assigned to dissimilar lineages.

9.8 Yinshanieae

This tribe is represented by two species, *Hiliella paradoxa* and *Yinshania acutangula*. The *Yinshania* species are all diploid, based on x = 6 (7). While *Yinshania acutangula,* which was utilised here, is mostly reported as one of the exceptions with 2n = 2x = 14 (Zhang, Y.

1995/1996, see reference in BrassiBase, cytogenetic tool), while KOCH (2000) suggests 2n = 12 as the ancestral state. However, all *Yinshania* species are described as diploids. Concerning the genus *Hilliella*, which is reported as polyploid, chromosome counts suggest a hexaploid state (AL-SHEHBAZ et al. 1998), with 2n = 6x = 42, based on x = 7, indicating an ancient genome triplication via autopolyploidy or allopolyploidy (WOLFE 2001).

Although this tribe has not been extensively reviewed, the state as polyploidy, at least in the genus *Hilliella* requires further regard.

As polyploidy is discussed as the primary mechanism for generated redundancy (WENDEL 2000), it has long been recognised as the most prominent speciation process (LEWIS 1979, SOLTIS et al. 1993, SOLTIS & SOLTIS 2000). Polyploid evolution in plants is a more dynamic process were many angiosperm genomes have reportedly experienced several rounds of polyploidisation at various times in their history and had later become diploid again. This defines the respective genomes as paleopolyploid, by means of sequence divergence between the duplicated chromosomes (WOLFE 2001). The modern angiosperm genome is hence characterised by a series of ancient as well as more recent polyploidisation events interspersed by rounds of diploidisation, superimposing the traces. Therefore it cannot reliably be illuminated whether the polyploidisation involved the merger of two differentiated genomes (allopolyploidisation), a simple doubling of the own genome (autopolyploidisation) or maybe even something in between, like segmental allopolyploidisation (JACKSON & CASEY 1979, MASTERSON 1994, SOLTIS et al. 1993).

It is argued that duplicated homoeologous copies of allopolyploids (chromosomes, segments or genes), as contributed by different donor taxa, result in descendant sequences (polyploidy) which are expected to arrange in a phylogenetic sister position to their diploid counterparts (WENDEL 2000) rather to each other (compare MICR **Figure 3**). Applied on the YINS this means that the hexaploid *Hilliella* would be expected to cluster close to the orthologous diploid taxa it derived from. As those are most likely not sampled, the arrangement apart from *Yinshania* corresponds the expectations.

This hypothesis of independent evolution is only assignable in case the genome was not affected by evolutionary events such as concerted evolution, loss of one homoeologous copy or other interaction among homoeologous sequences (WENDEL & DOYLE 1998, WENDEL 2000). An augmented sample size of that tribe could help to resolve the arrangement.

Results from this chapter have shown that the division of the discussed tribes is not due to polyphyly, but rather the result of distinct evolutionary events which can be traced awithin the genome. Even a small piece of nuclear encoded DNA thus demonstrates to be capable of delivering evidence for ancient impact on the species' development.

All clearly duplicated unexpected *chs* copies (see asterisks **Figure 3**) have therefore been removed for further investigations on the data.

However, the previous chapter suggests further ambiguous sequence arrangements within the phylogenies (see filled colour-coded circles in **Figure 3**), which could not be resolved with the exclusion of duplicated loci within the mentioned tribes. Results at hand, e.g. **Figure 10**, **Table 12** or **Table 15** suggest that other tribes do also hold proof for evolutionary deviations. Thus, tribes Arabideae, Cochlearieae, Dontostemoneae, Megacarpaeeae and Microlepidieae will be further investigated in order to detect distinctions which show proof for evolutionary patterns within the mustards.

Part 3. Done Twice is not Always Better - Duplicated Gene Pairs

Each tribe which contained two groups of sequences supposed to arrange within one monophyletic group was analysed in chapter two. To continue analysis on the *chalcone synthase* data without further detraction, the unexpected sequences were removed from the record to adjust the data set. The following chapter employs the remaining record (see colour-coded circles in **Figure 3**) in order to descry the impact of the excluded data on the expected data, as well as the characteristics of the remaining DNA sequence material.

10 Material and methods

10.1 Comparative phylogenetic reconstructions and analyses

Like previously deployed, different phylogenetic analysis methods have been applied on the data to assure the solidity and reproducibility. Therefore NJ and MP as well as ML analyses were utilised to compare the phylogenetic reconstructions with those from prior analysis. Bioinformatics programmes and settings are identical compared to previous applications.

10.2 Divergence time estimates

Divergence times have alternatively been generated using the Timetree (RelTime-ML) method (TAMURA et al. 2012). Divergence times for all branching points were calculated resting upon a ML method based on the JTT (Jones-Taylor-Thornton) matrix-based model (JONES et al. 1992). This algorithm was developed for larger phylogenetic datasets without assuming a specific model for lineage rate variation or specification of any clock. The analysis involved only the 72 amino acid sequences described above, depicting putative duplicated copies among 36 species.

10.3 Sequence analysis

10.3.1 Intra-pair identities

Scores for amino acid identity and similarity was retrieved from SIAS (Sequence identity and similarity) server. Inter-and intra-tribal and sequences from duplicated pairs were compared to find evidence for allelic variance or duplicated loci.

10.3.2 Sequence analysis of duplicated gene pairs

Evolver is a collection of programmes belonging to the software package PAML (YANG 1997) designed to reconstruct the evolution of the nucleotide sequence. The evolution of a representative gene or genome of a species compared with its generation time is simulated. To determine the relationship between DNA and amino acid identity relative to a model of strictly neutral evolution, simulated pairs of genes were created, also under neutrality. For simulations dN/dS was set to 1.0, the transition and transversion parameter was set to 3.0, while the codon frequency matrix was based on tabulated frequencies (ZHANG et al. 2002) from duplicated gene pairs (see supplementary material S11). Percent identity of DNA and protein sequences were calculated. Otherwise, the default parameters were used, setting ω to 0.3 (= dN/dS) and κ (=transition/transversion) to 5.0, as well as a uniform distribution of rate variation among the codon sites. For all simulated data, percent identity was calculated from both the DNA sequences and the translated amino acid sequences.

10.3.3 Sequence evolution in duplicated genes

Five tribes holding duplications of species could be detected within the dataset, which are to be found within the Arabideae, Cochlearieae, Dontostemoneae, Megacarpaeeae and Microlepidieae. Together 36 gene pairs could be discovered, most likely from different duplication events.

To test on the gene pairs, PAML’s (Phylogenetic Analysis by Maximum Likelihood) two models of evolution, 0 and 1 (YANG 1997), were initially tested. Model 0 forces every branch to be equal length, which implies an evolution at clock-like rates, whereas model 1 leaves the length of the branches unconstrained so that each sequence is free to evolve at their own rate.

10.3.3.1 Identification of gene regions

The coding (exon 1 and 2) and non-coding parts (promoter and intron) of the duplicated 36 gene pairs were torn apart in their respective component regions to compare the inter- and intra-paralog arrangements, identities and distinctions.

10.3.4 Genomic sequence blast

Started with the genome of *Arabidopsis thaliana*, several complete plant genomes have been sequenced during the last years, whereof a relevant number also belongs to the Brassicaceae. A certain number of these are publicly forthcoming others are only available via data bases and cannot be downloaded, not at least due to their immense size, which partly is up to 10 GB. Therefore miscellaneous genome browser like CoGe (LYONS & FREELING 2008), TAIR (LAMESCH et al. 2012) and EnsemblGenomes (KERSEY et al. 2014) and workbenches like

UGENE v.1.14.0 (OKONECHNIKOV et al. 2012) and CLC Genomics Workbench v.7.0.3 (http://www.clcbio.com) were strongly employed. Own blast pools had to be compiled as the number of genomes offering blast functions with the respective modulation functions for self-made data are limited. In the majority of cases only up to a maximum of five Brassicaceae genomes are available which can often only be searched for known regions or genes and not against imported data. All species duplicates were individually blasted against each of the provided genomes in the respective tool.

10.4 Synonymous substitution rates

Analysis of synonymous substitution rates were estimated, which equal the number of substitutions per synonymous site in a coding sequence. Synonymous substitution are nucleotide changes that do not alter the amino acid encoded and are therefore assumed to accumulate in a neutral manner - not under selection - at a constant rate that is similar to the background mutation rate. Substitutions at synonymous sites, being selectively neutral, should evolve at a rate similar to the mutation rate (LYNCH & FORCE 2000) and can thus be used to estimate the age of homolog divergence. Higher Ks values correspond to longer periods of time since the original duplication event. Therefore a Ks value higher than 1 results from the fact that a site can change over and over again during a longer evolutionary time. Ka and Ks between the duplicated gene pairs were estimated by the ML method implemented in PAML (YANG 1997). Afterwards a relative rate test (RRT) was utilised to ω values different from 1.

10.4.1 Relative rate test (Tajima's D)

This test, also known as Tajima's D (TAJIMA 1993), is implied in MEGA 5 and tests the hypothesis whether a clockwise rate, meaning a constant rate of molecular evolution, can be applied on two samples. Pairs of duplicated genes, each with an outgroup, are built and their differences in configurations are counted. The RRT uses a likelihood ratio (LR) statistic to test the null hypothesis. H_0 claims that the observed number of sites in both sequences are the same, irrespective of the model of substitution and the respective rate, meaning that two sequences evolve at equal rates (MUSE & WEIR 1992).

11 Results

11.1 Comparative phylogenetic reconstructions and analyses

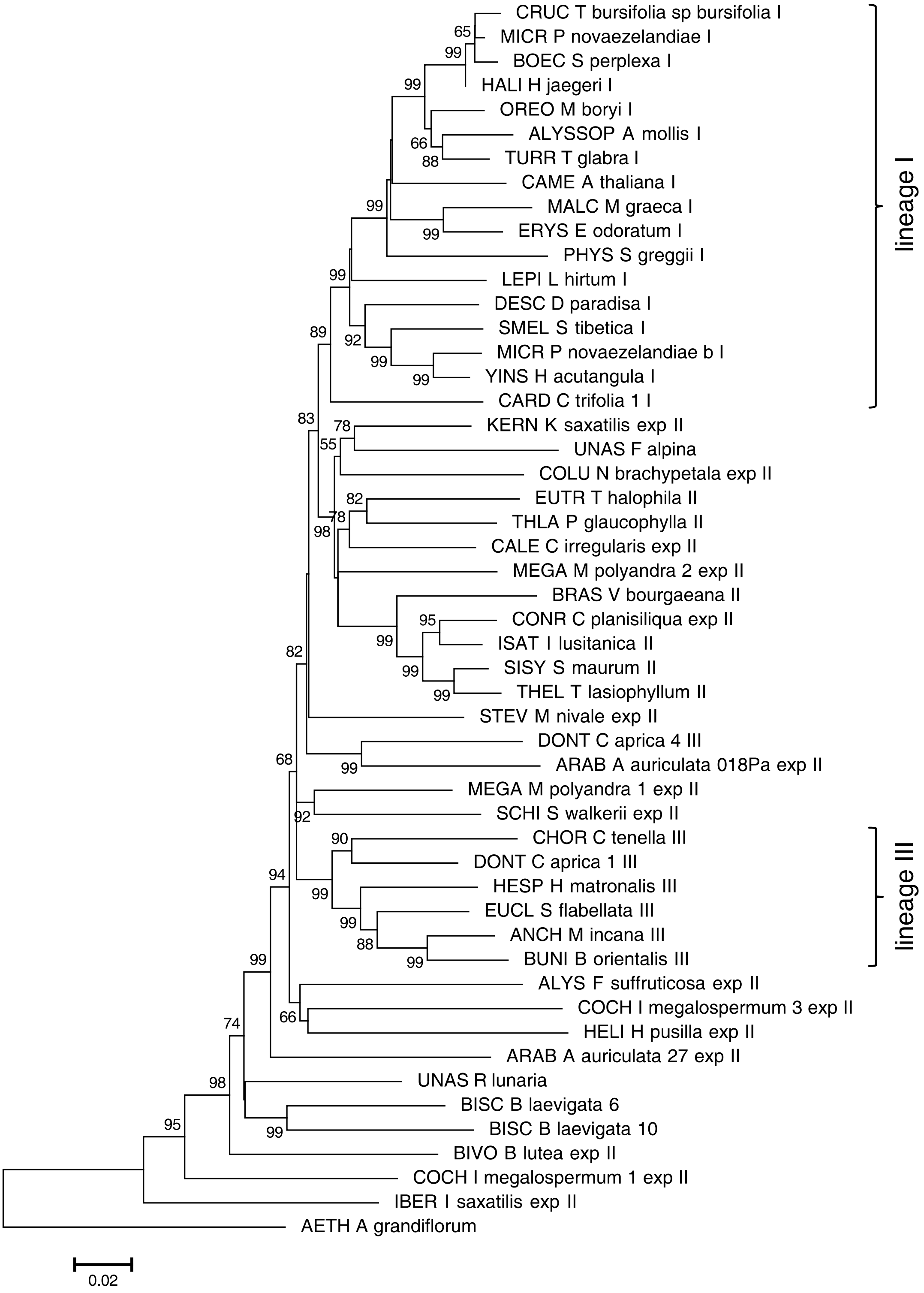


Figure 18. Phylogenetic reconstruction of the *chalcone synthase* gene displaying the adjusted data set (unexpected *chs* group was removed). Bootstrap support above 50%, of NJ are plotted next to the respective node. Lineage I and lineage III are displayed as monophyletic clades. While lineage II, as well as expanded lineage II contain additional *chs* sequences from other lineages. MP, as well as ML trees can be viewed in the appendix (S23 and S24).

The evolutionary history was inferred using the neighbour joining method which resulted in a well resolved illustration of the remaining data, with high bootstrap support at the majority of branches. The optimal tree with the sum of branch length = 2.2622 is shown with a topology in expected congruence to previous reconstructions. Lineage I is again well supported with 99%, as well as lineage III. This reassures the monophyly of those two lineages, although both hold sequences which do not cluster in the expected position. The majority of tree information equals its progenitor in chapter 2, although a set of disruptive data has been removed. Most of the tribes discussed do now display a monophyletic origin (YINS, TURR, PHYS, DONT, CAME, ANCH and MALC). But the remaining tribes, regardless, do not accumulate at one position. The affected tribes, not conspicuous yet, are Cochlearieae, Arabideae and Microlepidieae, while the already investigated ones are Dontostemoneae and Megacarpaeeae. While chapter 2 was engaged with DNA sequences suggesting a polyphyletic origin for a respective tribe, reasoned due to split groups not containing the same species, here tribes are highlighted which depict assigned species at two arrangements within the phylogenies. Exactly the same sequences are nested into two surroundings. It has to be emphasised that all sequences from one species derive from the same individuals. So, DONT and MEGA are investigated in both chapters as they fulfil both conditions. These five sequence doublets (*M. novaezelandiae*, *M. polyandra*, *C. aprica*, *I. megalospermum* and *A. auriculata*), displayed in the NJ genetree, are representatives for an additional 31 pairs found in the data set.

11.1.1 Divergence time estimates

The timetree analysis, conducted in MEGA6 (TAMURA et al. 2013), under the JTT+G model results in a rooted tree with an estimated SBL (sum of branch length) of 1.8114 and resulting in a tree log likelihood of -40988.93. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories: 0.1558, 0.4330, 0.7609, 1.2342, 2.4160 plus G, parameter = 1.2835). This alternative divergence time estimation approach was only applied on the gene pairs discussed here, not on the adjusted data set.

The topology depicted in **Figure 18** is in absolute congruence with estimations applied on BEAST for the same reduced dataset. Divergence times suggested within the phylogenetic reconstruction above indicate an ancient duplication event dated earlier than 35.14 mya (exact extrapolation for tmrca not available) whαmost likely is in coincidence with the latest WGD. What is highly demonstrative is the depiction of the two groups following the duplication event. The drafted comic **Figure 20** (A) shows the same data, depicted FigTree v1.4.0 (RAMBAUT 2012), were the clades of interest are accented and the remaining sequence pairs are collapsed to a small triangle at the top of the figure. COCH and MEGA exhibit the same pattern after the duplication suggesting, that the repeat event happened previous to the speciation.

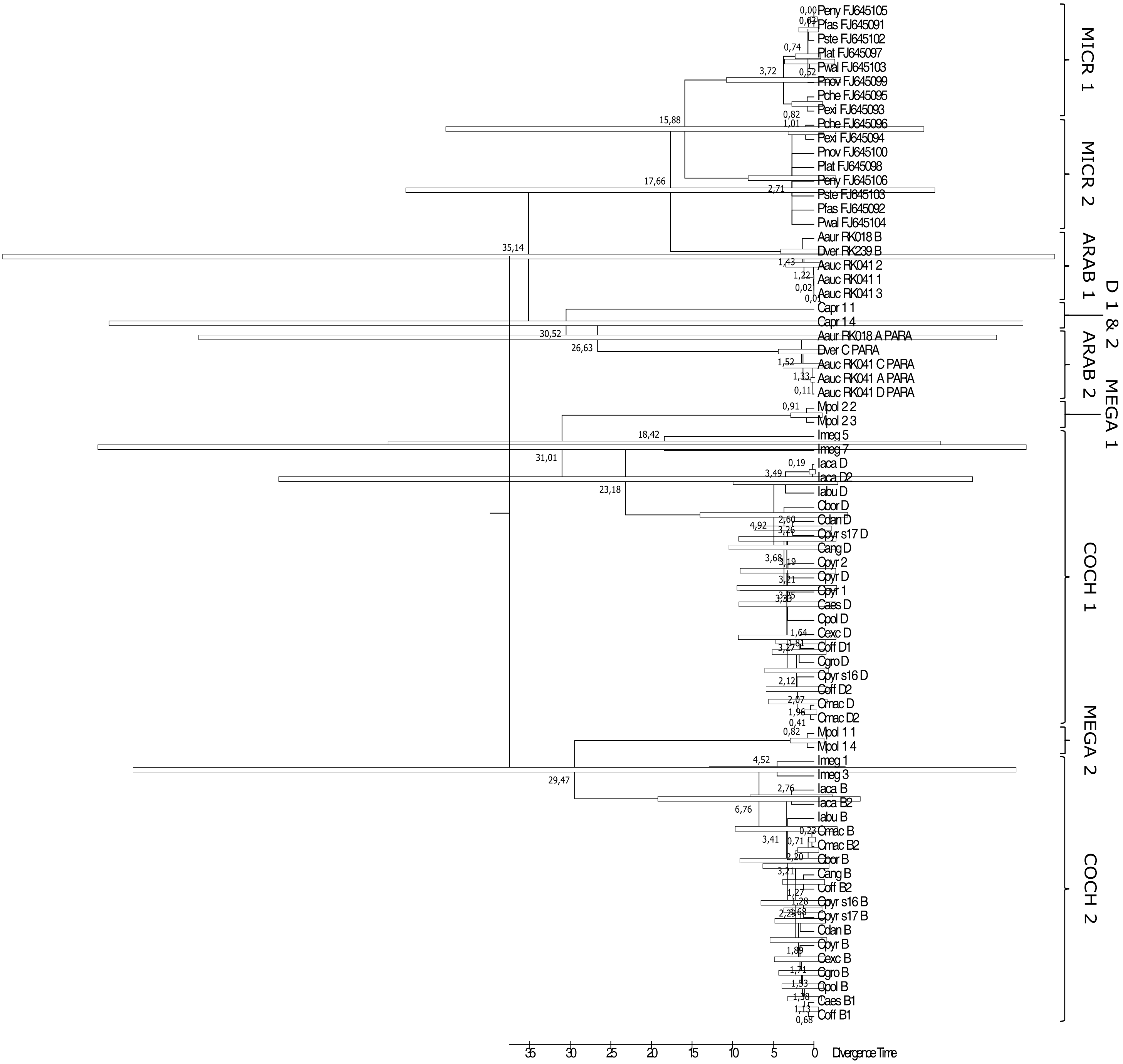


Figure 19. Divergence time estimates of duplicated *chs* genes among the Brassicaceae. Tribe 1 groups (e.g. COCH 1) contain all sequences which result in an expected grouping, while group 2 tribes (e.g. COCH 2) gather all sequences which arrange at unexpected positions. The bars around each node represent the 95% confidence intervals, using the method described in Tamura et al. (TAMURA et al. 2013). The tree is drawn to scale, with branch lengths measured in the relative number of substitutions per site.

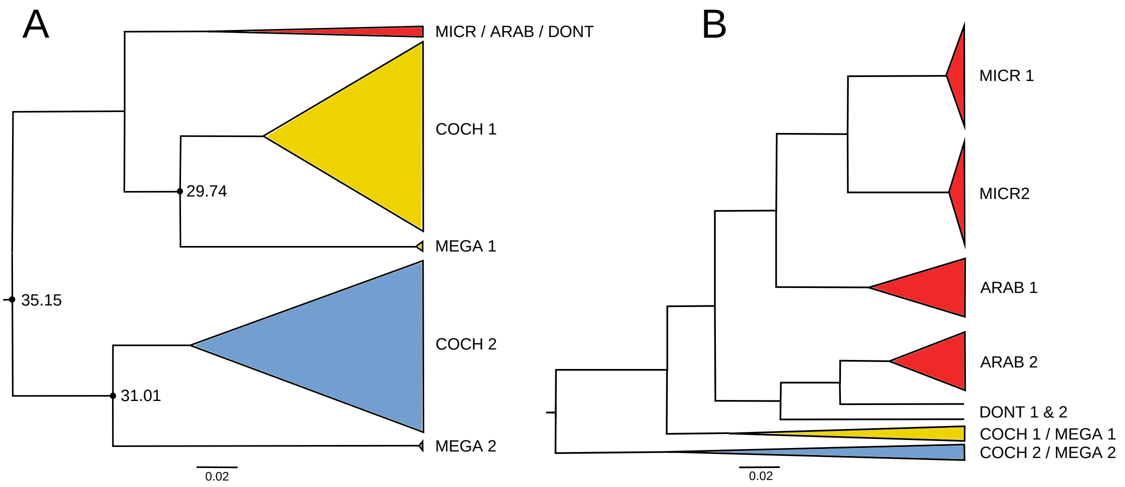


Figure 20. Simplified diagram of gene tree from *chalcone synthase* duplicated loci from the figure above. (A)The lower part of the tree is accented suggesting an older duplication event, with the upper part collapsed. (B) The upper part is magnified to display further additional polyploidisation events while the lower part is collapsed.

The root of the tree symbolises the duplication event of the most recent common ancestor. So, the duplication event precedes the speciation at least within the discussed tribes. It is also very striking that both ample triangles (see tree A), representing the tribe COCH, do show the diversification of the genera *Ionopsidium* and *Cochlearia* (for exact nodes see **Figure 19** or supplementary material S4-9) 6.76 mya (COCH 2) and 4.92 mya (COCH 1), after the divergence of MEGA, which was estimated 31.01 and 29.47 mya. The clade holding the paralogous duplicates evolved a short amount of time previous to the orthologous group.

In (B) the upper part of the tree is magnified and argues for further small-scale duplication events among several tribes (ARAB, DONT, MICR). The first node indicating divergence for those three tribes, also depicts an old duplication, estimated around 35 mya, separating duplicated tribe ARAB, what precedes an earlier assumed polyploidisation event expected at the basis of the tribe. It was hypothesised that the tribes ARAB and STEV, which are in a sister relationship, depict a duplication event concerning the common ancestor of those tribes (R. Schmickl and R. Karl, pers. comm.). The phylogenetic reconstruction suggests that after that doubling event tribe DONT proceeded with a tribe- or even genus- or species-internal polyploidisation, suggested by neighbouring arrangement of both *C. aprica* copies among that illustration. Anyway, this polyploidisation event is younger than the duplication of ARAB. Consequently, it even can be expected that the ARAB duplication equals the alpha WGD, as well.

Tribes DONT and MICR illustrate more recent doubling events. This hypothesis is supported for *Pachycladon* by JOLY et al. (2009) where a genus related hybridisation, an allopolyploidisation, was affirmed. The only difference can be applied to the suggested age of that respective event, which was dated 15.06 ± 6.38 mya for CHS and 8.18 ± 4.37 for the average of all five markers applied (CAD5, CHS, MS, MtN21 and PRK). Subsequently, the origin of the genus' radiation would be located in the Pleistocene between 1.6-0.8 mya (JOLY et al. 2009). However, the data calculated in this thesis suggests that the polyploidisation event has taken place 15.88 mya with the radiation of the genus between 3.72 and 2.71 mya, indicating a shift from the Pleistocene (2.588-0.012 mya) to the Pleiocene (5.332-2.588 mya) for the averaged data. In regard to the CHS data, results are in nearly perfect congruence, facilitating the estimations at hand.

11.2 Sequence analysis

11.2.1 Intra-pair identities

To check on the identities and similarities among and between the pairs, the SIAS sequences identities and similarities server (SIAS) was retrieved (RECHE 2008). For each affected of the five tribes, an intertribal-identity was calculated, including all genera from the respective tribe. Additionally, an overall-duplicates-value was estimated, displaying the similarity within all affected sequences, and, besides, values for the consistencies within each pair were gained. For those tribes, displaying more than one pair of doubles, the intra-copy identities were compared as well.

Tribe	all sequences	all pairs	range pairs	intra-copy exp	intra-copy unexp
ARAB	17.75-99.83	87.05-99.75	79.8-89.35	84.28-99.75	91.68-99.66
COCH	31,75-98.75	62.01-98.75	63.85-83.12	58.68-97.75	39.65-95.92
MEGA	88.78-99.83	89.19-99.83	89.19-89.6	99.83	99.58
DONT	71.1-99.3	89.34-99.03	89.34	n/a	n/a
MICR	87.53-99.33	87.53-99.33	87.47-91.78	97.33-99.91	95.92-100

Table 18. Sequence identities and similarities between and within duplicated gene pairs. All values are given in %. (un)exp = (un)expected, n/a = not available.

All surveyed tribes display a very low (17.75%) till moderate (88.78%) identity within each intra-tribal identity search. This was expected due to the actuality that, especially ARAB and COCH, are huge tribes each containing more than 70 sequences from several genera. This, in all probability, results in lower identity values than comparisons among a smaller amount of genera, like in MEGA or DONT. The image of all DNA sequence pairs from one tribe shows a range of which the end is always close to 100%, as expected and unexpected copies are grouped together. The range of the pairs therefore displays lower values, especially in the tribe COCH, with the lowest sequence identity of one pair with 63.85%. The most interesting and meaningful values are the intra-copy comparisons, although no clear conclusion can be drawn from this. In COCH, MICR and MEGA, the sequences that arrange at the expected positions in the phylogeny do display the higher inter-group identity, what seems to be manifest, while the values from the unexpected positions are lower. In ARAB it is vice versa, meaning that the duplicated sequences in the Arabideae share more identity within that group, suggesting a recent speciation. Note that DONT do not display some of the values as this tribes only exhibit one doublet and that MICR contains solely one genus, *Pachycladon*, of an allopolyploid origin. This weights the determination of the expected and unexpected group, as, besides, both bundles of pairs accumulate within lineage I. It cannot reliably be ascertained which group has to be removed as the additional copied locus. The same holds true for MEGA, as both *M. polyandra* sequences arrange well within other members from the expanded lineage II, which complicates the decision on which one has to be removed for further investigations.

11.2.2 Sequence analysis of duplicated gene pairs

From all 36 gene pairs, sequence identity of DNA ranged from 28.59% to 91.87% and amino acid identity ranged from 6.34% to 96.7%, while the entire gene pairs regions depict a dS range from 1.3201 to 3.323 synonymous substitutions per synonymous site.

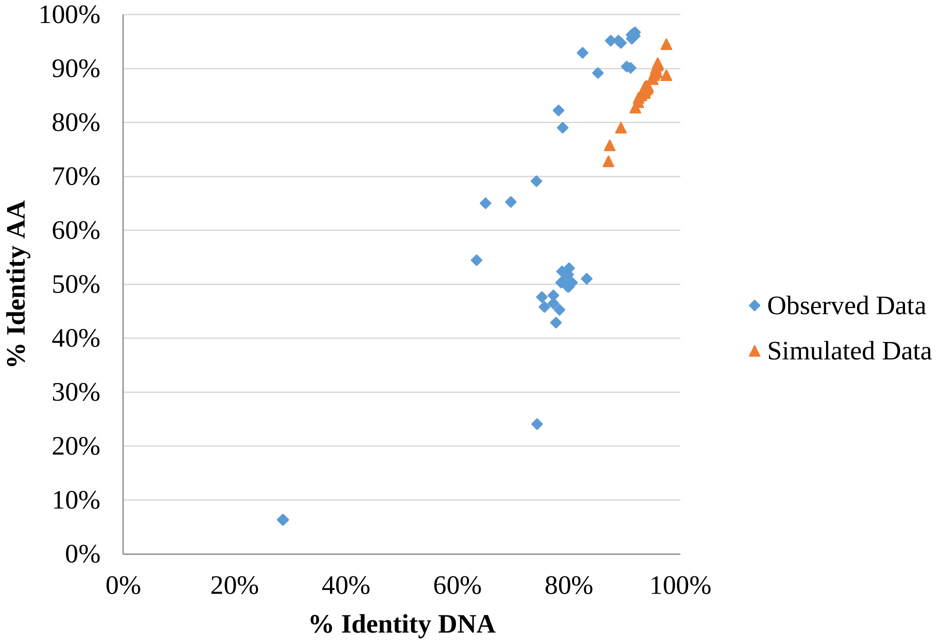


Figure 21. The relationship between DNA sequence identity and amino acid identity for all 36 putative duplicated gene pairs. Simulated data is represented via orange triangles, while the observed data is depicted by blue rhomb.

The simulated data, like expected, describes a more or less linear progress not absolutely close to a line through the origin. This devolution would be expected in case DNA and protein identity are always equal. This can result from a balanced frequency of radical and conservative base exchanges within DNA sequences. The simulations, however, indicate that DNA sequence identity was always above aa identity under the neutral model.

Surprisingly, a split result and no compact linear order can be recognised in the diagram concerning observed data. In prior research it was recommended that gene pairs within one locus exhibit a similar range of both DNA and protein identities to the entire region (ZHANG et al. 2002). This suggests that two loci are depicted, in case the two outliers are neglected. The first locus, described by the scatterplot below the imagined line, drawn by the simulated data, mostly contains genes from the tribe Cochlearieae. The second group, gathered above the simulated data points contains sequences with higher DNA and aa identity like all *Pachylcladon* sequences. Over the complete genes, DNA sequence identity was always higher than aa identity with exception of two pairs (Cpyr_s17_B and Cpyr_s17_D, Dver_RK239_B and Dver_C_PARA), where amino acid exceeded DNA identity. But, compared to the simulated data, one group (locus 1) displays lower, while the second group (locus 2), exhibits higher identity values. From this it can be reasoned that selective constraint appears to slow down the rate of nonsynonymous substitutions for those gene pairs with an amino acid identity above the simulated data (like MICR). This, in turn, results in an accelerated synonymous substitution rate, which can be viewed in **Table 21**, while those pairs with an observed amino acid identity below the simulate ones, consequently depict a lower rate. Rate variation among the duplicated gene pairs can be reasoned.

11.3 Identifying gene regions

11.3.1 Promoter

The promoter region within this small data set of 72 sequences is only available for a minority of them. This is mostly due to the fact that either alternative primers have been applied on the data, placed at a position only within exon 1 or applied gene bank accessions simply did not depict the complete gene. For these gene pairs sequences, were primers are analysable, they varied mostly to a significant degree. In MICR, for instance, two types of promoter regions could be detected, which are in absolute congruence to the two recognised loci on each *Pachylcladon* species. In other words: Promoter region type a always belongs to locus type a, resulting in two homogeneously groups with high intra-group identities (complete gene) of both above 95%, while the inter-group identities are around 10% lower. As it has been proven that each of the species' genome holds both loci (JOLY et al. 2009), these *chalcone synthase* copies are paralogous copies, which derived from a duplication (allopolyploidisation) event. A comparable situation concerning the promoter region can be observed in the DNA sequences from *M. polyandra* (MEGA). There are two types of promoter regions varying immensely in length and content, as displayed in **Figure 22**.

As previously suggested in chapter 1, the variability within non-coding regions can be correlated with evolutionary constraint.

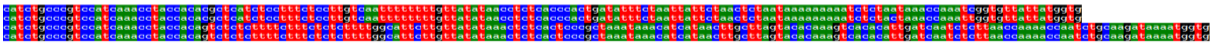


Figure 22. Scetch of nucleotide promoter region from *chs* of *Megacarpaea polyandra*. Base composition is colour-coded (red = T, blue = C, green = A, black = G).

Therefore it is argued that the longer promoter region belongs to the duplicated locus, most likely situated at an inappropriate position at phylogenetic illustrations. Again, the promoter divergence suggests paralogy on the inferred sequences. Absolutely comparable situations can be observed in the promoter regions of the remaining duplicated pairs.

11.3.2 Intron

Intron lengths polymorphisms (ILPs) have been used as genetic markers in several studies (WANG et al. 2005) and have been shown to yield substantial variability. The intronic region in duplicated gene pairs demonstrates a parallel behaviour to the promoter. In case the promoter region is variable compared to its paralog, then the intronic region is effected in the same way. In those pairs (e.g. *M. polyandra, C. aprica, I. megalospermum, C. tatrae, I. abulense*) one type of non-coding region displays an enhanced promoter with a consequently enhanced intronic region, while the same, vice versa, holds true for the second type. This is not remarkable as previous research has already suggested that genomic DNA sequences from a wide range of organisms show that the intron-exon structure of homologous genes in different organisms can variegate widely (RODRIGUEZ-TRELLES et al. 2006) and that eukaryotic genomes vary to a considerable extent in their length and density of introns between related species (MOURIER & JEFFARES 2003). This is documented with the argumentation that all eukaryotes are of common descent and subsequently underwent immense gain and loss of introns during their evolutionary history (DE SOUZA 2003, ROY & GILBERT 2006), mostly explained by selection factors. It is also postulated that larger species (in comparison to unicellular ones) show a tendency towards accumulation of new introns (LYNCH 2002).

But what has not been intensely investigated yet, as far as known, is the divergence of intronic regions within the same species, even the same individual. As it could be proved that the coding regions display utterly high identity, it seems meaningful to investigate the non-coding parts although they are spliced out and generally do not encode any polypeptide (COMERON & KREITMAN 2000). Their fast evolution indicates a general lack of function. However, this does still not explain the diverged evolutionary development of duplicated loci among single individuals. As separated physical locations are assumed, the loci obviously experience deceased evolutionary impact, due to their diverse genomic surrounding, which directs to different selective modes, resulting in variable exchange rates. This, besides, advocates for the gene functionality of both duplicates. If duplicated loci do not perform, they, in the long run, will be excluded from the genome.

11.3.3 Exon 1 and exon 2

The observed average evolutionary divergence of all amino acid sequence pairs per site is estimated to be 0.3, indicating a relatively high amount of sequence identities. Considering the fact that these sequences are putative duplicated gene pairs from different genera and even tribes which most likely originating from differing evolutionary polyploidisation processes, it is surprisingly that their identity is around 70%. Within the separated exons, identity values highlight that previous assumptions of divergence among the coding region can be supported (WANG et al. 2000). The duplicated loci therefore depict the identical pattern like the complete data set investigated in the previous chapters. The inter-species identity within exon 1 is always lower than the inter-species identity of the complete coding gene. Here exon 1 depicts an intra-exonic distance of 40.3%. The same commensurability holds true for the second exon, which displays an inter-species distance of only 0.159 and always depicts a higher intra-exon identity compared to the complete gene.

Within the second exon a 12 residue chalcone- and stilbene synthase signature motif, (W)GVLFGFGPGLT, is known, close to the carboxyl-terminus (MARTIN 1993). The conserved amino acid sequence is present in the CHS/STS (TROPF et al. 1994, WANG et al. 2007) family among all angiosperms. Four sequences, investigated in this chapter, hold changes in this region.

Motif sequence changes in the CHS protein imply functional divergence, although these functions are not known, yet (WANG et al. 2007). **Table 19** depicts the amino acid replacements in the duplicated gene pairs reviewed in this chapter, which can be categorised as conservative or radical changes (HUGHES et al. 2000).

Sequence	W	G	V	L	F	G	F	G	P	G	L	T	charge	polarity
Duplicated Species														
Cexc B									Q				neutral	nonpolar
Isav 1, 2, 3									S				neutral	nonpolar/polar
Acan RK214 A					S								neutral	nonpolar/polar
Aset RK025 B				S									neutral	nonpolar/polar

Table 19. Conserved motif at the carboxyl-terminus of the *chalcone synthase*. Each amino acid sequence was compared to the conserved.

Any change of category is counted as radical difference while a change within the category is evaluated as conservative. Concerning the charge of the amino acids, no radical categorical change could be observed, the neutral amino acids are changed to another aa neutral in its charge, while there were only four changes in the polarity were one change was conservative, while the remaining three changes all depict a switch in category from a nonpolar residue to a polar one. These amino acid substitutions may most likely result in a dramatically change of structure and chemical properties of the respective proteins (WANG et al. 2007). This can also result in the pseudogenisation of the gene within the named sequences. Those four sequences affected do belong to the tribes COCH and ARAB which are two out of five relevant tribes discussed. This strengthens the hypotheses of evolutionary divergent events within these tribes or of taxonomic inconsistencies not itemised yet.

11.4 Asymmetric rates of sequence evolution in duplicated genes

To characterise the evolutionary journey of the duplicated gene pairs within the Brassicaceae, triplets were constructed, containing a paralogous pair and an outgroup sequence. *Aethionema grandiflorum* was chosen for each pair to anchor the estimations. It is not completely clear where exactly these duplicated sequences derive from. Considering the phylogenetic placement of the duplicated genes of the Cochlearieae for example, it is supposable that these sequences depict material retaining from the palaeopolyploid whole genome duplication that stands at the origin of the family. Other duplicated pairs, like the *Pachycladon* couples, result from a proven recent palaeopolyploid allopolyploidisation event approximately 8 mya ago (JOLY et al. 2009). Apart from the differing point of departure concerning the age of the respective duplication occurrences, the initial situations are comparable and the applied methods deliver information on the copies retained. Therefore the resulting log likelihoods of both models have to be extracted and the result has to be taken twice

[2ΔlnL, where ΔlnL = lnL (no constraint) - lnL (clock-wise) (BLANC & WOLFE 2004)]. The degree of freedom (df) here is assigned to 1 and the test follows a chi-square distribution, with a critical value of 3.84 at a 5% probability level. The resulting table shows the 36 duplicated genes (supplementary material S12) of which 20 pairs (55.5%) are significant and, consequently, 16 pairs (44.5%) are not. Interesting to mention is, that tribes MEGA and DONT do not show significance. The remaining tribes result in 50% or more significant results. The paralogous pairs, which are not significant, indicate that the genes tested here evolve at a homogenous rate, so the assumption of a single evolutionary rate after duplication is only valid in this context. The significant results, testing whether this DNA segment evolved at a homogenous rate along its branches, indicate hence, that the null hypothesis for these pairs could probably be rejected. For these pairs the alternative hypothesis is valid, rates of substitution significantly vary among branches (and are meaningful) and a clock-wise rate is inappropriate (with α = 0.05 cut off of the tested triplets to falsely reject the model of symmetrical evolution). There are only four results, all within the Cochlearieae, depicting an omega value > 1.0, which is a hint for positive selection. Among the pairs with a constant level of substitution, no indication for selection could be detected. Therefore, model 2 under condition A (dN/dS of branch of interest is calculated while average dN/dS of remaining branches is assigned to those) and B were (dN/dS of branch of interest is set = 1, other branches see condition A) run, resulting in non-significant lnL results, tested again via LRT (supplementary material S12). This means that the branch of interest was not under positive Darwinian selection. Consequently, the 20 pairs with a significant result depict different rates in evolution, while the remaining 16 duplicated pairs show an identical rate among branches.

11.5 Genomic sequence blast

EnsemblGenomes (KERSEY et al. 2014) provides four Brassicaceae species' genomes, namely *Arabidopsis lyrata subsp. lyrata*, *Arabidopsis thaliana*, *Brassica oleracea var. oleracea* and *Brassica rapa subsp. pekinensis*. The blast output offered distinct results for the gene pairs which appear twice within phylogenetic reconstructions.

The reduced table (**Table 20**) proves that within the pictured pairs the concept of duplicated genes is evident within different Brassicaceae genome. In *Brassica rapa*, which is diploid, like the other genomes displayed here, the chalcone synthase gene is expected on the chromosome number A10, while some putative copies were expected on even other chromosomes.

	Genome Locus			
Sequence ID	**Brap (10)**	**Alyr (6)**	**Atha (5)**	**Bole (9)**
Blae_1	**A03**	6	5	**C3**
Blae_2	A10	6	5	C9
Capr_1_1	A10	6	5	C9
Capr_1_4	A10	6	5	C9
Cexc_B	**A03**	6	5	**C2**
Cexc_D	A10	6	5	C9
Cgro_B	**A02**	6	5	**C2**
Cgro_D	A10	6	5	C9
Coff_B1	A10	6	5	**C2**
Coff_B2	**A03**	6	5	**C2**
Coff_D1	A10	6	5	C9
Coff_D2	A10	6	5	C9
Dver_C	A10	6	5	C9
Dver_RK239	A10	6	5	C9
Iabu_B	**A02**	6	5	**C2**
Iabu_D	A10	6	5	C9
Iaca_B	**A02**	6	5	**C2**
Iaca_B2	**A03**	6	5	**C2**
Iaca_D	A10	6	5	C9
Iaca_D2	A10	6	5	C9
Imeg_1	**A03**	6	5	**C2**
Imeg_5	A10	6	5	**C2**
Mpol_1_4	**A09**	6	5	C9
Mpol_2_2	A10	6	5	C9

Table 20. Output for genomic blast in EnsemblGenomes (Kersey, Allen et al. 2014). Sequenced data was blasted against each genome. Reduced table (complete table see appendix S31) for blast results of four genomes. Genome names are abbreviated (see text). The respective chromosome numbers are added in brackets. Deviating results are displayed in bold letters.

Within the Cochlearieae and Biscutelleae, the additional copy could be observed on chromosomes A02 and A03, while the tribe Megacarpaeeae displayed is additional copy on chromosome A09. The other tribes holding duplicated gene pairs are not among this table, as their putative additional copies, if so, were discovered among the same chromosome. In *Arabidopsis lyrata subsp. lyrata* and *Arabidopsis thaliana* no second locus was detected. But among the *Brassica oleracea var. oleracea* genome three putative loci were spotted, which depict a sort of pattern. Every Cochlearieae sequence, assorted to the B-group, was detected on chromosome C2, while the expected locus is on C9. The Biscutelleae, however, described their additional locus on C3. The CoGe provides an interface to blast any query against the comprehended genomes. Blast search was successfully applied on the genomes of *Camelina sativa* (most likely 2n = 6x), *Sisymbrium irio* (most likely 2n = 2x/4x/6x) and *Eutrema salsugineum* (2n = 2x).

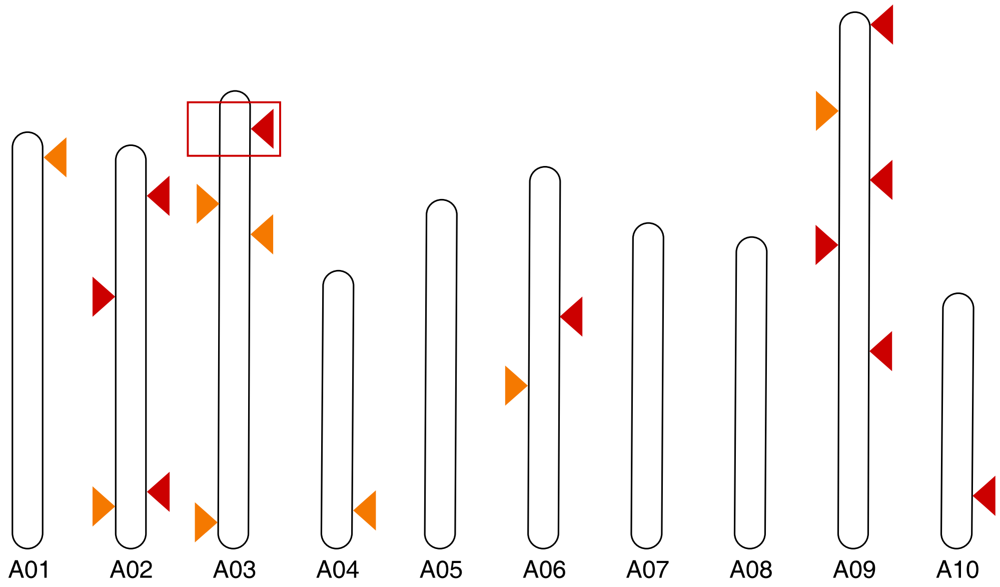


Figure 23. Output figure example for *Brassica rapa subsp. pekinensis* for query of Cexc_B. Most probable result is marked by a frame while other results can be neglected as the amount of sequence identity is less than 200 nucleotides with extensive gaps, suggesting random identity.

Within the genomes from the first two species mentioned, three putative *chs* loci each could be detected (supplementary material S31) in every single sequence, always at the same scaffolds. This suggests that both species sequenced here were of hexaploid origin and therefore showed three loci. *Eutrema* depicted, like both *Arabidopsis* species only one locus and the same locus (scaffold 2). UGENE v.1.14.0 (OKONECHNIKOV et al. 2012) was used for transcriptome data from deep-sequencing transcriptome analysis of *Chorispora bungeana* by ZHAO et al. (2012). This resulted in two hits for each and every of the 72 sequences of the specie's duplicates dataset, where the first hit was always located at locus KA059329, while the other could always be found at locus KA007050 (both with an average of 85% identity), suggesting two loci for chalcone synthase in diploid *Chorispora bungeana*, as well.

It has to be kept in mind that the plasmid sequence pairs listed above do derive from different genomes, which are unfortunately not sequenced yet. So this suggestions of reputed extra loci cannot be definitely assured, also sequences are well conserved.

11.5.1 Synonymous substitution rates

To answer questions concerning the evolutionary dynamics of the duplicated gene pairs, estimation of the level of synonymous substitutions between the pairs were performed according to the procedure employed by BLANC & WOLFE (2004).

Tribe	**Pair**	**dS**	**age (mya)**	**r = (dS/2T)**
ARAB	Aaur_RK018	2.664	1.85	7.2 x 10^{-8}
ARAB	Dver	2.521	1.85	6.81 x 10^{-8}
COCH	Cang	2.179	2.49	4.38 x 10^{-8}
COCH	Cmac	1.994	2.49	4.0 x 10^{-8}
COCH	Cmac_2	1.856	2.49	3.73 x 10^{-8}
COCH	Cpol	2.056	2.49	4.13 x 10^{-8}
COCH	Cpyr_B	2.074	2.49	4.16 x 10^{-8}
COCH	Cpyr_s16	2.476	2.49	4.97 x 10^{-8}
DONT	Capr	1.967	1.33	7.39 x 10^{-8}
MEGA	Mpol_1	1.976	1.68	5.88 x 10^{-8}
MEGA	Mpol_2	1.960	1.68	5.83 x 10^{-8}
MICR	Pche	2.157	1.15	9.38 x 10^{-8}
MICR	Pexi	2.166	1.15	9.42 x 10^{-8}
MICR	Pnov	2.147	1.15	9.34 x 10^{-8}
MICR	Pste	2.109	1.15	9.17 x 10^{-8}
MICR	Pwal	2.180	1.15	9.48 x 10^{-8}

Table 21. Duplicated gene pairs (each row stands for one pair with fused gene pair names) with tribal assignment, estimated dS values calculated with CODEML (YANG 1997) and age estimations. Listed pairs exhibit the identical synonymous substitution rate among branches.

Each duplicated pair of DNA sequences was aligned. DS sequences for each sequence pair were calculated based on codon alignments using the maximum likelihood method CODEML, under the F3x4 model, implemented in the PAML package (YANG 1997). Since it has already been investigated that the evolution among the branches of the single pairs evolves under different conditions, synonymous substitution rates for the 16 pairs with an identical rate of substitution were calculated. All calculated rates are accelerated in comparison to previously estimated ones. As the data compared depicts pairs which are not arranged close to each other but partly far from each other, the results match the expectations well. The highest rates can be observed within the five MICR pairs, which show a mutation rate between 9.17 x 10^{-8} and 9.48 x 10^{-8} .This is presumably tribute to the fact that the genus *Pachycladon* underwent two separate radiation events after its allopolyploidisation. The gained substitutions within the COCH pairs result in the slowest rate, as the age estimation for the split of each of the pairs are relatively old, while the dS values are moderately low. This suggests that the pairs of Cochlearieae sequences resemble each other more and accumulated less mutations. Consequently, these duplicated gene pairs both have an impact on the divergence time estimates as well as on the phylogenetic calculations, as both methods are based on sequence divergence.

Distributions of dN/dS were estimated to examine whether selection contributed to sequence divergence between the doublets. Calculated ω values did neither exceed 1.0 (positive selection) nor depict strict neutral evolution (dN/dS = 1.0), as values between 0.02073 and 0.04613 were calculated. Hence, neither positive nor neutral evolution were governing the sequence divergence of the gene pairs, which is in congruence with the analysis of DNA and amino acid identity.

11.5.2 Relative rate test (Tajima's D)

The test of equality of evolutionary rate between sequences resulted in partly significant values, with $p \leq 0.05$. Sequence pairs from the Cochlearieae (p between 0.00004 and 0.00432) as well as all Arabideae (p between 0.0 and 0.00284) doublets are affected. For these pairs the null hypothesis is rejected in favour of the alternate hypothesis, meaning that rates among that pairs deviate significantly from each other. Note that the sequences from COCH, which display a significant RRT are those with differing mutation rates between the pairs and are therefore not listed in **Table 21**. For the remaining pairs, the null hypothesis, meaning there is no significant difference between the doublets, is valid. This effect underpins the hypothesis, that the tested duplicates do not derive from the same duplication event, e.g. At α WGD, but from at least two events, varying significantly in their date of appearance, which is reflected in the variation of sequence similarity. It can be concluded that the significant values can be assigned to a more ancient event, illustrating an appropriate time frame to allow one of the copies to diversify significantly from its counterpart.

11.5.3 Amino acid composition

A surprising outcome can be observed while comparing the amino acid occurrence frequencies (compositions) of all sites between the particular gene pair parts. The resulting output data suggests an intensive use of only five out of 20 amino acids (alanine, cysteine, glycine, asparagine and threonine) among all 36 sequences among the expected group with a percentaged use between 18.2% (threonine) and 23.3% (glycine). The other half of the duplicated sequence pairs show only a slightly increased amount of amino acid bias, suggesting the use of an additional two amino acids (histidine and aspartate), but only with a marginal usage of each 0.0149% and only among duplicated COCH sequences. The evolutionary origin of amino acid occurrence frequencies is yet not fully understood and divers theories, like protein composition work alongside the genetic code (HORMOZ 2013), have been brought up. Amino acid composition have proven to be well-conserved from species to species (GILIS et al. 2001, ITZKOVITZ & ALON 2007). Therefore any given organism is expected to display a representative averaged composition similar to the amino acid frequency in its proteome set. Deviations from this average composition have been found and linked to cellular organisation and enhancement of protein stability (LOBRY & GAUTIER 1994, MAZEL & MARLIERE 1989). But the rapid reduction of amino acid use observed here cannot be connected to simple aa exchange events, although functional constraints of course influence the interchangeability and its respective impact on the functionality. Though it most likely cannot solely explain the finding. It is not in question that the COCH sequences here are truncated, although they all at least depict the complete second exon (around 1,000 bp) which forecloses the length factor as initiator. Besides, both gene pair groups are affected to a similar extend and, moreover, the synonymous substitution rate seems not to be vastly accelerated, ranging from 3.73 x 10^{-8} to 4.97 x 10^{-8}. Although this heavy reduction of amino acid bias suggests a vast amount of accumulated mutations or the loss of functionality, no evidence for negative inducement of the gene's function could be found.

12 Discussion

Analyses within this chapter have demonstrated that the *chalcone synthase* gene could be synthesised twice in the discussed species within five (ARAB, COCH, DONT, MEGA, MICR) of the 39 investigated tribes. Divergence time estimates of those taxa pairs support the theory of a huge-scaled duplication preceding the speciation of those tribes. Tribes COCH, MEGA and ARAB are assigned to expanded lineage II, what immediately directs to the hypothesis that this reduced delineation illustrates the fate of all expanded lineage II members meaning that two loci were existend within those tribes. On that aasumption, the duplication event should have occurred prior to the lineage divergence. It can be argued that all other tribes assigned to that lineage have undergone a subsequent diploidisation event resulting in the loss of the additional gene loci. Whether this duplication is identical with the *At-α* cannot certainly be confirmed.

Tribes DONT and MICR, indicated by the *chs* gene tree phylogenies, as well as by divergence time estimates, suggestedly illustrate more recent doubling events, effecting the tribe if not only the genera, at maximum. This hypothesis is supported for *Pachycladon* by JOLY et al. (2009) where a genus related hybridisation, an allopolyploidisation, was affirmed. High sequence identities and similarities (compare **Table 18**) additionally support the hypothesis of recent duplications within those two tribes, as well as highly conserved coding regions, especially among the carboxyl terminus. Whereas the diverged non-coding regions are evidence for duplication events which have happend recently (XU et al. 2012), although not much is known about structural divergence among duplicated genes.

Within the following passages, individual scenarios will be discussed for every investigated tribe from this chapter.

12.1 Arabideae

The tribe Arabideae is assigned to the expanded lineage II (FRANZKE et al. 2009), which still contains a huge amount of unresolved relationships between and within this lineage. Besides, this tribe is the largest in the Brassicaceae family (AL-SHEHBAZ 2012) and varies widely in evolutionary character traits like life cycle history or amount of polyploid species. A profound insight into the tribe's phylogenetics, biogeography and trait evolution was recently demonstrated (KARL & KOCH 2013). Discovered internal evolutionary patterns, like shifts in life cycles, are potentially capable to be applied on the whole family (KARL et al. 2012).

Concerning phylogeny, it could be demonstrated that tribe Stevenieae is sister to ARAB (GERMAN et al. 2009), what is supported via *chs* data at hand. This validated information, as well as the location of the additional ARAB clade in the gene tree reconstruction simplify the commitment on whether which group represents the orthologous loci, derived via speciation. The duplicated clade in the complete data set is associated to DNA sequences from other tribes (ANAS, DONT) assigned to different lineages (see **Figure 3**), as well as the putative outgroup, showing no noticeable structure or allegeable phylogeny. All members of this very group, supported by a bootstrap value of 99%, have been excluded from the data. Supposedly all members of that clade derive from a paralogous origin. The nuclear encoded *chs* therefore hints to have undergone a polyploidisation event precedingly to the respective tribal speciation, defining those paralogs, sensu KOONIN (2005), as outparalogs. The duplication event was previously discussed to have happened to the common ancestor of sistered tribes ARAB and STEV (R. Schmickl, pers. comm.), individually. Gene tree reconstructions present argue for a postponed duplication event, including an increased amount of tribes.

As members from lineage III, as well as expanded lineage II are associated with *Cleome*, detached from all remaining groups, this could evidence not only for ancient gene copies of *chs* deriving from the *At*-α WGD, but maybe already from the more ancient *At*-β. The diverged DNA sequences from the ARAB paralogs, the association with *Cleome*, as well as the increased dS values argue for older duplications of the locus.

Sequences from DONT demonstrate highly divergence as well, which will be discussed in 12.3. Like previously explained, ARAB paralogs could only be sequenced due to modified primer combinations which could not be applied on any other tribe employed here, once again arguing for an older age of the loci and demonstrating inter-as well as intra-species divergence.

In other words: Paralogous ARAB copies vary to such an amount from their orthologs, as well as further paralogs that individual primer pairs needed to be designed for putatively ever tribe. Therefore it is conceivable that any of the tribes implemented still contains additional degraded *chs* information remaining within the genome. However, the primer pairs employed for sequencing were not capable of amplifying those highly diverged loci. Assumed that the promoter region primarily accumulates mutational changes, this presumption accounts for the reputed presence of ancient highly mutated *chs* loci within a certain amount of Brassicaceae genomes.

12.2 Cochlearieae

The tribe Cochlearieae comprises only genus Cochlearia and Ionopsidium and is with circa 30 species moderately sized. Although the tribe has long obtained minor interest, the application of nuclear chalcone synthase on those genera has revealed an active history of, at least, that respective locus.

Although *B. luteae* has been proven to both describe a monophyletic tribe on its own (BIVO) and, additionally, to be not closely related to tribe COCH (KOCH 2012), demonstrated via the application of chloroplast *trnLF* and nuclear ribosomal ITS, the original *chs* data set (**Figure 3**) suggested a neighbouring position to one group of COCH. After thorough investigations this very group was excluded from the *chs* data as putatively duplicated and interacting with the remaining sequences. Admittedly, KOCH & MARHOLD (2012) demonstrated that the utilisation of markers ITS and *trn*LF result in a congruent single locus illustration of the tribe Cochlearieae at a very basal position not associated with BIVO. The tribe has been placed to expanded lineage II as a synopsis of various reviews suggested a placing outside lineages I to III. This argues for the correct respectively orthologous *chs* locus which was facilitated in the work at hand. Together with HELI and ALYS the Cochlearieae are associated in a small expanded lineage II group. Consequently, either the two applied markers do also demonstrate duplication events of the respective loci or the diverse marker systems do not depict congruent outcome due to additional impact not concerned here. It is argued that those conflicting results originate from deployment of DNA sequence information descendent from different genome types (mt, cp, n) (KOCH 2012), which are also known to show high variety among silent substitution rates (WOLFE et al. 1987). Further investigations suggest diverse outcome for the placement of the COCH. Different sister relationships were observed, either to tribe COLU (COUVREUR et al. 2010) or to ISAT (BEILSTEIN et al. 2010, LYSAK et al. 2009), which can neither be underpinned with *chs* data. Splits of COCH from any other tribe are dated between 23.6 mya (fossil calibration) and 11.5 mya (synonymous substitution rate) according to the respective BEAST analysis and are therefore dated forward compared to prior estimations which dated the split to a minimum of 30 mya. This supports the hypothesis that the tribe describes a position along a yet unresolved basal polytomy (FRANZKE et al. 2011) drawing support to its expected antiquity (KOCH 2012). Moreover, the tendency of the genus *Cochlearia* to polyploidisation, which is based on a well-defined diploid species level of 2n = 2x = 12, results in species with tetra-, hexa- and even octoploid cytogenetic data (KOCH 2012). This as well as uncertain or variable ploidiy levels in some species handicap a thoroughgoing designation of orthologous and paralogous gene copies. The supposed polyploidisation in *Ionopsidium* demonstrated by a duplicated base chromosome number of n = 12, compared to *Cochlearia* with n = 6 (7) is indicative for further duplication events.

It has to be reminded that within this *chs* estimations the basal COCH group was excluded which expectedly results in younger estimations. In case the removed COCH clade should really depict the original *chs* locus, than the estimated divergence of that split is dated to 31.15 mya ago (see **Figure 19**) suggested by estimations via RelTime ML and arranging among the recommended split age. However, argumentations cited earlier in this thesis propose that this postponed split from any other Brassicaceae tribe equals the most recent WGD event as origin of all Brassicaceae (SCHRANZ & MITCHELL-OLDS 2006). The subsequent gene duplication in COCH, at least in *chs*, argues for the facilitations of both copies with the more recent dated one as functionally working and encoding the orthologous gene. For this hypothesis the divergence between genus *Cochlearia* and *Ionpsidium* is dated 9.64 mya (see

Table 22), based on angiosperm calibrations, to an after borne age. Although in otherwise complete analogy to the *chs* chronogram of the coding region presented by KOCH & MARHOLD (2012) the placement of that tribe seems not absolutely verified. However, the *chs* data here argues for an eraly duplication event in the respective tribe, leaving at least two functional loci within the genome. Actually, no tendency for silencing or gene loss to any extant could be detected.

12.3 Dontostemoneae

This tribe and its situation has already been touched in the previous chapter. As genes are continuously being created, mostly via polyploidisation, they also get rapidly destroyed by mutations, resulting in the re-diploidisation of the genome, mostly keeping only the smallest percentage of the duplicated gene pairs. The calculated half-value period is supposed to arrange within 3-7 mya (SANKOFF & NADEAU 2000). As the DONT did not only demonstrate a split up tribe, arranging at two diverse standings within the gene tree what indicates different scenarios like the remaining of different gene versions within different species, it also shows a diverse arrangement of one species, namely *Clausia aprica*. The *real* Dontostemoneae copy was closely associated with Hesperideae (*Hesperis matronalis*), which already was demonstrated by Liu et al., (2012), as well as the close relationship between the tribes Anchonieae and Euclideae. The Buniadeae, which arrange even closer to the Anchonieae as any genera from Euclideae, were not discussed extensively before.

There is evidence that an exclusive duplication event can be found in this tribe, dated around 13.3 mya, suggested by BEAST analyses (see supplementary S6). Referring to that estimated date stamping this polyploidisation event is supposed to appear after the last whole genome duplication. If this holds true, then the *Clausia aprica* copies obtained here retained considerably longer within the genome than expected. This, however, indicates, that the two loci for chalcone synthase among at least that species, if not genus or even tribe are functional and are essential for the organism. Unfortunately, there is only evidence for *Clausia aprica* to depict an additional locus, which is the reason for the suggestion of an intra-genus (*Clausia*) duplication event, which would postpone the duplication to have taken place after the speciation of the genus. But positive evidence for the extent to which the data is affected by this event is not available. This last-mentioned species moreover depicts different recess from several chromosome counts (BELAEVA & SIPLIVINSKY 1975, YURTSEV & ZHUKOVA 1982) which all derive from samplings from Russia. Here the outcome suggests that *Clausia aprica* is of neopolyploid origin with one count 2n = 2x = 14 and another count revealing a tatraploid origin with 2n = 4x = 28. Additional information on chromosome numbers for species discussed were obtained from a database (WARWICK & AL-SHEHBAZ 2006) as well as by the cytogenetic search tool implemented in BrassiBase (KIEFER et al. 2014). This shows a fourth count done as part of the BrassiBase project, which offers additional information of the haploid genome size (1C), which here arranged at 3.97 and a monoploid size of 1.99 (1Cx) suggesting an even diploid origin of the respective species. If this specimen is of tetraploid origin, up to four varying alleles are expected, which the additional loci are most likely the outcome from the polyploidisation event, which, without difficulty, can be arranged on two different loci.

As is commonly believed that strict autosyndesis in allopolyploids leads to independent segregations of alleles on homeologous chromosomes and hence fixed heterozygosity is inherited in a disomic manner, split arrangement of polyploidy genera seems substantial.

Granted, but in case the genus is diploid these are additional loci which are kept for a remarkably long time span, as the Brassicaceae show a tendency for relatively rapid diploidisation after polyploidisation occurred (LYSAK et al. 2009). To sum it up, an independent duplication event, prior to the speciation of the species (at least) is suggested resulting in two groups, of which one exclusively holds *Clausia aprica*, while the other additionally contains extra species. Most likely this tribe experienced a duplication event previous to its speciation.

If the duplication took place after the speciation, it would be expected that the sequences arrange relatively close to each other in the gene tree, as a) the duplication must be very recent and b) the duplicates are redundant immediately after its duplication. If the sequence data is compared, this additional, votes for the polyploidisation after the alpha WGD but before speciation of the genus.

12.4 Megacarpaeeae

The Megacarpaeeae have also already been reviewed in 9.5. As they depict no clear evolutionary pattern, they show up in both large-scale analysis drafts. They not only can be observed at the expected but also at an unexpected placing in the cladogram. They, moreover, are not clearly diversified via species boundaries, meaning, that one species nests at one surrounding, while the second employed species arranges at the second surrounding. This tribe, additionally shows a split species (*M. polyandra*) where one splinter is clustering with the second MEGA species (*P. pterocarpum*), while the other splinter stands on its own. The situation resembles this of the DONT. In case the tribe underwent an additional duplication why is it that only *Megacarpaea polyandra* shows a second locus? If the second locus derives from WGD α, how come it still remains within the genome? And why only exclusively within one species? The evolutionary development of *chs* within this tribe, on the first sight, seems to be very thoroughgoing, but if the questions above are considered, the journey of the gene does not seem to be that straightforward.

12.5 Microlepidieae

One genus from this tribe, namely *Pachycladon*, has already been extensively investigated by JOLY et al. (2009)demonstrating that *Pachycladon* is of an allopolyploid origin (2n = 20), thus keeping two genome copies with duplicated *chs* (and other) genes. The A (rabidopsis) lineage holds one, while the B(rassica) lineage (JOLY et al. 2009) contains the second copy. The lineages received their name due to the phylogenetic placement of both copies detected. The A genome copy depicts *Pachycladon* in a close relationship to the

Crucihimalayeae and the Boechereae, which can immediately be supported from the results presented here (**Figure 3**, **Figure 8** and **Figure 18**). While the B genome copy is propagated do be close to the split between the *Arabidopsis* and *Brassica* lineage, which cannot be affirmed here. In the phylogenetic representation presented in this study, the second copy is well within lineage I sensu BEILSTEIN et al. (2008) and not even close to the split suggested by JOLY et al. (2009). The placement of the B-copy indicates a close relationship to the tribe Yinshanieae, sistered by the Smelowskieae and the Descuraineae. This group of four tribes is most likely to reveal the real placement of the second genomic copy of *chs*. The split of this group and the group occupying the other copy, supported with a bootstrap value of 97, presumably show the hybridisation event of the genus *Pachycladon* dated 8 mya. The different results of the "Brassica" copy originate from data sampling. As references for *Pachycladon* are mostly genera from the same lineage, namely lineage I, were consulted, resulting in a phylogeny which is not representative for the complete family. Genera from the tribe Lepidieae are confirmed to pose at a position basal and sister to the lineage (BEILSTEIN et al. 2006, BEILSTEIN et al. 2008, JOLY et al. 2009). And there, additionally, is some evidence (in study at hand), that the Cardamineae (typified by the genera *Rorippa*, *Cardamine* and *Barbarea*) are as well standing at a basal position outside the core of lineage I. The four genera representing lineage II (*Brassica*, *Sisymbrium*, *Thlaspi*, *Thellungiella*) are nested within the phylogeny which does not underpin the expected resolution.

The polyploidy event of the Australian *Pachycladon* genera most likely occurred before the genus diverged. If the duplication event would have happened independently after the divergence of *Pachycladon*, it would be very unlikely that the genome copies were in similar positions in the respective gene trees. Besides, the tribes closely related to the genus are not affected by signs of paralogy, i.e. a second divergent genome copy. This image of *Pachycladon* could still be explained by the fact that a small-scale duplication of a part of the chromosome took place is confuted by the fact that all five markers employed in this study were duplicated and are distantly located on the complete genome (JOLY et al. 2009). So, consequently, the allopolyploid *Pachycladon* genera underwent a tribal internal polyploidisation, respectively allopolyploidisation, event before the genus but after the lineage diversification.

The duplicated gene pairs investigated presumably describe duplicated gene loci, on a physically different location. This is one factor cited as contributing to rate variation among genes. GC content, as well as codon usage bias do also count as a factors showing impact, while differing levels of selective constraint on amino acid substitutions are named as the fourth reason (TICHER & GRAUR 1989, TSUNOYAMA et al. 2001, ZHANG et al. 2002).

As a consequence, the parts of the duplicated 36 gene pairs (corresponding to 72 DNA sequences) causing disorder among the data analysis were removed (compare **Figure 3**, colour-coded squares) for further investigations. This resulted in the delimitation of 36 sequences while the other half, as putative orthologous locus was kept. The analysis applied on the data at hand implicitly suggests that great importance has to be attached to the material worked with. It is beneficial to compare only accurately investigated data in order to assure comparability. This analysis demonstrates the impact of genetic sequences, on which evolution had worked to a certain extent.

The following chapter will focus on the remaining colour-coded sequences, namely the squares. Asterisks (see part 2: Trouble with the Tribal Arrangement), as well as circles (see part 3: Done Twice is not Always Better) have been discharged.

Part 4: No End of Trouble

After the exclusion of around 70 functional DNA sequences from the original data set utilised (representatives were symbolised with colour-coded squares, see **Figure 3**), a final set of 600 sequences is left. In case any disorders and derangements could be organised, a thoroughgoing data set, holding only homologous gene copies, resulting in straightforward output data is expected. A first impression should be gained via the phylogenetic reconstructions.

13 Material und methods

13.1.1 Comparative phylogenetic reconstructions and analyses

Like previously deployed, different phylogenetic analysis methods have been applied on the data to assure the solidity and reproducibility. Therefore NJ and MP as well as ML analyses were utilised to compare the phylogenetic reconstructions with those from prior analysis. Bioinformatic programmes and settings are identical compared to previous applications.

13.1.2 Divergence time estimates

The identical three approaches, described in 4.3.2.1 (synonymous substitution rate, fossil calibration and angiosperm split ages), were (direct and secondary calibration) applied on the reduced data set, to produce comparable output estimations. All settings were in absolute congruence to previous computations. For each approach four files were created which were each run for 50 million generations on the CIPRES science gateway in the San Diego Supercomputer centre (MILLER et al. 2010).

13.1.3 Transition/transversion ratio

As the amount and pattern of homoplasy influences the accuracy of the phylogeny a further test on the DNA evolution with DAMBE (XIA 2013) was employed. The dataset was stepwise cleaned from distortion via exclusion of evolutionary “leftovers”. As high levels of genetic divergence result in a decreased transition/transversion ratio, like it could be examined in 0, the illustration from the reduced data is expected to depict a much steeper diagram, because the genetic distance is shorter. Therefore the transition/transversion bias was calculated using DAMBE’s 5.3.115 (XIA 2013) tool for sequence analysis on third codon position estimating nucleotide substitution patterns, depicting a detailed output with patterns and lines.

13.1.4 Lineage through time plot

All disruptive data, still present, was excluded and a LTT plots was constructed with the divergence time estimate trees generated in BEAST, using the ape package (PARADIS et al. 2004) of the R software environment (IHAKA & GENTLEMAN 1996). This plot (**Figure 25**) contains the purified data, as well as the original with all identified outliers. That plot illustrates an overall pattern of diversification of gene evolution among the Brassicaceae.

14 Results

14.1 Bioinformatic data analysis

14.2 Comparative phylogenetic reconstructions and analyses

The evolutionary relationship among the taxa was inferred using the neighbor joining (SAITOU & NEI 1987), as well as the maximum likelihood and maximum parsimony method. The evolutionary distances for the NJ analysis were computed using the Kimura 2-parameter method (KIMURA 1980) and are in the units of the number of base substitutions per site. The reconstruction pictured displays the NJ algorithm, which, in the majority of nodes and topology, is in absolute congruence with the further applied methods. The sum of the branch length is 2.2622 and the bootstrap replicates are drawn next to the branches.

Now, the phylogenetic reconstruction of the *chalcone synthas*e gene displays a representative and well resolved gene tree phylogeny in most instances, where the majority branches are well supported. Eight groups can be categorised of which three are the moderately to soundly supported lineages I to III, of which lineage I and three are still definitely monophyletic. Lineage II bears two disruptive sequences (*Calepina irregularis* and *Conringia planisiliqua*).

Expanded lineage II among the *chs* gene is not of monophyletic origin. At least three distinct groups can be identified being scattered among the reproduction. Expanded lineage II consists of three sequences, assigned to two tribes, namely COLU and KERN, while the third sequence, *Fourraea alpina* is yet not allotted to a tribal group. The gene tree reconstruction at hand (**Figure 24**) recommends this genus as member of the expanded lineage most closely related to *Kernera saxatilis*. The expanded lineage II group consists of STEV and ARAB, supported via 99% and MEGA with SCHI, also with a high bootstrap value of 95%. The first named group is displayed as sister to lineage III, sharing the most recent common ancestor. *M. polyandra* and *S. walkeri*, however, build a small separate group between lineages II and III.

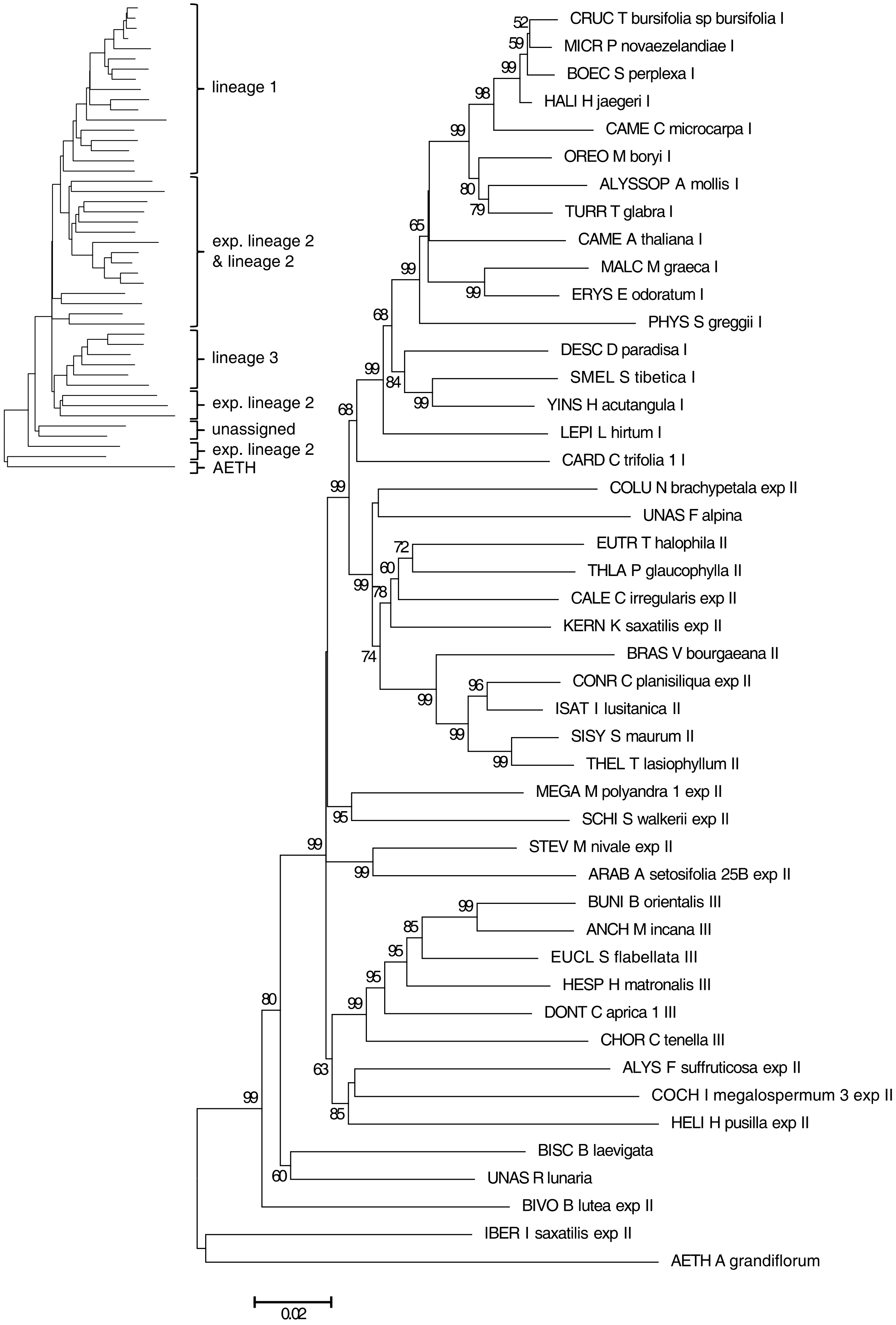


Figure 24. Gene tree (NJ) of adjusted data of the *chalcone synthase* gene among the Brassicaceae. All unexpexted data (duplicated tribes and gene pairs) were excluded. Bootstrap values are only displayed for nodes > 50% and are drawn next to the nodes. Lineages affiliation is shown in the small scheme top left. MP and ML analyses can be viewed in the appendix (S25 and S 26).

Expanded lineage II is a basal group of three with a moderate support value of 75% and a divergence support of 99%. HELI, ALYS and COCH are arranged in a sister group relationship to lineage III. *Iberis saxatilis*, also expanded lineage II, displays a much unexpected basal positioning close to *Aethionema grandiflorum*. In the aggregate, the adjusted phylogenetic reconstructions of the *chalcone synthase* gene represent in most instances a congruent appearance compared to other molecular marker phylogenies like ITS.

Thus, the addressed data from tribes BISC, BIVO, CALE, CONR, IBER and the unassigned genera, all marked with colour-coded squares in **Figure 3** will be investigated in this chapter.

14.3 Divergence time estimates

The results of the divergence time calculations are listed in **Table 22**. The most probable origin of the Brassicaceae as well as different divergence estimations cannot be set to certain age. This is due to the fact that both varying approaches have been applied on varying data sets. The indication of this effort was to demonstrate the impact of either of the factors. It is essential to investigate the employed data accurately prior to perform calculations upon it and before drawing conclusions. It can persuadingly be observed what immense impact even one imprecise chosen DNA sequence (*C. spinosa*) can have on the whole estimations (compare results 669 and 668). But still, after justification and exclusion of inappropriate sequences, the remaining data does demonstrate a vast range of estimated divergence times generated by the diverse drafts applied on the identical data. Note that the divergence estimates induced by synonymous substitution rates always resulted in the most recent calculations while the fossil constraints always postponed the divergence even up to double the age, e.g.from 19.6 mya (tmcra Bras, synonymous substitution rate) to 37,89 mya (tmrca Bras, fossil constraint). The estimations via angiosperm splits seems to be highly appropriate as they constantly arrange between the other deliverables and also are relatively stable. Therefore, the suggested crown age of the Brassicaceae arranges between 37.89 and 19.60 mya, most likely settles somewhere between those boundaries. It can be concluded that divergence times via synonymous substitution rates underestimate while fossil constraints supposable result in overestimations of age estimations.

Divergence times from the nuclear *chalcone synthase* gene tend to be somewhat older compared to plastid or mitochondrial genome data. Even compared to other nuclear single-or low-copy nuclear genes, *chs* produces by far the most predated age estimations (JOLY et al. 2009). This might indicate a different evolutionary history of the plastid or mitochondrial

Applied Data	669			668			600		
Constraint	Rate	Angio	Fossil	Rate	Angio	Fossil	Rate	Angio	Fossil
Runs	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Generations	5.00E+07	5.00E+07	5.00E+07	5.00E+07	5.00E+07	5.00E+07	5.00E+07	5.00E+07	5.00E+07
Likelihood	-66623.65	-60367.82	-56494.74	-56134,85	-59942.44	-55872.19	47190.28	-50861.62	-47181.22
Divergence *C. spinosa*	28.05	42.01	18.10	n/a	n/a	n/a	n/a	n/a	n/a
tmrca Brassicaceae	25.50	34.48	39.18	24.90	29.66	39.30	19.60	26.06	37.89
tmrca Lineage I	14.10	21.05	20.92	14.70	17.60	20.48	11.70	17.39	22.41
tmrca Lineage II	11.80	17.99	18.06	8.64	10.64	13.99	11.70	10.88	18.49
tmrca Lineage III	11.50	16.48	17.05	10.10	15.93	16.46	10.30	16.68	25.88
Divergence DONT	23.60	34.48	27.35	13.30	15.93	22.83	7.31	8.76	14.05
Radiation DONT	4.75 – 3.46	11.49 – 11.35	10.95-7.11	8.29 – 6.48	9.94 – 0.71	5.14 – 0.91	n/a	n/a	n/a
Divergence MICR	9.94	14.86	15.26	11.50	12.00	14.91	12.90	1.4	3.98
Radiation MICR	0.59 – 0.56	2.26 – 1.59	1.01 – 1.0	0.59 – 0.56	0.68 – 0.65	0.87 – 0.85	0.48	n/a	1.09
Divergence ARAB	23.60	31.44	28.57	18.50	22.38	29.69	8.35	11.66	19.58
Radiation ARAB	6.98 – 6.56	14.41 – 10.18	16.71 – 14.09	7.1 – 5.77	11.51 – 6.89	10.73 – 9.29	6.34	8.67	14.19
Divergence COCH	21.70	34.48	39.18	24.90	26.16	39.30	11.50	14.28	23.60
Radiation COCH	11.6 – 10.7	20.76 – 18.9	17.37 – 12.65	12.2 – 8.42	14.2 – 12.52	16.57 – 13.08	7.52	9.64	15.1
Divergence MEGA	21.70	31.44	35.52	16.80	20.98	39.30	13.90	16.06	21.05
Divergence YINS	21.70	31.44	27.35	18.50	22.38	25.60	1.84	2.36	3.98
Divergence PHYS	21.70	29.36	39.18	24.90	23.60	28.13	3.36	6.87	10.70
Divergence TURR	21.70	31.44	27.27	11.30	23.60	28.13	2.16	2.94	4.40

Table 22. Divergence time estimates calculated with BEAST. The table depicts the original data set (669) and two adjusted sets (668 and 600). Parameters and results of the divergence time estimate calculations usingthe three approaches introduced in chapter 1. Divergence (split age of respective tribe) and radiation values (one for each of the polyphyletic arranged groups) are listed, as well as estimations for the most recent common ancestors (tmrca). Radiation values are only given for those tribes containing at least two different species in each group.

compared to the nuclear genome and hint to not universal but rather specific mutational rates among nuclear and other genes.

14.4 Transition/transversion bias

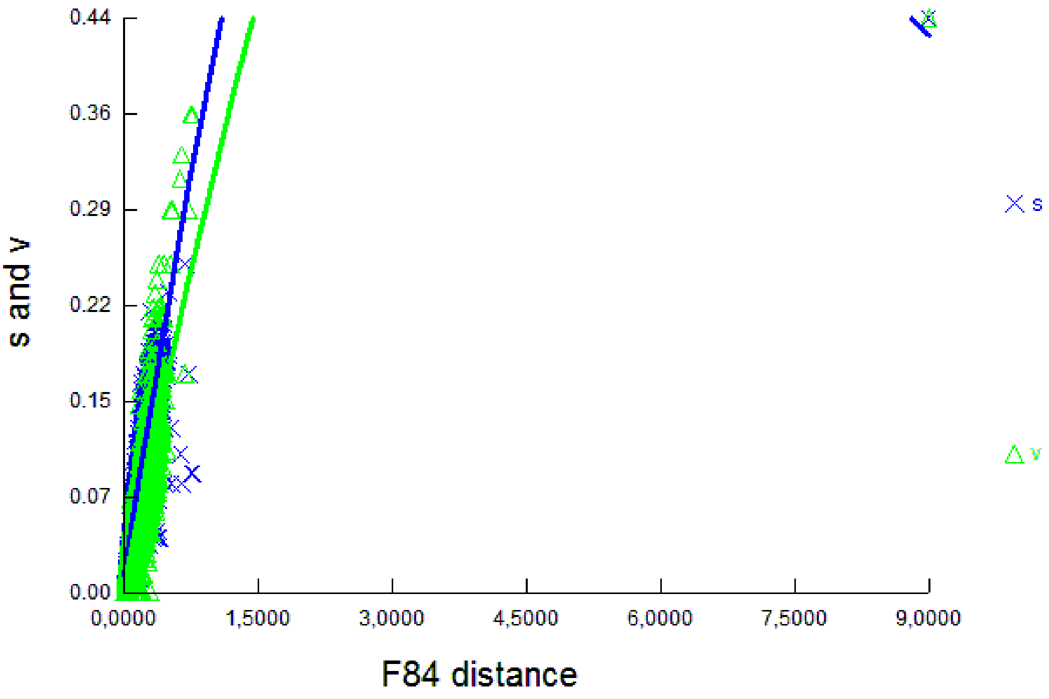


Figure 25. Transition/transversion plot (DAMBE) of the coding *chalcone synthase* genes' third codon position. Distances are plotted against transitions (blue crosses) relative to transversions (green triangles).

The transitions and transversions plotted against the distances result in a steep representation of the data. The substitution types do not approach because the number of transitions does in parallel increase to the number of transversions, while the later ones do not exceed transitions. Therefore, no tendency for saturation can be observed and a plateau can most obviously not be identified. Therefore, no saturation, which defines the point at which multiple substitutions have occurred at the same degenerate codon position, such that it is not possible to accurately estimate sequence divergence (BROUGHTON et al. 2000), can be observed. As the relatively steep increase in transitions and transversion is not adjunctive with the growth in distance, this suggests lower genetic divergence among the data utilised here, compared to that from **Figure 14**.

Although reclusively the third codon position is employed and an apparent slow-down in the accumulation of substitutions is expected due to the respectively greater saturation level of the highly variable third codon positions in the protein-coding sequences, no substitution saturation can be observed here. As substitution saturation decreases the phylogenetic information due to the loss of phylogenetic genuine signals this outcome of the plot is preferred because it implies the fact that the net data set is phylogenetically informative and contains unique signals. Although there is one data point at an extreme position within the plot, this codon can be neglected as statistical outlier.

It can be concluded, that the purged data was distracting the analysis to a commensurate level.

14.5 Lineage through time plot

The lineage through time plot describes a relatively uncommon result, which is due to data composition. The curves describe the original data set from the first chapter and the adjusted data which resulted from sequence exclusion during the second and third chapter. Conventionally, a plot which diverges in the number of species would be expected as both compared groups variegate with 70 sequences, which would result in two curves originating at the same age of origin and split during their gain in sequence amount. This plot depicts more or less the opposite exposure. The two groups start at different ages and converge with the acquirement of sequence numbers. This suggests, that the original data describes an older age of origin, while the reduced sequences seem to be younger. However, this is not exactly the information displayed. As it was already evidenced here, the excluded sequence material influenced the divergence time estimates to a certain degree. The synonymous substitution rates, as well as estimations via BEAST always resulted in conservative suggestions for the evolution of this group. This can be observed in the diagram, depicted by the light blue curve. Therefore, the estimations of the origin of the complete data mounts up to ~25.5 mya, while the reduced group was calculated to originate around 19.6 mya ago. The values on which this plot is based derive from the divergence time estimates via synonymous substation rate range carried out in BEAST. Here, the absolute age divergence is not in the focus concerning this analysis, but the proportion these two groups are divided by. It can be concluded that the DNA sequences removed from the material executed a high impact on the data concerning its divergence times, postponing its origin. As it was already discussed, the removed data most likely results from diverse duplication events and therefore poses high variability within its DNA sequences, resulting in an overestimation of the calculated origin. Hence, the exclusion and separate treatment is legitimate.

Disregarding this unexpected behaviour of the two data sets compared, the absolute illustration of the birth rate of Brassicaceae species can be observed here. Especially the number of lineages from the adjusted data (all_600) increases rapidly but constantly after the origination of the family (here 19.6 mya) displaying a relative even rate through time. Then, the increase

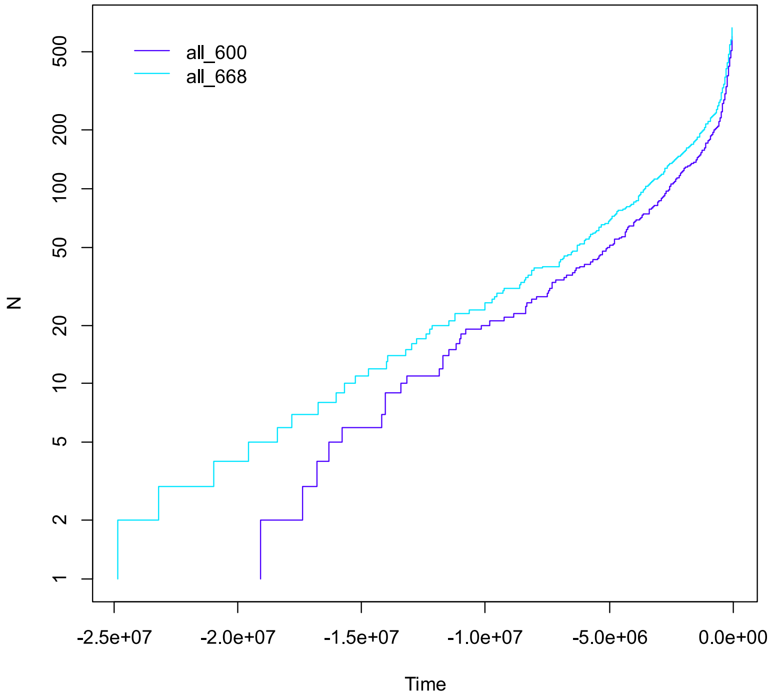


Figure 26. Lineage through time plot based on divergence time estimates calculated from synonymous substitution rates.

is delayed demonstrated by a moderately rising graph (~12.0-5.0 mya). Starting in the Early Pliocene (~ 5 mya) the blue curve rises steeper again, as the formation of new lineages increase considerably. During the Late Pleistocene (2.588-0.012 mya), the graph rises rapidly to a momentarily peak, indicating the formation of new lineages to increase fundamentally. Radiation events within most of the tribes can be dated to a relatively recent age, most of those falling within the Pleistocene or Pliocene (5.332-2.558 mya). However, both graphs display a parallel development and merge at a relatively recent time span.

14.6 Investigation of outliers

14.6.1 Biscutella laevigata

Within BISC the sets of sequences are relatively similar, especially among the coding regions what makes it challenging to distinguish between two scenarios. Firstly, the sequences diverged only recently from one another via duplication or, secondly, the sequences have diverged in concert.

To find support for the different drafts, *Biscutella laevigata* was examined with MEME v. 4.9.1 (Bailey et al. 2009) for motifs among the complete gene. The promoter region displayed in **Figure 27** depicts a minor number of motifs, arranged in a peculiar manner.

There are two types of promoter sequences, either in the motif order 1-2-3 or 1-3-2-1. Even more striking to detect by the order of the motif numbers, a partly inversed sequence arrangement could be detected here. Essential elements of the region are all faultless although some severe changes must have taken place.

Important to mention is, that the exonic regions are very similar within this two sequence types indicated in the promoter. Exon 1 exhibits only four motifs, 2-1-4-1-3, occurring thoroughgoing among all plasmids. Within the second exon, the number of motifs is oddly high, indicating that 18 different motifs could be detected. The order suggested is 3-13-3-16/18-1-8-

2-14-6-4-2-11-2-15/17-1-7-3-5-3-10-1-4-12-1-9-5. Two positions display different motifs (16/18 and 15/17). Motifs 1, 2, 3 and 5 are repetitive.

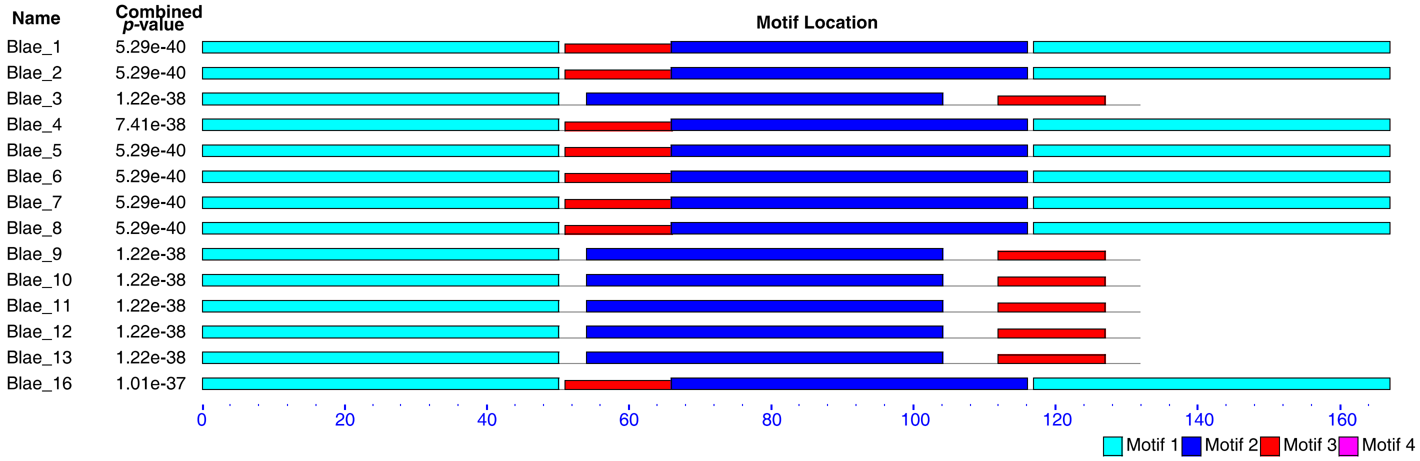


Figure 27. Combined block diagram of notified motifs from promoter region of *Biscutella laevigata*. Non-overlapping sites with a p-value better than 0.0001 are displayed. The height of the respective motif block is proportional to -log (p-value), truncated at the height for a motif with a p-value of 1e-10 (BAILEY et al. 2009).

The intron shows again two distinctive sorts of sequences, parallel to that of the promoter. There is either the motif order 2-5-3 or 2-1-4 to detect, indicating that the beginning of the intron resembles in both sequence types, while the remaining sequence is more diverse. These results hint to the fact that *Biscutella laevigata* possesses two *chs* copies, each of them very conserved, but with an estimated p-distance of 0.253.

14.6.2 Bivonaea lutea

This specimen shows an intra-species identity of 99.48% among the complete as well as for the coding gene, indicating a very high identity between the sequences, which most likely display the two alleles of the *B. lutea*. It is even possible, due to the high similarity between the DNA data that even one allele is displayed holding some sequencing or PCR based differences, which are no actual mutations. This species is assigned to expanded lineage II, which, together with some other tribe representatives (IBER, COCH, MEGA), arranges at this basal position. Previously reviewed affinities tribes from lineage II (SISY, BRAS, THEL, and ISAT) or closely associated EUTR and THLA could not be affirmed. The uncertain position of *Bivonaea lutea* therefore seems still to be not revealed. The sequences do not display any peculiarities which provide a common explanation for the unexpected position within the phylogenetic representations.

14.6.3 Calepina irregularis and Conringia planisiliqua

Calepina irregularis displays a divergence between the plasmids of the coding region between 97.63 and 99.57% and 95.44-99.65% within the complete gene, suggesting a high similarity between the respective sequences. However, divergence above 1% is suggested to imply not only allelic variation but diverged loci within the genome. This is also supported by the DNA sequences itself, depicting highly conserved coding, as well as promoter region, while the intron displays a variable length. The deletion of 15 nucleotides proves that it is most likely that a duplication event took place. It is assumed that *Calepina* underwent this event very recently, as firstly, only the intronic region is moderately effected and, secondly, this variability cannot be observed within the phylogenetic reconstructions. Therefore, the discussed arrangement of that genus is not going to change due to the fact of a duplication.

Conringia planisiliqua, moreover, displays a sequence similarity above 99%, suggesting allelic variation among the data. Its placement is consequently legitimated.

14.6.4 Iberis saxatilis

The Iberideae are represented by the species *Iberis saxatilis*, sharing mediocre similarity, of 78% within the complete gene, with the second species from the same genus, *I. semperflorens*. Comparison of the coding regions of both species resulted in a much higher output similarity of 94%. Hence, both DNA sequence groups cluster closely together. While *I. saxatilis* displays only minor difference between its plasmid sequences, *I. semperflorens* owns two highly divers intronic regions and promoters, varying immensely in length and composition. Therefore it is also suggested, like explained in *C. irregularis*, that this species underwent a recent gene duplication event, maybe also present in other species of this tribe, which could not be observed. As neither the coding region is effected by mutational influences to any extend nor is the promoter, a close-by silencing resulting in the exclusion of the locus from the genome is not expected. A species-specific duplication could also derive from hybridisation events (allopolyploidisation), but clear evidence can neither be named due to not appropriate methods applied here nor are they central to the investigation goals.

14.6.5 Unassigned taxa Fourraea alpina and Ricotia lunaria

Both unassigned genera display a highly intra-species identity above 98%, which are moderately distributed among the complete gene. This indicates that the divergence verisimilar originates from sequencing lapses, showing mutational accumulations which are not authentic. As the promoter regions a nearly sequence identical, functionality, as well as single locus appearance are confirmed. The arrangement among phylogenies can neither be approved nor rejected because the taxonomic surrounding is not yet ascertained.

15 Discussion

15.1 Biscutella laevigata

In case the *B. laevigata* copies have only diverged recently what is suggested via phylogenetic and divergence-times approach, then the reason for their resemblance is simply a lack of time as duplicates initially are redundant. Within the last 8 mya (see supplementary material S1-S9) *Biscutella laevigata* copies separated and started to diverge only around 0.5 mya. This time-span was not sufficient to accumulate an appropriate number of mutations to apparently let the sequences appear diverged.

If they have evolved via concerted evolution, the two sequence groups of *Biscutella laevigate* depict a more ancient duplication event followed by recombination and gene conversion. Ectopic or nonallelic gene conversion is said to be found among all species among all loci examined in detail (GRAUR 1985), where biased gene conversion appears more often than unbiased, meaning that chances of converting a sequence into another are equally distributed. This mode of evolution describes the fact that repeats within a genome appear more related to each other than the respective orthologs in neighbouring species. The molecular process which is responsible for this is homogenisation (DOVER 1982) of a set of nonallelic (ectopic) homologous sequences.

The third hypothesis which could be applied on the history of *B. laevigata* derives from an observation already executed in the late 1960s (BRESINSKY & GRAU 1970, SCHÖNFELDER 1968). It could be observed that *Biscutella laevigata* depicts two patterns with reference to its ploidy level. Outside the Alps any *B. laevigata* examined showed a simple diploid genome with 2n = 2x = 18, while populations distributed among the Alps depict a tetraploid genome, most likely due to autopolyploidisation (mesoneopolyploid). Therefore it is expected that the two sets of *Biscutella* present in the analysis at hand depict allelic variation of the *chalcone synthase* gene. As duplication events are accompanied by chromosomal rearrangements, it is suggested that the variance among the two sets is due to the effect. Alternatively, selective pressure acts differently on the duplicated loci after the polyploidisation event.

One gene is not in responsibility for only one function, but is capably of multifunctionalisation (CONRAD & ANTONARAKIS 2007). Hence, subfunctionalisation, the division of the ancestral functions by maintaining different parts of the gene, is thought to appear more often than previously expected (DE SMET & VAN DE PEER 2012). This partitioning event is accompanied by segmental gene silencing subsequently leading to the formation of paralogs that are no longer duplicates (CONRAD & ANTONARAKIS 2007), as each gene is carrying its individual function. Both outcomes of functional divergence support the draft that either *B. laevigata* versions will be kept within the genome. The two sequence types deviate from each other but within each type they show highly conserved progression.

This suggests that both copies are functional versions, either coding a slightly different function, suggesting that the *chs* gene in *Biscutella* underwent functional divergence leading to neofunctionalisation (DICKINSON et al. 2012). This adaptive mutational process resulting in the preservation of an additional function, not abounding the ancestral gene (OHNO et al. 1968). As this occurrence is thought to be free of selective pressure due to the fact that one copy accumulates the mutations while the other retains the ancestral gene function (RUBY et al. 2007, SEMON & WOLFE 2008). This is promoted by the fact that ten point mutations in the coding region could be detected, all perfectly assignable to the two respective sequence types. An amount of 75% sequence identity is definitely speaking for the fact that allelic variation among one locus is not sufficient to explain the gene tree phylogeny regarding BISC.

15.2 Bivonaea lutea

Bivonaea is assigned to the monotypic tribe Bivoneeae and its unique status was recently demonstrated outside the tribe Cochlearieae (KOCH 2012, KOCH & MARHOLD 2012, WARWICK et al. 2010). However, it was shown that *B. lutaea* regardless describes affinities to *Cochlearia* and *Ionopsidium* and tribes from lineage II. This, partly, can be confirmed here, as BIVO and COCH depict a close sister relationship with a bootstrap value of 96% (NJ), but only within the not adjusted datasets. One arrangement of Cochlearieae and Bivoneeae are placed at a relatively basal position outside the core lineages I to III. As COCH is officially assigned to the expanded lineage II (FRANZKE et al. 2011). In the data at hand only one *B. lutaea* collocation could be observed what consequently leads to the question which of the supposed arrangement situations describes the authentic *chalcone synthase*. Argumentations can be drawn from different angles of view. Either the basal position outside the majority of expanded lineage II members is correct and, consequently, the rejected COCH positioning was misleadingly be excluded or the establishment of *Bivonaea* describes a *chs*-like copy while the expected position is near the other COCH DNA sequence within the majority of expanded lineage II members. A third argumentation would be in favour of the duplication theory. In case *Bivonaea lutea* had undergone an individual duplication event or maybe in concerted manner with COCH, than its arrangement would suggest that the second locus is still available in the genome but was plainly not attained in the experimental phase. This rather suggests that both loci are functional as remaining in the genome. Considering the fact that the sequence locality of *Bivonaea* gained, displays an actuality, then this locus is rather unexpected while most likely resulting from an ancient WGD. For an unsettled reason this tribe retained the duplicated *chs* locus while the standard locus was excluded from the genome.

It has already been suggested that the most evident back drop for this situation derives from either the fact that both *chs* loci of that species or even tribe were kept within genome. This hides to functional divergence, more precise, subfunctionalisation.

But as the phylogenetic positions was recently proposed to be still uncertain (WARWICK et al. 2010), no terminal conclusions can be drawn, least concerning taxonomic consequences.

15.3 Calepina irregularis and Conringia planisiliqua

The monotypic genus *Calepina* was traditionally placed outside the Brassiceae, while *Conringia* has been separated from the tribe. Both have been demonstrated to not have undergone the allohexaploidy event (triplication) which could be verified for the BRAS (KOCH et al. 2001, WARWICK & SAUDER 2005) around 7.9-14.6 mya (LYSAK et al. 2005). *Calepina* as well as *Conringia* are assigned to the expanded lineage II which, in ITS phylogeny, arranges most obviously not within lineage II. The *chalcone synthase* phylogeny suggests that both tribes integrate into lineage II but as independent tribes. The internal branching pattern of that group confirms the core lineage (BRAS, ISAT, SISY, THEL), approved with 99% bootstrap and the corresponding sister relationship of the tribes EUTR and THLA (90%). *Calepina* arranges among the sister group, while *Conringia* clusters next to ISAT, supporting previous hypotheses based on *trn*F (KOCH et al. 2007). *C. planisiliqua* has been discussed intensively in previous studies (ANDERSON & WARWICK 1999, WARWICK & SAUDER 2005) but could not be meaningfully be associated to another position than a member of expanded lineage II.

15.4 Iberis saxatilis

Although diverse sequence residues among *I. semperflorens* could be detected and a duplication event is suggested, the tribe IBER is monophyletic in all illustrations and estimations and consequently holds the identical basal rank in all models applied. IBER arranges outside the core phylogeny and argues for a shared most recent common ancestor with Aethionemeae, which is quite unexpected, as the latter named is expected to be the most basal genus among the Brassicaceae. Although it was recently suggested that the AETH also underwent the last whole genome duplication (HAUDRY et al. 2013), his indicates that both *chalcone synthase* copies from IBER and AETH derive from the same whole genome duplication event (*At*-α). Both tribes do not come up with additional loci arranged at another position within the gene trees. Because the Iberideae are assigned to expanded lineage II, a more recent directive, near other tribes determined as expanded lineage II members, would be awaited. This either implies that the sequences of the genus *Iberis,* or even tribe IBER, hold an additional locus for the *chalcone synthase* gene which could not be isolated and identified during the experimental phase, or, this tribe (or genus) plainly maintained one of the paralogs after the duplication event, like most of the other tribes, as well. The difference with IBER therefore is, that the other locus, compared to the majority of all mustards, remained within the genome. Immediately after the polyploidisation, paralogs are redundant, as they are genes related via duplication (KOONIN 2005). Therefore, it should not have any consequences which of the loci is chosen to remain within the genome. The phylogenetic estimations argue for the fact that the decision which locus to keep does not happen accidentally, otherwise a complete rearrangement of the gene tree would be the outcome. The original locus, which serves as matrix, is determined as the preferred one. As the tribe stood out with its high AT content, it can be argued that this resulted in lower amount of possible nucleotide combinations yielding to a diverged DNA sequence. However, the source for this increased AT content cannot be appointed. Cause and effect cannot clearly be distinguished.

In case the available *chalcone synthase* sequences display only one member of a gene family and another assumed member would be co-resident, the here available data would expose an accelerated synonymous substitution rate, due to the fact that evolutionary pressure would not appeal on the paralog. However, the estimated rate mounts up to 4.77 x 10^{-9} synonymous substitutions per site per year. This rate is even slower than calculated rates from the study at hand and close to the slowest rate (8 x 10^{-9}) published for that gene.

To make meaningful interpretation of the outcome, a duplication event leaving both copies among the genome can be excluded while it is argued for a single copy locus, which exclusively experienced evolutionary incidents, leaving its traces via mutations on the discussed copy.

15.5 Unassigned taxa Fourraea alpina and Ricotia lunaria

Whether *Fourraea alpina*'s arrangement among the *chalcone synthase* cladogram complies its real position within the Brassicaceae family cannot be revealed here. This is due to the fact, that the nuclear marker gene, like demonstrated in the employment at hand, has to be handled with a certain care and should not be addressed as stand-alone reference for phylogenetic re-arrangements. Moreover, the amount of data for that species is only moderate and therefore not appropriate to draw valid conclusions from. Though, *Fourraea alpine* has been employed in previous reviews, its position is not completely unravelled, yet. Besides, this is not meant to be a phylogenetic study, so no additional information about morphological data is available to conclude with reasonable statements. The majority of research, although the species has not been intensively under examination, suggests an arrangement in the nearer surrounding of lineage II, close to Sisymbrieae (HEENAN et al. 2002, KOCH et al. 2001). Rotation of the sequences around the node from lineage II also results in the arrangement of *Fourraea* close to SISY.

Ricotia lunaria, which is also net assigned to a tribe yet. Judging on amount of released announcements, *Ricotia* is not in the centre of interest, which hampers the evaluation of the findings received here. From the results gained in this study, the molecular marker *chs* suggests for the unassigned specimen *Ricotia lunaria* an arrangement close to BISC, which does also not account to a lineage or tribe. *Biscutella laevigata*, the representative utilised, arranges at a very basal position outside the core phylogeny.

Part 5: All Done and Dusted – the End of Trouble?

16 Pitfalls – do always look twice

All tribes and genera causing ambiguities at the phylogenetic level have been investigated and, where necessary, been excluded from the analysis. This effort resulted in an adjusted data set, which is supposed to unravel inter- and intra-tribal relationships among the Brassicaceae family. Some undissolved taxonomic and phylogenetic peculiarities have been remaining in the data at hand, leaving projection for further investigations.

However, this is due to the fact that phylogenetic reconstructions were all based on the nuclear-coding *chs* gene, as this is state of the art. Although it has already been figured out in the employment at hand that variability, especially among the non-coding gene parts, are present in some if not in many of the sustained sequences. Therefore, a second view on the investigated data is inevitable in case it is designated to attach importance to the complete information provided by the DNA material.

Thus all DNA sequences were screened separately to find intra-species delimitation. This initially was done by visual inspection of each group of sequences assigned to one species. Especially the comparison of the complete gene promptly reveals potential candidates showing off sequence divergence. This, firstly, can be spotted by variations in the sequence length (mostly insertions and/or deletions) and, secondly by nucleotide composition. An absolute threshold can hardly be defined, as the range of DNA sequences at hand displays a huge amount of species with no appropriate background information. No rule of thumb or definitions are reliably confirmed to clearly discriminate whether gene sequence divergence derives from a recent or ancient duplication event or from a single ancestral gene in the last common ancestor. The evolutionary history of genes and genomes depicts combinations of speciation and duplication events, intermingled with horizontal gene transfer (HGT), gene gain and loss as well as rearrangement events aggravating to keep track with individual incidences. These complex webs of relationships can best be understood if the usage and application of imprinted key terms proceed coherently.

Thus, it is of fundamental priority to distinct at least between the two subcategories of homologs, namely orthologs and paralogs, as it is critical for reconstructions of robust evolutionary classifications of genes.

tribe	species	n A/P	id cd	id com	effected region	n	2n	1C
ALYSSOP	*A. mollis*	4A	98.6	98	complete gene slightly variable	8	16	0.19
ARAB	*A. soyeri*	2A	99.2	99.2	complete gene slightly variable	8	16	0.32
ARAB	*A. alpina*	2P	93.2	94.6	high variability among complete gene	8	16	0.38
CALE	*C. irregularis*	2P	98.1	93.0	promoter and intron variable	14, 21	14, 28	0.21-0.38
CARD	*C. glacialis*	2P	98.0	98.9	promoter and intron variable	36, 72	64, 72	1.06
CARD	*L. alabamica*	3P	91.2	90.1	complete gene variable	11	n/a	n/a
COLU	*N. brachypetala*	2P	85.8	99.4	promoter and intron variable	n/a	14	0.14-0.35
DESC	*D. sophia*	2P	97.2	97.4	complete gene variable	7, 14	14, 28	0.31
HESP	*H. matronalis*	2P	98.2	99.3	promoter variable	7, 12, 14, 24	14, 24, 28	8.3
IBER	*I. semperflorens*	2P	98.2	99.5	promoter and intron variable	11	22, 44	0.46
LEPI	*L. hirtum*	2P/A?	99.6	99.5	coding region variable	4, 8	16	0.2
MEGA	*M. polyandra*	2P	92.4	57.1	promoter and intron variable, coding region slightly	n/a	n/a	n/a
MICR	*P. cheesemanii*	2P	90.2	91.2	promoter and intron variable, coding region slightly	n/a	20	n/a
SISY	*S. maurum*	2P	98.2	99.3	promoter and intron variable	n/a	14	0.39
STEV	*P. turrita*	2P	79.1	82.7	promoter and intron variable	8	16	0.38
THLA	*P. glaucophylla*	2P	95.4	99.9	promoter and intron variable	7	14	0.29
TURR	*T. laxa*	3A	98.8	99.8	slight variability among coding region	6	14	n/a
COCH	*C. tatrae*	3P	48.4	88.5	high variability among complete gene	7	34, 42	0.99-1.05
COCH	*I. abulense*	2P	85.1	88.9	high variability among complete gene	n/a	28	0.53

Table 23. Species showing divergence among the *chs* gene not conspicuous in gene tree phylogenies. Listed are numbers of alleles or paralogs respectively, as well as their identity (in %) among the coding and complete gene. Haploid (n) and diploid (2n) chromosome number counts as well as the haploid genome size (1C value in pg). Data collected from BrassiBase's cytogenetics tool (Kiefer et al. 2014). N/a = not available.

First hint to decide whether a sequence infers orthology is the functionality of the promoter region which was investigated prior in the work at hand. Intra-species diverged promoter regions therefore allude to conflicting evolutionary scenarios requiring further investigations ("promoter-argument"). In addition to that, intra-species sequence identities are supposed to be very high among orthologous sequences. As already mentioned, a value could not be finalised to a certain boundary thoroughly indicating vertical descent from an single ancestral gene (KOONIN 2005). But previous work (KOCH et al. 2000) argues for a differentiation boarder dividing sequences via estimated identities into alleles or diverged loci. An intra-species distance $p < 1.0$ argues for allelic variation among two DNA sequences of one species, meaning that both slightly diverged homologs display two alleles of one locus and therefore are orthologs ("identity-argument"). In case the sequences divergence exceeds this latitude, it can be indicated that two diverged loci within a gene family are present among one genome, automatically plead for a duplication event. Thus, these sequences typically characterise paralogous genes. Though, a clear distinction, even coherently defined in theory, is not absolutely decisive.

Table 23 investigated species which stood out after visual inspection. Note that a certain amount of tribes listed has already been investigated within another context (ARAB, MEGA, MICR, IBER, COCH) due to the fact that their moderately identity was obviously present in gene tree representations. However, examples listed in that table are all those copies which were not suspected to bear troublesome content.

A. soyeri, A. mollis, as well as *T. laxa,* marked in dark grey did not appear to hold unexpected evolutionary incident resulting from its phylogenetic gene tree reconstructions. Although ARAB as well as TURR, however, demonstrated intra-tribal complications, those species arranged well within the tree. Only via visual inspection differences became peculiar. In spite of variability among the complete gene, including the coding parts, it is concluded that those species solely show allelic variation as no additional evidence could be gathered to argument for the opposite.

The collected data hints to several, partly contradicting theories. All species in the non-marked fields depict variations within the non-coding regions, namely the promoter as well as the intron in the majority of species. Following the argumentations made, these species depict paralogous *chs* copies and therefore underwent a duplication event. It can be argued that the amount of divergence, here identity, can be adducted for a rough determination for the duplication event. As the accumulation of mutations takes a certain amount of time, a thoroughgoing argumentation can be drawn: the more the genes are diverged, the longer the duplication event dates back. Obviously, no absolute numbers can be named here. Therefore *Pseudoturritis turrita (*82.7% identity), as well as *Calepina irregularis* (93% identity) underwent a more ancient duplication than the remaining marked species. Actually, some of these data show identities above the suggested 99% agreeing in one argument, disagreeing in the other. However, the identities of the complete gene show slightly up to moderately less identity, most likely indicating recent polyploidisation events. The sequences of the duplicated loci are also named inparalogs (symparalogs) as they evolved recently and subsequent to a speciation event.

All species highlighted in light grey display further diverged sequences ranging from slight variability among the complete gene, over modifications only concerning the coding region up to complete highly diverged intra-species sequences. They were combined in one group as they all demonstrate variability having reached the coding exonic gene parts. As the exonic region encodes the protein, changes and substitutions within its sequences can result in severe transformations of the gene's function and functionality. Hence, mutations can only be accumulated in an evolutionary neglected copy of the functional gene wrecking its capacity. With exception of *L. hirtum*, which a) shows a very high sequence identity and b) depicts only variability within the coding region, all other marked data from that grouping most definitely portrays two, partly even three, paralogous sequences., while *Lepidium* could also illustrate allelic variation. Within these species *chs* is actually not single-copy. For *Leavenworthia alabamica*, e.g., three syntenic *chs* loci have been detected which derive from three genomic regions describing the outcome of a triplication event (Schranz, pers. comm.), also supported via *adh* (alcohol dehydrogenase). It is argued that the genus *Leavenworthia* (CHARLESWORTH et al. 1998) underwent the polyploidisation event after its speciation (KOCH et al. 2000), making it symparalogs. Granted, but measured on their advanced divergence, it is feasible that the duplicated copies will undergo functional divergence resulting in pseudogenisation and gene loss. With *Arabis alpina*, the interpretation is straight forward. The distinct *chs* loci derive from different accessions originating from Europe (AF112084) and Africa (AF112083), comparing different populations instead of intra-genomic data (KOCH et al. 2000).

To sum this up, patterns of diversity cannot directly be applied on certain evolutionary events. As is also cannot immediately be concluded from the known ploidy level whether a locus can or cannot be existent as duplicated. As it has been proven that the Brassicaceae depict a tendency for polyploidisation as well as subsequent polyploidisation (KASHKUSH et al. 2002, LYNCH & CONERY 2000, MA & GUSTAFSON 2005, WOLFE 2001) it can be argued that single gene loci demonstrate a comparable behaviour. A certain trend can be observed showing that species with unknown (*M. polyandra, M. cheesemanii*) or unclear (*C. irregularis, C. glacialis, H. matronalis*) chromosome amount and consequently unknown ploidy level, as well as those, where polyploidy is reported, more often tend to demonstrate duplicated loci. And those sequences which are known to be of diploid origin (*A. mollis, A. soyeri, T. laxa*) display higher intra-species identity indicating that the data displays rather allelic variation than duplicated gene loci.

The overall rule of thumb, summarising this table, seems to be that non-coding regions diverge prior to coding regions, starting with the promoter, while lower identities indicate more ancient polyploidisations. In other words: A promoter region with 99% or less intra-species identity indicates the youngest duplicated loci, while a complete diverged gene (non-coding and coding) with lower intra-species identities (~80%) argues for a more ancient duplication event. However, the data presented does not demonstrate a thoroughgoing strand revealing blatantly evolutionary rules applicable on every kind of sequence.

17 Conclusion

From the data investigated it became clear that the inspection of nuclear genomic DNA adheres exceedingly more direct information, as well as indirect suggestions or hints, than previously thought. The most assistant approaches were gathered in the following table.

what you find	what it means
1) AT% > GC%	very basal phylogenetic arrangement, old maintained copy
2) bp intron$_A$ (species$_A$) ≠ bp intron$_B$ (species$_A$)	different loci, longer intron suggests paralogy
3) if 2 is valid than bp promoter$_A$ (species$_A$) ≠ bp promoter$_B$ (species$_A$)	different loci, longer intron and diverse promoter suggests paralogy
4) codon 2 in exon 1 = G	orthologous species assigned to lineage I
5) bp exon1 $_A$ (species$_A$) ≠ bp exon1$_B$ (species$_A$)	different loci
6) exon 2 ≠ 995	no orthologous copy
7) (exon 1 + 2)$_{speciesA}$ = (exon 1 + 2)$_{speciesA}$	not necessarily one locus, consider non-coding region
8) only promoter diverged	Most recent duplication

Table 24. Checklist for simple manual sequence screening of the *chalcone synthase* gene. Note that the meaning cannot unconditionally be applied on any data.

The results from the compiled *chs* data validated to bigger or minor parts in many cases previous results from *ndh*F, *phy*A, IST, *mat*K and mitochondrial *nad*4 intron analysis. The overall tree topology based on *chs* sequences agreed, after selectively sorting the data, quite well with molecular phylogenies of the family published to date. In other words: the majority of the tribes were assigned to lineages I to III, which represent the most well supported groups above the tribal level in any family-based phylogenetic study these days (LIU et al. 2012). However, this indicates that the molecular marker discussed here is not perfectly appropriate to reveal phylogenetic relationships on the genus and species level, at least not without additional marker systems resolving the data on a small-scale register. *Chs* is not suitable for resolving deep phylogenetic relationships between tribes and between groups of tribes. But, as mentioned right at the beginning, it was never intended to employ a single-locus study for phylogenetic resolutions.

Moreover, it was intended to combine phylogenetic and molecular genetic perspectives to gain insight into the gene and genome evolution of the Brassicaceeae. Fundamental mechanisms were illuminated which could have led to the present situations investigated at the data at hand. This nuclear encoded gene is capable of delivering further insight into processes affecting the evolutionary fate of duplicated genome regions and to draw conclusion from. Divers evolutionary steps within the cyclical process of duplication and divergence can be described within the tribes of the mustard family, which is of major help to resolve and redraw the tribal or even taxa specific evolutionary history.

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An anagram of nuclear is unclear (Karl, pers. comm.).

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Appendix

provided on DVD

Figures

All figures listed in this thesis (see list of figures) are displayed in graphical format (svg or png)

Tables

All tables listed in this thesis (see list of tables) are displayed as pdf.

Supplementary Material

Supplementary material contains figures, as well as tables, referred to in this thesis. Data format see above. Supplementary material is numbered consecutively with prefixed capital S.