The phylogeny of smut fungi (Ustilaginomycotina)

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‘Show me a plant pathogen and I will show you a species complex’

(Crous & Groenewald 2005)

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Statement of Original Authorship

The work contained in this thesis has not been previously submitted for a degree or diploma in any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by any other person, expect where specific references are made.

Signed:

Date:

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Keywords

asexual smuts, cryptic species*, Entyloma,* ITS*, Moesziomyces*, molecular analysis, multilocus, new species, one fungus on name, phylogeny, protein coding genes, smut fungi, smut specific primer, species complex, taxonomy, *Ustilago*, Ustilaginomycotina, yeasts

Summary

Smut fungi (Ustilaginomycotina) were previously defined as plant parasites that produced blackish or brownish masses of teliospores in or on various organs of plants. Each teliospore germinates to form a single basidium with usually four basidiospores that subsequently grow as a saprobic, yeast-like, haploid stage. The Ustilaginomycotina are a highly diverse group with about 1,700 species in 115 different genera. All of the species were united in a single order, the Ustilaginales, in late 19th century. These teliospore producing fungi are now considered the classic smut fungi. Towards the end of the 20th century, new ideas were brought into this classification system. Most notable was the comparative work regarding the ultrastructure of septal pores and the anatomy of the interaction zones between host and parasite. This work changed the whole concept of smut fungi and their evolutionary relationships. These results were subsequently supported by molecular phylogenetic studies. Both lines of investigation led to the classification of the smut fungi into four different classes, Ustilaginomycetes, Exobasidiomycetes, Malasseziomycetes and Moniliellomycetes (see chapter 1.3).

A reliable taxonomy that reflects phylogenies needed in order to estimate the diversity and the relationships between the diverse groups of smut fungi. In the last 20 years, molecular investigations based mostly on rDNA loci, e.g. ITS (internal transcribed spacer) or LSU (large subunit), have revealed the evolutionary relationships between many taxa of smut fungi. However, there are few phylogenetic studies available for smut fungi (see chapter 1.5.1), and much work is needed to develop backbone phylogenetic trees and to resolve species complexes of many smut fungi.

This thesis reports the results of six different studies that aimed to develop new and improved tools for the phylogenetic analyses of smut fungi, and then apply these methods to selected groups of smut fungi. The first study (Kruse et al. 2017a, Chapter 3) developed a method to improve the amplification of ITS sequences of some smut fungi. Due to its high discrimination value, the ITS gene region is widely used as a barcoding locus for species delimitation of fungi. For this purpose, the general ITS primers ITS1 and ITS4 or more specific modifications, e.g. ITS1F for Ascomycota, ITS4B for Basidiomycota or M-ITS1 for smut fungi, were used. As these primer combinations often yielded unsatisfactory results, due to coamplification of other

(contaminant) fungi or the host plant DNA, improvement of the amplification of the ITS region was needed. In order to design new smut specific primers for the ITS region, a representative set of several sequences of the flanking regions of the ITS region (LSU and SSU) of smut fungi, plants and other fungi were downloaded from GenBank. A set of primers was designed on this dataset. These primers were tested on a representative set of about 70 different smut genera under different PCR conditions. Finally, three different primers, one forward primer, smITS-F, and two reverse primers, smITS-R1 and -R2, were selected as the best ones. The following tests with different combinations of these primers, and also under inclusion of the M-ITS1 primer, showed only slight differences in the number of different genera that successfully amplified. But there were some differences regarding the genera that amplified. A broader test on 205 samples in 39 genera showed that the PCR efficiency of the newly designed primers was much better than the primer set ITS4/M-ITS1. With the primers designed in this study almost no non-target ITS was amplified, giving new opportunities especially for amplifying ancient DNA or DNA from older herbarium samples. However, many species groups remain unresolved by only one gene region.

The second study (Kruse et al. 2017c, Chapter 4) found new loci and suitable primers that better resolved multi-locus trees. To date, the most frequently used loci for making multi-locus trees are SSU (small subunit), LSU (large subunit) and ITS (internal transcribed spacer). While the LSU is not always sufficient to distinguish between closely related species, it is highly discriminative above the species level. In an effort to increase the phylogenetic resolution of smut phylogenies, some protein-coding genes were used, including *rpb*1, *rpb*2, and *atp*6 with varying success (see Chapter 2.1.2). As most of these loci are seldom used or sometimes only work on pure cultures because of their low specifity, new protein-coding loci were identified that produced reliable phylogenetic trees. Based on five available genomes, potential gene loci were filtered for possible primers. Initially, 40 different primer combinations for 14 gene loci were tested on a set of twelve different genera of smut fungi. The best candidates were selected and optimized during further tests. Finally, 22 different forward primers and 17 different reverse primers for nine different gene regions were developed, with each differentiating at least one genus of smut fungi (preferably for Ustilaginomycetes). The different primers showed varying discriminative power for different smut genera. They worked best for the Ustilaginaceae, based on the primer designed from Ustilaginomycetes genomes. These new primer sets and loci have the

potential to resolve different species groups within the smut fungi and furthermore to produce reliable phylogenetic trees with high resolution. To prove their applicability, three species complexes were investigated in-depth, two from the Ustilaginomycetes and one from the Exobasidiomycetes.

The smut genus *Moesziomyces*, which was previously monotypic with *Moesziomyces bullatus*, revealed some interesting links between asexual yeasts and sexual smut fungi (Kruse et al. 2017b, Chapter 5). A recent study recombined some species of the asexual yeast-like genus *Pseudozyma* to the sexual smut genus *Moesziomyces*. Our aim was to resolve the species complex of *Moesziomyces*, and apply a phylogenetic species concept, thereby linking asexual yeasts to sexual smuts. For that purpose we examined the phylogenetic position of several different *Moesziomyces* species on different host plants, based on ITS sequences from herbarium samples and cultures, together with reference sequences from GenBank. The phylogenetic tree supported conspecifity of both *Moesziomyces aphidis* and *M. rugulosus* (synonyms *Pseudozyma aphidis* and *P. rugulosa*, respectively) with *Moesziomyces bullatus*. These yeasts likely represent the asexual and apathogenic stage of *Moesziomyces bullatus* which only becomes pathogenic and produces its sexual stage on a specific host. The conspecifity of *M. aphidis* and *M. rugulosus* with *M. bullatus* had remained unnoticed as sequences of *M. bullatus* on the type host *Echinochloa crus-galli*, are unavailable on GenBank.

Ustilago striiformis, a leaf stripe forming smut with single spores on several different Poaceae host species, has received little systematic attention. It is difficult to discriminate different species within this complex based on morphology alone (macroscopic and microscopic characters). The advantages of the newly designed primers for the Ustilaginales (Kruse et al. 2018a, see Chapter 6) were seen when applied to the *U. striiformis* species comlex. For this purpose we extracted DNA from about 90 different smut species in the *U. striiformis* complex and several other related species and genera, including some other leaf stripe forming smuts in *Ustilago*. Amplification and sequencing was done with the newly designed primer sets (Kruse et al. 2017a, 2018a). The outcome allowed a construction of two different phylogenies, one based on nine different loci with 93 samples and another one based on three different loci with 70 samples. The topology of both trees was similar in that *U. striiformis* was comprised of several polyphyletic species. *Ustilago serpens* was also found to be a species complex. Other stripe smuts from the genus *Ustilago* formed

sister clades. Within these species groups, the new primer sets were able to distinguish between different smuts on various host plant species, with mostly high statistical support. As many smut fungi are known to be host specific (see Chapter 1.5.3), the different species within this complex were distinguished by a combination of host species, spore morphology, recognition of monophyletic groups and molecular phylogeny. Three new species were described (two from the *U. striiformis*-complex, one from the *U. serpens*-complex) and some rarely used names were resurrected. During the literature review, it was found that *U. salweyi* had priority over *U. striiformis* on the type host species *Holcus lanatus*, because this name was published two years earlier.

The Exobasidiomycetes contain several species complexes, which await resolution. Such complexes hide many cryptic species and diverse fungi (see Chapter 1.5.3). One example is the genus *Entyloma*, which has five different species on *Ranunculus* (incl. *Ficaria*). Three of the species have a broad host range (*E. microsporum*, *E. ranunculi-repentis* and *E. verruculosum*). The other two species only occur on *Ficaria verna*. As the newly developed primer sets only worked on a small set of Exobasidiomycetes, new primers were designed, based on the methods of Kruse et al. (2018a) (see Chapter 8). With the availability of the *Microstroma album* genome as representative of the Exobasidiomycetes, primers were designed within the gene loci already used for *Ustilago striiformis*. New primers for two of the gene regions (*ssc*1 and *map*) from Kruse et al. (2018a) amplified the target DNA. Based on the combination of morphological, ecological and molecular markers, known as Consolidated Species Concept (CSC), different smut species could be distinguished within two different species complexes, *Entyloma ranunculi-repentis* and *E. microsporum* (Kruse et al. 2018b). Two multilocus trees, one based on two loci and the other on four loci, revealed several monophyletic clades with medium to high statistical support. After in-depth analysis of macroscopic and microscopic characters and phylogentic analyses, 12 different lineages representing six new species were found and formally described within these complexes. Furthermore, several neglected names were resurrected from the literature.

Following the abolishment of the dual naming of pleomorphic fungi (one fungus one name, see Chapter 1.4.2), a nomenclatural error was found concerning the name *E. ranunculi-repentis*, which occurs on *Ranunculus repens* (type). This species was recombined to *E. gibbum*, but the type collection of this species represents a chimeric

collection and description of two different smut fungi. Thus this name was proposed for rejection (Kruse & Thines 2017, Chapter 7). *Entyloma eburneum* is the valid name for this species and should be used in future. It was not the first time that such taxonomic problems have occurred during taxonomic research on smut fungi. As previously discussed, the *Ustilago striiformis* complex is represented by the type species *Ustilago striiformis* on *Holcus lanatus.* Following the priority rules this species has to be named *U. salweyi*.

This study on smut fungi resulted in six published papers that show how much work remains to be done in the systematics of smut fungi. Since many smut fungi only have subtle morphological differences, the host range and molecular phylogenetic approaches are essential tools for defining species. The findings outlined in this thesis verify the importance of molecular phylogenetics based on multilocus analysis to discriminate species complexes and cryptic species. With the improved tools reflected by the new primer sets (ITS and protein coding loci) it is now possible to delve deeper into the evolutionary history of smut fungi and reveal their diversity. Further, the description of new species has practical impacts for biological control, disease management and biosecurity.

Zusammenfassung

Brandpilze (Ustilaginomycotina) wurden früher als Parasiten definiert, die schwarze oder braune Sporenmasse an oder in unterschiedlichen Pflanzenteilen oder Pflanzenorganen ausbilden. Sie keimen durch die Ausbildung einer Basidie mit Basidiosporen, die danach als saprophytisches Hefestadium in ihrer Haplophase wachsen. Die Ustilaginomycotina sind eine sehr artenreiche Gruppe mit weltweit bisher ca. 1.700 bekannten Arten aus insgesamt 115 verschiedenen Gattungen. Alle diese Arten wurden früher (im späten 19. Jahrhundert) unter einer einzigen Ordnung vereint – den Ustilaginales. Pilze mit diesem Aussehen und dieser Entwicklung gehören zu den Brandpilzen, die Teliosporen produzieren, oder den so genannten „klassischen Brandpilzen“. Erst viele Jahre später, seit Ende des 20. Jahrhunderts, kamen neue Ideen zur Klassifizierung dieser Brände auf. Ein großer Meilenstein war die vergleichende Arbeit über die Septalporen und Interaktionszonen zwischen Wirt und Parasit. Diese Entdeckung einer enormen Strukturdiversität innerhalb der Brandpilze veränderte das gesamte Konzept der Brandpilze und ihrer Verwandten. Die Ergebnisse wurden weiterhin durch phylogenetische Studien bestätigt. Die Unterschiede, die in beiden Studien aufgezeigt wurden, führten zu einer Aufspaltung der Ustilaginales in zwei verschiedene Linien. Es wurde gezeigt, dass innerhalb der Brandpilze derzeit vier verschiedene Klassen existieren: Ustilaginomycetes, Exobasidiomycetes, Malasseziomycetes und Moniliellomycetes (vgl. Kapitel 1.3).

Eine glaubwürdige Taxonomie und plausible phylogenetische Stammbäume sind sehr wichtig, um die Diversität und die Verwandtschaftsverhältnisse der verschiedenen Gruppen der Brandpilze zu verstehen. Seit den letzten 20 Jahren werden molekulargenetische Untersuchungen immer populärer und auch für Brandpilze gibt es bereits mehrere Ansätze, die Verwandtschaftsverhältnisse dieser Gruppe aufzudecken, meist basierend auf ribosomalen DNA-Genorten wie ITS (internal transcribed spacer) oder LSU (large subunit). Obwohl mehrere Studien zu Brandpilzen im Allgemeinen oder zu spezifische Gruppen innerhalb dieser existieren (vgl. Kapitel 1.5.1), gibt es dennoch viel zu tun in diesem Themenfeld, um sowohl innerhalb der phylogenetischen Stammbäume bessere Auflösungen vom Rückgrat zu bekommen, als auch die Auflösung zahlreicher Artkomplexe innerhalb der Brandpilze zu verbessern.

Aktuell gibt es auf dem Feld der phylogenetischen Stammbäume nur wenig Bewegung innerhalb der Brandpilze, um diese zu verbessern. Deshalb dient diese Arbeit vor allem der Verbesserung der Werkzeuge, Methoden und Ideen für bestimmte Brandpilzgruppen. Die Untersuchungsergebnisse wurden in insgesamt sechs verschiedenen Publikationen zusammengefasst und dargestellt, mit jeweils unterschiedlichen Themen innerhalb der Brandpilze als Schwerpunkt.

In der ersten Ausarbeitung (Kruse et al. 2017a, Kapitel 3) geht es um die Verbesserung der Amplifikation der ITS-Sequenzen verschiedener Brandpilze. Wegen seiner hohen Unterscheidungsrate wird dieser Genort vorzugsweise als barcoding locus für die Artabgrenzung innerhalb der Pilze verwendet. Meist wurden dafür vor allem die allgemeinen ITS-Primer ITS1 und ITS4 oder die vielfach spezifischeren Modifikationen, z. B. ITS1 für Ascomyzeten, ITS4B für Basidiomyzeten oder M-ITS1 für Brandpilze, benutzt. Da diese Primer-Kombinationen öfters Ergebnisse liefern, die nicht zufriedenstellen, weil andere Pilze (Kontamination) oder die Wirtspflanze amplifiziert werden, war eine Verbesserung der Amplifikation der ITS-Regionen dringend notwendig. Für das Design neuer brandpilzspezifischer Primer für die ITS-Region wurde ein repräsentatives Set verschiedener Sequenzen (flankierende Regionen der ITS: LSU und SSU) von Brandpilzen, Pflanzen und anderen Pilzen von GenBank heruntergeladen und ein neues Set verschiedener potentieller neuer ITS-Primer designt. Diese wurden an einem repräsentativen Datenset von ungefähr 70 verschiedenen Brandpilzgattungen unter verschiedenen PCR-Bedingungen getestet. Schlussendlich wurden drei verschiedene Primer als vielversprechend ausgewählt: ein Vorwärtsprimer (smITS-F) und zwei Rückwärtsprimer (smITS-R1 und R2). Die nachfolgenden Tests mit verschiedenen Kombinationen dieser drei Primer und auch Kombinationen mit dem brandpilzspezifischen Primer M-ITS1 zeigten nur geringe Unterschiede in der Menge der verschiedenen amplifizierten Gattungen. Dennoch zeigten sich Unterschiede darin, welche Gattungen bevorzugt amplifiziert wurden. Ein weiterer, größerer angelegter Test mit insgesamt 205 verschiedenen Brandpilzarten aus insgesamt 39 verschiedenen Gattungen zeigte, dass die PCR-Effizienz bei der Amplifikation mit den neu designten Primern vielfach besser war als mit dem bisher verwendeten Primerset ITS4/M-ITS1. Mit Hilfe der in dieser Studie neu entwickelten Primer wurde fast keine Nicht-Ziel-ITS amplifiziert, was vor allem im Bereich von historischen Proben oder Proben aus Sammlungen (Herbarium) neue Möglichkeiten

bietet. Dennoch ist ein Genort für die meisten Artkomplexe innerhalb der Brandpilze nicht ausreichend, um diese aufzulösen.

Deshalb wurde in der zweiten Studie (Kruse et al. 2017c, Kapitel 4) der Versuch unternommen, neue Genorte und vielversprechende Primer zu finden, um die Auflösung von Multigen-Bäumen zu verbessern. Momentan sind die am häufigsten verwendeten Genorte für Multilocus-Studien die SSU (kleine Untereinheit), LSU (große Untereinheit) und ITS (internal transcribed spacer). Während die LSU meist nicht fähig ist, innerhalb nahe verwandter Arten oder in Artkomplexen zu unterscheiden, ist dieser Locus sehr gut für die Unterscheidungen außerhalb des Artniveaus geeignet. Bemühungen, die phylogenetische Auflösung innerhalb der Stammbäume von Brandpilzen zu verbessern, führten dazu, dass zusätzlich auch einige protein-kodierende Gene benutzt wurden wie z. B. *rpb*1, *rpb*2, *atp*6 etc. (vgl. Kapitel 2.1.2) mit jeweils unterschiedlichen Erfolgsquoten. Ein Großteil dieser Orte wird bisher eher selten genutzt oder die Amplifikation funktioniert nur bei DNA, die aus Reinkulturen gewonnen wurde. Wegen der geringen Spezifität dieser Orte haben wir neue proteinkodierende Orte identifiziert, die für die Erstellung glaubwürdiger phylogenetischer Stammbäume genutzt werden können. Auf Basis von fünf verfügbaren Genomen haben wir nach potentiellen Genorten und möglichen Primern für die Amplifikation von Brandpilzen gesucht. Dabei wurden zuerst 40 verschiedene Primerkombinationen für 14 verschiedene Genorte an einem Set von zwölf verschiedenen Brandpilzgattungen getestet. Zuletzt konnten insgesamt 22 verschiedene Vorwärtsprimer und 17 verschiedene Rückwärtsprimer für neun verschiedene Genregionen (z.B. *map*, *atp*2, *tif*2 etc.) entwickelt werden, die zumindest für eine Gattung innerhalb der Brandpilze positive Ergebnisse lieferten (vorrangig für die Ustilaginomycetes). Die verschiedenen Primer zeigten unterschiedliche Auflösungsraten für verschiedene Brandpilzgattungen. Für die Familie der Ustilaginaceae konnten die besten Ergebnisse erzielt werden, da die Primer auf Basis von Ustilaginomyceten-Genomen entwickelt wurden. Die neuen Primersets und Genorte bieten neue Möglichkeiten, um verschiedene Artenkomplexe innerhalb der Brandpilze aufzulösen und glaubwürdige phylogenetische Stammbäume mit hoher Auslösung zu erstellen. Um die Wirksamkeit dieser neuen Primer und Genorte aufzuzeigen, wurden exemplarisch drei verschiedene Artenkomplexe (zwei aus den Ustilaginomyceten und einer aus den Exobasidiomyceten) eingehend analysiert.

Die Untersuchung der Gattung *Moesziomyces*, die bisher monotypisch mit *Moesziomyces bullatus* war, zeigte interessante Verbindungen zwischen asexuellen und sexuellen Hefen (Kruse et al. 2017b, Kapitel 5). In neuester Zeit wurden mehrere Arten der asexuellen Hefegattung *Pseudozyma* in die sexuelle Brandpilzgattung *Moesziomyces* überführt. Unser Ziel war es, den Artenkomplex um *Moesziomyces bullatus* aufzulösen, das bereits existierende Artenkonzept aufzuzeigen und Verbindungen zwischen asexuellen und sexuellen Stadien zu finden. Dafür haben wir, basierend auf ITS-Sequenzen von Herbarproben, Kulturen und Genbank, die phylogenetische Position mehrerer verschiedener *Moesziomyces*-Arten von verschiedenen Wirtspflanzen untersucht. Der daraus resultierende phylogenetische Stammbaum zeigte verschiedene *Moesziomyces*-Arten auf unterschiedlichen Wirtspflanzen, aber ebenso eine Konspezifität von *Moesziomyces aphidis*/*M. rugulosus* [Syn. *Pseudozyma aphidis*/*P. rugulosa*] mit *Moesziomyces bullatus*. Vermutlich stellen diese Hefen das asexuelle und nichtpathogene Stadium von *Moesziomyces bullatus* dar und die Art wird nur bei Vorhandensein eines spezifischen Wirtes pathogen und reproduziert sich dann sexuell. Unglücklicherweise wurde diese Konspezifität bisher nicht erkannt, denn Sequenzen vom Typuswirt von *Moesziomyces bullatus*, *Echinochloa crus-galli*, fehlen in Genbank. Aus diesem Grund wurden *Moesziomyces aphidis* und *M. rugulosus* vormals als eigene Arten beschrieben, obwohl sie identisch mit *Moesziomyces bullatus* sind.

Für *Ustilago striiformis*, einen Streifenbrand mit einzelnen Sporen auf verschiedenen Poaceae als Wirtspflanzen, gibt es bisher nur eine einzige Publikation, die versucht, die systematische Stellung der einzelnen Arten dieses Artenkomplexes aufzulösen. Da diese Auflösung bisher unzureichend ist, haben wir ihn zur Präsentation der Vorteile der von uns neu entwickelten Primer für die Ustilaginales benutzt (Kruse et al. 2018a, Kapitel 6). Es ist sehr schwierig, Arten in diesem Komplex allein auf Basis der Morphologie zu unterscheiden (makroskopische und mikroskopische Merkmale). Deshalb haben wir von ungefähr 90 verschiedenen Aufsammlungen von *Ustilago striiformis* und mehreren weiteren verwandten Arten und Gattungen inklusive weiterer Brandpilze der Gattung *Ustilago* mit streifenartigen Ausprägungen DNA extrahiert. Die Amplifikation und Sequenzierung wurde mit den neuen Primersets durchgeführt (Kruse et al. 2017a, 2018a). Die Ergebnisse erlaubten die Konstruktion zweier verschiedener phylogenetischer Stammbäume – einer basierend auf neun verschiedenen Genorten von 93 Aufsammlungen und ein weiterer

auf Basis dreier verschiedener Genorte von 70 Proben. Beide Bäume führten zur gleichen Schlussfolgerung: *Ustilago striiformis* ist ein polyphyletischer Artenkomplex und auch *U. serpens* stellt an sich einen Artenkomplex dar. Andere Brandpilze mit ähnlichen streifenartigen Brandsymptomen gruppierten als benachbarte Äste. Innerhalb dieser Artengruppe waren die neuen Primersets fähig, zwischen verschiedenen Brandpilzarten auf unterschiedlichen Wirtspflanzen zu unterscheiden, mit oftmals hohen Unterstützungswerten. Da bekannt ist, dass ein Großteil der Brandpilze hochgradig wirtsspezifisch ist (vgl. Kapitel 1.5.3), haben wir die verschiedenen Arten innerhalb dieses Artkomplexes auf Basis einer Kombination von Wirtsart, Sporenmorphologie, Bildung monophyletischer Gruppen und diagnostischer Basen in der Phylogenie unterschieden. Insgesamt haben wir drei neue Arten beschrieben (zwei aus dem *Ustilago-striiformis*-Komplex und eine aus dem *Ustilago-serpens*-Komplex) und mehrere in Vergessenheit geratene Namen wieder aufleben lassen. Während der Literaturrecherche ist uns außerdem aufgefallen, dass *Ustilago salweyi* Priorität vor dem Namen *U. striiformis* auf dem Typuswirt *Holcus lanatus* hat, da letzterer Name zwei Jahre jünger ist.

Aber auch innerhalb der Exobasidiomyceten existieren mehrere Artenkomplexe, die auf eine Auflösung warten. Diese Komplexe beherbergen eine große Anzahl kryptischer Arten und werden einen hohen Einfluss auf die Diversität der Pilz haben (vgl. Kapitel 1.5.3). Ein Beispiel ist die Gattung *Entyloma,* die auf Hahnenfuß (inkl. *Ficaria*) vorkommt, auf die wir uns in einer weiteren Studie fokussieren (Kruse et al. 2018b, Kapitel 8). Derzeit sind fünf verschiedene *Entyloma*-Arten bekannt, die die Gattung *Ranunculus* parasitieren, von denen drei ein weites Wirtsartenspektrum aufweisen (*Entyloma microsporum*, *E. ranunculi-repentis* und *E. verruculosum*). Die anderen beiden Arten sind mit ihrem Vorkommen auf die Gattung *Ficaria* beschränkt. Da das Set von neu entwickelten Primern nur für eine kleine Anzahl von Exobasidiomyceten funktioniert, haben wir neue Primer für diese Brandpilzklasse mit den bereits erläuterten Methoden entwickelt. Mit der zusätzlichen Verfügbarkeit des *Microstroma-album*-Genoms, repräsentativ für die Exobasidiomyceten, waren wir fähig, neue Primer für die bereits erfolgreich bei *Ustilago striiformis* angewandten Genorte zu entwickeln. Wir haben letztendlich neue Primer für zwei der Genregionen (*ssc*1 und *map*) aus Kruse et al. (2018a) entwickelt, die fähig waren, die Ziel-DNA zu amplifizieren. Auf Basis der Kombination von morphologischen, ökologischen und molekularen Markern, bekannt als „Consolidated

Species Concept (CSC)“, konnten unterschiedliche Brandpilzarten in zwei Artenkomplexen unterschieden werden: *Entyloma ranunculi-repentis* und *E. microsporum*. Zwei Multilocus-Bäume, einer basierend auf zwei Genorten und einer auf vier Genorten, zeigten mehre mittel bis hoch unterstützte Äste auf. Nach eingehenden Untersuchungen der makroskopischen und mikroskopischen Merkmale und der Ermittlung diagnostischer Basen konnten wir immerhin zwölf verschiedene Linien finden, die ihrerseits eigene Arten repräsentieren. Von denen konnten wir sechs als neue Arten beschreiben. Weiterhin konnten wir zahlreiche vernachlässigte Namen aus den Synonymlisten wiederbeleben.

Doch auch nomenklatorische Probleme tauchten auf. Da die Regel („Ein Pilz ein Name“, vgl. Kapitel 1.4.2) mittlerweile eine Doppelbenennung pleomorpher Pilze verbietet, konnte ein Fehler bei der Kombination von *Entyloma ranunculi-repentis* auf dem Typus-Wirt *Ranunculus repens* aufgedeckt werden. Diese Art wurde zu *Entyloma gibbum* umkombiniert. Unglücklicherweise repräsentiert die Typuskollektion dieser Art eine chimäre Kollektion und Beschreibung zweier verschiedener Brandpilze. Deshalb haben wir diesen Namen für eine Ablehnung (rejection) vorgeschlagen (Kruse & Thines 2017, vgl. Kapitel 7). *Entyloma eburneum* ist der nächste gültige Name für diese Art und sollte in Zukunft verwendet werden. Es war nicht das erste Mal, dass innerhalb unserer Analysen solche taxonomischen Probleme auftauchten. Der *Ustilago-striiformis*-Komplex ist durch den Typus *Ustilago striiformis* auf *Holcus lanatus* repräsentiert. Wenn man den Prioritätenregeln folgt, muss die Art jedoch *Ustilago salweyi* heißen.

Insgesamt lässt sich sagen, dass diese Arbeit über Brandpilze, die hier in sechs verschiedenen Ausfertigungen dargestellt ist, zeigt, wie fließend das System der Brandpilze momentan ist und dass immer noch viel zu tun ist, um die phylogenetischen Werkzeuge zu verbessern. Da viele Brandpilze nur geringe morphologische Unterschiede haben, sind phylogenetische Ansätze in Kombination mit der Wirtspflanze gute Werkzeuge, um eine Art zu definieren. Die Untersuchungsergebnisse dieser Arbeit zeigen, wie wichtig molekulare Phylogenien basierend auf Multilocus-Analysen sind, um glaubwürdige Bäume zu erhalten und Artenkomplexe sowie kryptische Arten zu unterscheiden. Mit den verbesserten Werkzeugen, die durch die neuen und verbesserten Primersets präsentiert werden (ITS und proteinkodierende Orte), ist es jetzt möglich, noch tiefer in verschiedene Brandpilzarten oder -artenkomplexe einzutauchen und ihre phylogenetische Position

im System zu klären. Eine glaubwürdige Taxonomie ist sehr wichtig, um die Diversität der Brandpilze zu verstehen. Weiterhin haben diese neuen Artdefinitionen einen praktischen Nutzen für die biologische Bekämpfung von Pflanzenkrankheiten.

Preliminary Remarks

The work presented in this thesis was supervised by Prof. Dr. Marco Thines from the Johann Wolfgang Goethe University, Frankfurt am Main, and was carried out in his working group at the Biodiversity and Climate Research Centre (BiK-F) Senckenberg Frankfurt in the period of October 2013 to March 2018. All parts of this work have already been published in international peer-reviewed journals. These articles and further articles can be found in the following publication list.

Publications (included in this thesis)

1. **Kruse J**, Choi YJ, Thines M (2017a) New smut-specific primers for the ITS barcoding of Ustilaginomycotina. *Mycological Progress* **16**: 213–221.

2. **Kruse J**, Doehlemann G, Kemen E, Thines M (2017b) Asexual and sexual morphs of *Moesziomyces* revisited. *IMA Fungus* **8**: 117–129.

3. **Kruse J**, Mishra B, Choi YJ, Sharma R, Thines M (2017c) New smut-specific primers for multilocus genotyping and phylogenetics of Ustilaginaceae. *Mycological Progress* **16**: 917–925.

4. **Kruse J**, Thines M (2017) (2507) Proposal to reject the name *Ramularia gibba* (Ustilaginomycotina: Entylomatales). *Taxon* **66**: 515–516.

5. **Kruse J**, Dietrich W, Zimmermann H, Klenke F, Richter U, et al. (2018a) *Ustilago* species causing leaf-stripe smut revisited. *IMA Fungus* **9**: 49–73.

6. **Kruse J**, Piątek M, Lutz M, Thines M (2018b) Broad host range species in specialised pathogen groups should be treated with suspicion–a case study on *Entyloma* infecting *Ranunculus*. *Persoonia* **41**: 175-201.

Publications (not included in the thesis)

7. Choi YJ, **Kruse J**, Thines M (2017) *Hyaloperonospora erucae* sp. nov. (Peronosporaceae; Oomycota), the downy mildew pathogen of arugula (*Eruca sativa*). *European Journal of Plant Pathology*: https://doi.org/10.1007/s10658-017-1389-0.
8. Görg M, Ploch S, **Kruse J**, Kummer V, Runge F, et al. (2017) Revision of *Plasmopara* (Oomycota, Peronosporales) parasitic to *Impatiens*. *Mycological Progress* **16**: 791–799.

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1 Introduction

1.1 Fungi

All living organisms are classified into one of six Kingdoms, namely, Protista, Eubacteria, Aarchaebacteria, Animalia, Plantae and Fungi. This study considers the classification of some plant parasitic microfungi in the Kingdom Fungi. Fungi are defined as heterotrophic eukaryotes that often form filamentous threads (hyphae). Fungi includes organisms commonly known as mushrooms, yeasts, molds, rusts, smuts, puffballs, truffles, morels, and moulds. These organisms have common morphological and taxonomic traits. Phylogenetic analyses also supports their classification. Fungi also share ecological and physiological characteristics with other organisms including Chromista and Protozoa (Barr 1992).

Presently the number of described fungi (members of the kingdom Fungi, Chromista and Protozoa) is estimated at about 70.000-100.000 species (Hawksworth & Rossman 1997, Kirk et al. 2008). Following Webster & Weber (2007) this number renders the fungi as one of the least explored group of organisms in the world. Many estimates of fungal diversity have been published. Hawksworth (1991) considered that about 1.5 million species of fungi occurred worldwide. Ten years later Hawksworth (2001) revised this to 2.3 million species on the basis that there was greater diversity in tropical regions (Hawksworth 2012) realised by the discovery of numerous cryptic species revealed by molecular phylogenetic analyses (e. g. Bauer et al. 2008, Beenken et al. 2012, Göker et al. 2009, Kemler et al. 2009, Kruse et al. 2018a,b, Liu & Hambleton 2013, Piątek et al. 2013, Ploch et al. 2011, Rouxel et al. 2013, Runge et al. 2011, Telle et al. 2011, Voglmayr & Göker 2011, see chapter 1.5.3).

Studies of new fungal habitats, such as those within animal guts (Griffith et al. 2009, Liggenstoffer et al. 2010) and rocks (Ruibal et al. 2009) or water as habitats (Shearer et al. 2007) lead to recalculations of the global diversity of fungi. There is a lack of information about fungi associated with insects (e. g. Kubatova & Dvorak 2005, Sun & Liu 2008), which Hawskworth (2012) considered significant. Next generation sequencing methods that have often been used to examine the microbiome of soil samples, have shown a huge hidden diversity of fungi, as evidenced by the number of unassignable environmental sequences deposited in GenBank (https://www.ncbi.nlm.nih.gov/). In many cases these unassigned sequences

represent new species or new lineages (e. g. Fouts et al. 2012, Hawskworth et al. 2012, Mardis 2008, Medinger et al. 2010). Recently, the oceanic crust was found to represent an important and large fungal habitat (Ivarsson et al. 2016). Unresolved species complexes also have a huge potential for the discovery of fungal diversity. Crous & Groenewald (2005) realized when they stated, *"Show me a plant pathogen and I will show you a species complex*." Just recently, Hawksworth & Lücking (2017) recalculated the probably number of fungal species worldwide as between 2.2 to 3.8 million species. This means that with 120.000 known fungal species, more than 90 % of fungi still await discovery and description (Hawksworth & Lücking 2017).

Currently, there is much effort assigned to resolving the phylogenetic relationships between Fungi s. str. (without Chromista), often with the help of multilocus sequence analyses (Hibbett et al. 2007, James et al. 2006, Lutzoni et al. 2004, Matheny et al. 2007, Spatafora et al. 2006).

The phyla Basidiomycota and Ascomycota were established as monophyletic groups (Bruns et al. 1992, Hibbett et al. 2007, Lutzoni et al. 2004, Swann & Taylor 1993, 1995), belonging to the subkingdom Dikarya (James et al. 2006) with the synapomorphy of dikaryophytic hyphae (Theler 1988). Furthermore the Basidiomycota were divided into four main groups (monophyletic subphyla): macroscopic mushrooms (Agaricomycotina), Wallemiomycotina, smut fungi (Ustilaginomycotina) and rust fungi (Pucciniomycotina) (Aime et al. 2006, Begerow et al. 2004b, Begerow et al. 2006, Swann & Taylor 1993, Zhao et al. 2017). The Chytridiomycota were considered by James et al. (2006) to be basal to the remaining fungi but also belonging to this kingdom. Many different phylogenetic analyses followed to determine the phylogenetic relationships between these groups (Aime et al. 2006, Bauer et al. 2006, Begerow et al. 2006, Swann & Taylor 1993, Wang et al. 2015).

The plant parasitic fungi belong to different phylogenetic groups scattered across the Fungi (Kirk et al. 2008). Some of these groups are well-known, for example, powdery mildews (Erysiphales), rust fungi (Pucciniomycotina), and smut fungi (Ustilaginomycotina). Less than 10 % of all known fungi are able to colonize plants and even fewer can cause infections, but their diversity is increasing since many species groups were detected (Knogge 1996).

The fungi studied in this research belong to the monophyletic subphyla Ustilaginomycotina. Sequence analysis support the monophyly, but with varying statistical support (Bauer et al. 2006, Swann & Taylor 1993, 1995). The

Ustilaginomycotina differ from the Pucciniomycotina and Agaricomycotina in having a dominance of glucose and an absence of xylose in their cell wall (Prillinger et al. 1990, 1993) and by the producing of two types of spores (teliospores and basidiospores) whereas the rust fungi can produce up to five different spore stages (spermogonia, aecia, uredia, telia and basidia). It is unique for the Pucciniomycotina that many species within this group have a pleomorphic lifecycle and undergo a host shift between two different hosts, often only distantly related (Conifers to ferns, or herbaceous plants to shrubs or trees, Gäumann 1959).

1.2 Smut fungi (Ustilaginomycotina)

Smut fungi are a species-rich group, which are typically plant parasites that occur in a variety of habitats (Begerow et al. 2014, Vánky 2012). Many are rare, with one third found only once. The majority of smut fungi produce blackish spore masses in different organs of plants. Some species produce a light coloured spore mass in the anthers of plants or white to yellow spots in the leaves or galls of various plants. Many smut fungi are undescribed and many cryptic species and species complexes exist (e. g. Kruse et al. 2017a, Piątek et al. 2013). Correct species identification of smut fungi is important for sound plant health management and effective biosecurity (Choi et al. 2015, Göker et al. 2009, Lutz & Piątek 2016). Due to previously allowed dual naming of several fungi, many uncertainties exist belonging the naming or treatment of several fungi (McNeill et al. 2012).

Because many smut species are very rare it is really important to use herbarium collections, to check morphology and of course if possible the phylogenetic features. Some phylogenetic analysis within the smut fungi, also a small portion of that based on multigene analyses have shown, that many smut fungi still have an unclear position within the system (Begerow et al. 2006).

1.2.1 Taxonomic concepts in smut fungi

Smut fungi are biotrophic parasites that mostly produce black to brown masses of teliospores in different organs of plants. The teliospores germinate to form basidia and basidiospores that grow as a saprophytic haploid yeasts. These fungi were placed in the order Ustilaginales (Clinton 1906, Schröter 1889, Tulasne & Tulasne 1847, Zundel

1953). In this order, two families were described, the Ustilaginaceae and Tilletiaceae, based on the presence of phragmobasidia and holobasidia, respectively (Vánky 1987).

Vánky (2001) named teliospores-producing smuts as classical smut fungi and Begerow et al. (2006) proposed the name teliosporic smuts for these fungi. This classification system remained unchanged until Bauer et al. (1997) studied the septal pores and interaction zones between fungus and host cell. Bauer et al. (1997) recommended a new classification system for the smut fungi that challenged most of the accepted familial relationships. Begerow et al. (1997) supported this work based on genetic evaluations. Begerow et al. (1997) split the Ustilaginales into several lineages (see chapter 1.3.2). Based on these analyses the smut fungi were divided into two different classes, the teliosporic Ustilaginomycetes and the non-teliosporic Exobasidiomycetes, both belonging to the Ustilaginomycotina (Bauer et al. 2006). Recently two further classes, the Malasseziomycetes and Monilliomycetes were included in the smut fungi (Wang et al. 2014).

Furthermore, these studies excluded some smut fungi from the Ustilaginomycotina. For example, the Microbotryales, which mostly occur in the anthers of host species in the Caryophyllaceae, were found more closely related to rust fungi (Pucciniales) than to the Ustilaginomycotina (Bauer et al. 1997, Begerow et al. 1997). Some non-basidiomycetes fungi appear similar to smut fungi. For example, the genus *Schroeteria* G. Winter that occurs in the seeds of some host plants in *Veronica* L. (Scrophulariaceae) produce black to dark blue spore masses (Vánky 1981) but belongs to the Ascomycota (Nagler 1989). Another example are the Protomycetaceae that produce yellow to white thickened spots (Reddy & Kramer 1975) that resemble smut fungi in the non-teliosporic genus *Entyloma* de Bary. Two further genera, *Entorrhiza* C.A. Weber and *Talbotiomyces* Vánky, R. Bauer & Begerow, were excluded from the Ustilaginomycotina, and transferred to a new phylum Entorrhizomycota (Bauer et al. 2015, Riess et al. 2015).

There are currently about 115 different genera with about 1.700 different species of Ustilaginomycotina that occur worldwide (Begerow et al. 2014). Vánky (2012) noted that one third of all smut fungi had only been found once, which demonstrates the rarity of many species. Most species of smut fungi infect only a few susceptible host species or often only one host plant species. Begerow et al. (2004a) evaluated the host lists from the European smut monograph (Vánky 1994) and found that only elven of 600 smut species had more than 20 hosts. The narrow host ranges of smut fungi

have been supported by phylogenetic analyses (e. g. Kruse et al. 2017 b, 2018 a,b, Li et al. 2017 a,b, Piątek et al. 2013, Stoll et al. 2003).

Most of the members of the Ustilaginomycotina, apart from some asexual taxa (see chapter 1.2.3) are plant parasites, especially on Poaceae (45%) and Cyperaceae (13%) (Begerow et al. 2014). Only a few species are associated with other tracheophytes, for example, *Melaniella* R. Bauer, Vánky, Begerow & Oberw. on spike mosses (*Selaginella* P. Beauv.), *Exoteliospora* R. Bauer, Oberw. & Vánky and *Violaceomyces* S.A. Albu, M. Toome & M.C. Aime on ferns, and *Uleiella* J. Schröt. on conifers (Albu et al. 2015, Vánky 2012).

There are some well-known economically important genera of smut fungi, for example, *Ustilago* (Pers.) Roussel (*Ustilago-Sporisorium-Macalpinomyces*-complex, Li et al. 2017b, McTaggart et al. 2012, 2016)*, Urocystis* Rabenh. ex A.A. Fisch. Waldh. (Lotze-Engelhard 2010) and *Tilletia* Tul. & C. Tul. (Castlebury et al. 2005). Some of the most significant smuts that cause great economic losses on graminicolous crops are *Ustilago hordei* (Pers.) Lagerh. (covered smut of barley), *U. nuda* (C.N. Jensen) Rostr. (loose smut of barley), *U. tritici* (Pers.) Rostr. (loose smut of wheat), *U. maydis* (DC.) Corda (corn smut), *Urocystis agropyri* (Preuss) A.A. Fisch. Waldh. (flag smut of wheat), *Sporisorium reilianum* (J.G. Kühn) Langdon & Full. (sorghum head smut), *Sporisorium scitamineum* (Syd.) M. Piepenbr., M. Stoll & Oberw. (sugar cane smut), *Tilletia indica* Mitra (karnal bunt of wheat and triticale), *T. caries* (DC.) Tul. & C. Tul. (common bunt of wheat), and *T. controversa* J.G. Kühn (dwarf bunt of wheat) (Carris et al. 2006, Fischer & Holton 1957, Knogge 1996, Murray & Brennan 2009, 2010, Wilcoxson & Saari 1996).

1.2.2 Life cycle

Smut fungi are characterized by a dimorphic life cycle, i.e. a saprobic haploid yeast phase followed by a parasitic dicaryotic teliospore phase (de Bary 1884). The saprobic phase allows the smut fungus to survive away from their host as free living asexual anamorphic yeast that can be cultured. A hyphal growth within this phase has been observed for some Ustilaginomycotina (Begerow et al. 2014). Saprobic states of smut fungi occur on plants (Begerow et al. 2000, Sampaio 2004) but also on other substrates, e.g. soil, blood (Boekhout 2011, see chapter 1.2.3). The saprobic state ends with the conjugation of compatible haploid cells (plasmogamy). The parasitic

teliospore phase starts with mating (Kämper et al. 2006, Kellner et al. 2011) that precedes infection of the host plant, which is followed by intercellular mycelial growth. Haustoria are produced in host cells that provide nutrition for the fungus. The formation of sori and production of teliospores concludes this parasitic phase. Some smut fungi have modified life cycles, e.g. do not produce teliospores (e.g. Exobasidiales, Microstromatales and Ceraceosorales) or do not have a diploid phase, which is typical for some yeasts (e. g. *Malassezia*).

1.2.3 Asexual smut fungi (Yeasts)

Besides the sexual smut fungi there is also an increasing knowledge since the beginning of the 21st century regarding lipophilic yeasts, occurring on endotherm species or various marine substrates or habitats. The genus *Malassezia* Baill., also belonging to the asexual yeasts, is the only one which is able to infect humans (Amend 2014, Ashbee 2007, Begerow et al. 2000, Boekhout et al. 2010, Cabañes 2014; Wang et al. 2014). Saprophytic ustilaginomycetous yeasts belong to many different genera and have been isolated from many ecosystems as well as from healthy plants (Albu et al. 2015, Amin et al. 2010, Begerow et al. 2000, Nasr et al. 2014, Padhi & Tayung 2013, Piątek et al. 2016, 2017, Rush & Aime 2013, Takahashi et al. 2011, Tanaka & Honda 2017, Wang et al. 2014). These yeasts are not restricted to the smut fungi. Worldwide there occur several different genera of asexual free living yeast in different habitats spreading over different fungal groups (Albu 2012, Aime et al. 2014, Avis et al. 2001, Boekhout 1995, Branda et al. 2010, Gai et al. 2009, Golubev & Sampaio 2009, Golubev et al. 2007, Inacio et al. 2002, Nakase 2000, Piątek et al. 2017, Rodriguez et al. 2008, Toome et al. 2013).

Wang et al. (2015) linked many different asexual ustilaginomycetous yeasts to teleomorphic stages. Currently, yeasts and yeast-like organisms belonging to the smut fungi (Ustilaginomycotina) are known from eleven different orders, namely, Entylomatales, Exobasidiales, Georgefischeriales, Golubeviales, Malasseziales, Microstromatales, Moniliellales, Robbauerales, Urocystidales, Ustilaginales, Violaceomycetales (Albu et al. 2015, Begerow et al. 2000; Boekhout et al. 2011, Nasr et al. 2014, Piątek et al. 2017, Rush & Aime 2013, Sampaio 2004, Tanaka & Honda 2017, Wang et al. 2014, 2015). Kruse et al. (2017b) and Wang et al. (2015) noted that some asexual smut yeasts were closely related to known sexual stages of smut fungi.

The distribution of asexual yeasts is strongly correlated with abiotic factors, especially the availability of metabolites (Fonseca & Inácio 2006).

1.3 Classification of smut fungi (Ustilaginomycotina)

The modern taxonomy and classification of smut fungi begins with the world-monograph of Vánky (2012), which owes much to the work of Bauer et al. (1997) and Begerow et al. (2006). Within Vánky's (2012) monumental work, he recognised about 1.700 species of smut fungi (including the Microbotryales). Several more recent molecular phylogenies have augmented Vánky's classification (Piątek et al. 2016, Wang et al. 2015).

The genus *Entorrhiza* (Entorrhizales, Entorrhizomycota) was excluded from the Ustilaginomycotina and raised in a new phylum (Bauer et al. 2015, Hibbett et al. 2007, Matheny et al. 2007, Riess et al. 2015). The smut fungi (Ustilaginomycotina) currently comprise four classes, Exobasidiomycetes, Ustilaginomycetes, Monilliomycetes and Malasseziomycetes (Begerow et al. 2006, 2014, Piątek et al. 2016, Vánky 2012, Wang et al. 2014, 2015).

1.3.1 Exobasidiomycetes

The Exobasidiomycetes differ from all other Ustilaginomycetes in the structure of the local interaction zones. Except for the Tilletiariaceae, all species in the Exobasidiomycetes produce ballistosporic holobasidia (Begerow et al. 2014). The Exobasidiomycetes contain several anamorphic yeast species, which were linked by Wang et al. (2015, see chapter 1.2.3) to their related teleomorphic stage, for example *Meira* Boekhout, Scorzetti, Gerson & Sztejnb., *Jaminaea* Sipiczki & Kajdacsi and *Tilletiopsis* Derx. At present, seven orders are included in the Exobasidiomycetes (Piątek et al. 2016, Wang et al. 2015), which are discussed as follows.

Ceraceosorales

The Ceraceosorales producce intracellular hyphae with a simple interaction apparatus (Begerow et al. 2006). There is only one family, Ceraceosoraceae (Denchev & Moore 2009) included in this class, with three species, namely, *Ceraceosorus africanus* Piątek, K. Riess, Karasiński & M. Lutz (Piątek et al. 2016), C. *bombacis* (B.K. Bakshi)

B.K. Bakshi, and *C. guamensis* T. Kijporn. & Aime (Kijpornyongpan & Aime 2016). The last species is only known from its asexual stage. The first two species occur on Malvaceae, while the third species was isolated from an indeterminate dicotyledonous plant. At present the phylogenetic position of this order is unresolved. In the study of Piątek et al. (2016), the Ceraceosorales were closely related to *Exobasidium* Woronin and *Entyloma* de Bary, indicating its position within the Exobasidiomycetes.

Georgefischeriales

The Georgefischeriales is characterized by poreless septa in the soral hyphae (Begerow et al. 2014). Most of the species occur on leaves of Poales, with the exception of *Georgefischeria* Thirum. & Naras. on Convolvulaceae (Vánky 2012). Four different families belong to this order, namely, Eballistraceae, Georgefischeriaceae, Gjaerumiaceae and Tilletiariaceae. *Gjaerumia* R. Bauer, M. Lutz & Oberw. has a special position, differing from all species of this order by having dolipores (Bauer et al. 2005). Wang et al. (2015) found two new *Gjaerumia* species as well as four species of *Phragmotaenium* R. Bauer, Begerow, A. Nagler & Oberw., within the asexual genus *Tilletiopsis* Derx. *Tilletiaria anomala* Bandoni & B.N. Johri was the first member of the order Georgefischeriales to have its genome sequenced (Toome et al. 2014).

Tilletiales

The Tilletiales are characterised by the presence of dolipores in the mature septa (Bauer et al. 1997). Most species occur on Poaceae, except the *Erratomycetaceae*, which are restricted to Fabaceae (Piepenbring & Bauer 1997). Most species have ornamented spores and some produce trimethylamine (Castlebury et al. 2005, Vánky 2012). Sori are mostly located in the ovaries and rarely on vegetative plant organs such as leaves. The Tilletiales contains two families, the Tilletiaceae and the monotypic Erratomycetaceae (Denchev & Denchev 2013).

Microstromatales

The Microstromatales are characterised by simple pores and local interaction zones without an interaction apparatus (Bauer et al. 1997). Teliospores are not produced and the fungi mostly occur on woody plants. Three families belong to this order, Microstromataceae, Quambalariaceae and Volvocisporiaceae (de Beer et al. 2006, Begerow et al. 2001). The genus *Jaminaea* Sipiczki & Kajdacsi was also proposed to

belong to the Microstromatales, but its phylogenetic position is unresolved. Begerow et al. (2014) proposed that *Jaminaea* belonged to the Quambalariaceae. Wang et al. (2015) was unable to support this relationship in their phylogenetic analysis. Kijpornyongpan & Aime (2017) show a narrow relationship to other Microstromatales, and a splitting of the genus into *Jaminaea* and *Parajaminaea* T. Kij. & Aim.

Entylomatales

The Entylomatales is characterised by a simple interaction apparatus and septal pores (Bauer et al. 1997). All species in the Entylomatales have simple hyaline spores, which are often thick-walled (Vánky 2012). The highest diversity in the Entylomatales is found on host plants in the Ranunculales and Asteridae. Typical disease symptoms are white to yellow or light brown flattened or thickened spots in the leaves or as galls on the stems (Vánky 2012). Some species of the asexual genus *Tilletiopsis*, namely *T. cremea* Tubaki, *T. lilacina* Tubaki and *T. washingtonensis Nyland,* were linked to the Entylomatales, occupying a position within the Entylomataceae (Wang et al. 2015). Many host jumps have taken place in the Entylomatales (Begerow et al. 2002b). The genus *Entyloma* is very host specific, and several different species may occur on the same host plant species (e. g. *Entyloma ranunculi-repentis* and *E. microsporum*, Kruse et al. 2018b, Savchenko et al. 2014b).

Doassansiales

The Doassansiales have a complex interaction apparatus, including cytoplasmic compartments (Bauer et al. 1997). Many genera produce complex spore balls. *Doassinga* Vánky, R. Bauer & Begerow, *Melaniella* R. Bauer, Vánky, Begerow & Oberw. and *Rhamphospora* D.D. Cunn. produce single spores. Within this order three different families are described, namely, Melaniellaceae, monotypic with the genus *Melaniella*, occuring as black spots in leaves or stems of spike mosses (Bauer et al. 1999); Rhamphosporaceae, also monotypic with the genus *Rhamphospora*, occuring as white spots on the leaves of Nymphaeaceae; and Doassansiaceae with eleven different genera. All members of the Doassansiales occur on paludal or aquatic plants sharing the same niche (Begerow et al. 2014).

Exobasidiales

The Exobasidiales are characterised by interaction tubes produced in a complex interaction apparatus (Bauer et al. 1997). The Exobasidiales form a well-supported monophyletic clade (Begerow et al. 2002a, 2006) and infect species of the Ericaceae. At present, the Exobasidiales contains four families. The Brachybasidiaceae contains species that mostly occur on monocotyledonous hosts (Cunningham et al. 1976, Oberwinkler 1978, 1982, 1993), whereas the Graphiolaceae are parasites of palms (Piepenbring et al. 2012). The Cryptobasidiaceae mostly occur on Laurales, except for the genus *Coniodictyum* Har. & Pat. (Begerow et al. 2014). The Exobasidiaceae are the biggest family, with about 128 different species (Begerow et al. 2002a). There also occur some asexual yeasts in the Exobasidiales, namely, *Acaromyces* Boekhout, Scorzetti, Gerson & Sztejnb. and *Meira* Boekhout, Scorzetti, Gerson & Sztejnb. (Wang et al. 2015), both known to occur on mites (Boekhout et al. 2003). The position of *Cladosterigma* Pat. in the Exobasidiales is unclear (Seifert & Bandoni 2001).

1.3.2 Ustilaginomycetes

The Ustilaginomycetes is characterised by enlarged interaction zones (Bauer et al. 1997). They contain most of the smut fungi that produce black spore masses, together with some genera of asexual yeasts. All of the asexual *Farysizyma* species and most of the asexual *Pseudozyma* species have been linked to sexual genera (Wang et al. 2015, Tanaka & Honda 2017). Only four species within *Pseudozyma* remain unlinked and unresolved (*Pseudozyma alboarmeniaca* Sugita, Takash., Poonwan & Mekha, *P. hubeiensis* F.Y. Bai & Q.M. Wang, *P. pruni* G.Y. Liou, Y.H. Wei & F.L. Lee, *P. thailandica* Sugita, M. Takash., Mekha & Poonwan).

Urocystidales

The Urocystidales contain five families, characterised by spore balls or single spores, and often haustoria and pores in the septa of hyphae (Begerow et al. 2006, Wang et al. 2015). The Doassansiopsidaceae has only one genus, *Doassansiopsis* (Setch.) Dietel, characterised by the formation of complex spore balls (Piątek et al. 2008) and host plants found in moist or aquatic habitats (Vánky 2012). The Floromycetaceae is characterised by the occurrence of sori with single spores or spore balls that form in the flowers of host plants in the Asparagaceae (Bauer et al. 2008, Vánky et al. 2008b).

In contrast the Glomosporiaceae parasitise eudicot plants, and form solid to slack spore balls or even single spores (Vánky et al. 2008a). The members of the Mycosyringaceae are monotypic with *Mycosyrinx* Beck and restricted to the Vitaceae, producing spores in pairs (Vánky 1996, 2012). The Urocystidaceae is the largest family and contains about 180 species (Vánky 2012), which all have coloured teliospores. Some genera produce single spores and other genera produce spore balls comprising both sterile and fertile cells, e.g. *Urocystis* Rabenh. ex Fuckel (Vánky 1987, 2012). Recently, the first urocystidalean yeast lineage, *Fereydounia* S. Nasr, M.R. Soudi, H.D.T. Nguyen, M. Lutz & Piątek, was described from plant remnants (Nasr et al. 2014).

Ustilaginales

The Ustilaginales are the largest order within the Ustilaginomycotina. The Ustilaginales are characterized by poreless septa (Begerow et al. 2014). The phylogenetic relationships between many genera in the Ustilaginales has not been resolved. The Ustilaginales contains about four families. The largest family is the Ustilaginaceae, which is mostly restricted to the Poaceae, with an occasional host jump to other plant families, for example, *Pattersoniomyces* Piątek, M. Lutz & C.A. Rosa on Bromeliaceae (Piątek et al. 2017). Molecular phylogenetic analyses have split this family in several smaller monophyletic genera that are further supported by host specifity and morphological features (McTaggart et al. 2012, 2016). Another approaches is to retain *Ustilago* as a catch-all for species in the *Sporisorium-Macalpinomyces* complex (Kruse et al. 2018a).

There are at least three further familes in the Ustilaginales. The Anthracoideaceae occur on Juncaceae and Cyperaceae (Vánky 2012). Two further families are the Melanotaeniaceae on eudicots and Websdaneaceae on host plants in the Anathriaceae and Restionaceae (Vánky 2012). Following Begerow et al. (2014), the genera, *Clintamra* Cordas & Durán, *Pericladium* Pass., and *Websdanea* Vánky are treated here as *incertae sedis*, since molecular data and other characteristics that might differentiate them have not been identified. Some anamorphic yeast species belonging to the Ustilaginales were recently transferred to their corresponding sexual

genera (Kruse et al. 2017b, Wang et al. 2015). Only five yeasts, all belonging to the genus *Pseudozyma* pro temp., are still unresolved.

Uleiellales

The Uleiellales infects gymnosperms and produces haustoria and poreless septa. The Uleiellales contains only one family, the Uleiellaceae, with one genus *Uleiella* J. Schröt. (Schröter 1884) and two species. Riess et al. (2016) showed that this order was closely related to the Violaceomycetales.

Violaceomycetales

The Violaceomycetales contains only one family with one species, *Violaceomyces palustris* Albu, Toome & Aime, associated with palustrine plants, mostly in the genus *Salvinia* Ség. The anamorphic yeast stage is two celled, forming ballistoconidia, varying in colour from greyish violet, dark violet to blue, or sometimes yellow (Albu et al. 2015). It is not known whether *Violaceomyces palustris* is a pathogen, because Albu et al. (2015) did not observe any symptoms of disease on the colonized leaves.

1.3.3 Malasseziomycetes

The Malasseziomycetes was proposed by Wang et al. (2014) for this sister group to the Ustilaginomycetes and Exobasidiomycetes. The Malasseziomycetes is restricted to human or animal skin (Findley et al. 2013, Gaitanis et al. 2012, Guého-Kellermann et al. 2010, Sugita et al. 2010). A sexual stage is not known (Wang et al. 2014). It contains only one order (Malasseziales) with one family.

1.3.4 Moniliellomycetes

The Moniliellomycetes contains only one order (Moniliellales) with one family and was proposed by Wang et al. (2014) for this sister group to Ustilaginomycetes and Exobasidiomycetes. A sexual stage is not known. The Moniliellomycetes have been found in diverse habitats.

1.4 Importance of systematics

Systematics is the study of the classification, taxonomy and description of organisms. The aim of systematics is to define and identify organisms, which thereby provides a means to discover and ultimately protect species of animals, plants and other organisms. Systematics seeks to reflect the evolutionary relationships between organisms, which allows a natural (rather than artificial) classification system to be built. Ideally, a natural classification system will consider several different attributes, and arrive at natural groups based on a common ancestry (monophyly).

Linnaeus established the binominal system of nomenclature, which assigns a genus name and a species name to every living organism (von Linné et al. 1792). Linnaeus first used his nomenclatural system for plants in *Species Plantarum,* and applied his system later to other groups of organisms. This system is now the foundation of modern systematics.

1.4.1 International Code of Nomenclature for algae, fungi and plants (ICNafp)

In order to prevent different authors giving the same fungus different names, a set of rules was established for the naming of organisms. The first widely accepted set of rules for the naming of plants was established in 1930, under the name *Cambridge Code* (Rendle 1934)

One of the rules (McNeill et al. (2006), Art. 59) in ealier versions of the international code was that different morphs of the same fungus could have different legitimate names, despite that this contravened one of the basic principles on which the code was built, i.e. every species must only have one name. This rule has now been replaced and each fungal species is only allowed one name (Braun 2012).

1.4.2 One Fungus- One Name

As there was no regulation for the naming of asexual and sexual morphs for many years, a large number of redundant names exist in the literature for the same fungus. Presently the code has changed and one paragraph within the ICNafp clearly regulates this problem "*11.4. For any taxon below the rank of genus, the correct name is the combination of the final epithet of the earliest legitimate name of the taxon at the*

same rank, with the correct name of the genus or species to which it is assigned" (ICNafp; https://www.iaptglobal.org/). This means, that only one legitimate and valid scientific name has to be used for each species of a fungus. That stands in contrast to the old version of the ICNafp, where it was legitimate, to name the asexual morph and the sexual morph with different names. The current problem is now two names for the same fungus which still exist. To reduce the confusion with the naming, anamorphic and teleomorphic states of fungi were proposed by Rossman (2014) to be replaced by the terms sexual state or morph and asexual state or morph. Furthermore Gams et al. (2012) highlighted important changes resulting in the new ICNafp.

But there is also the opportunity to write proposals and submit them as lists, to conserve or reject certain names, considered and recommended for acceptance by the Nomenclature Committee for Fungi (NCF). The conservation or protection of species names is especially important if the name is well known by mycologists (Rossman 2014). How this could look like and some current application samples, could be found in Braun (2012), Rossman (2014) and Rossman et al. (2016).

Currently two different tools for resolving systematics exists. The classical systematics deals with the taxonomy, nomenclature and determination of species. The modern systematics includes furthermore the phylogenies and evolutionary progresses.

1.5 Phylogenies in general

As mentioned above, systematics is structured in two fields – classical and modern (phylogenetic systematics). The latter method aims to find evolutionary relationships, specifically monophyletic groups (Knoop & Müller 2009). The general tools for this are molecular genetics and phylogenetic reconstructions. Phylogenetic trees are searched with different assessment criteria. The most important ones are Maximum Likelihood (ML), Minimum Evolution (ME) and Bayesian Inferrence (BA). Of course, there are also some methods to calculate distances, like neighbour joining, but they are not so often used. It is important to have in mind that the results can vary, depending on the locus used. As Maddison (1997) has already shown, various gene regions evolve at different rates. But not only the choice of a locus has an impact on

the quality and result of the calculated trees – other parameters like number of taxa and outgroup are also important.

Ribosomal DNA sequences (rDNA) are suitable for calculating phylogenetic trees. They are some of the most conserved and highly repeated genetic regions in all cellular organisms, which is owed to their important role for the formation of ribosomes and protein synthesis in the cell (Bruns et al. 1991). Among others, rDNA sequences are useful for species identification of fungi and have been used as barcode loci (Badotti et al. 2017, Kurtzman 2014, Scorzetti et al. 2002). Herein the ITS1-5.8S-ITS2 (internal transcribed spacer) region is used for fungi in general (Schoch et al. 2012), whereas the D1 and D2 region of the ribosomal LSU (large subunit) is often used for yeasts (Kurtzman & Robnett 1998). These loci were successfully used to address different issues, e.g. solving relationships in Ustilaginomycotina mostly occuring as asexual stage from environmental samples or to go deeper in the systematics of smut fungi in general (e.g. Albu et al. 2015, Bellemain et al. 2010, Boekhout et al. 2003, Nasr et al. 2014, Piątek et al. 2016, Rush & Aime 2013, Wang et al. 2015).

While the nrLSU is sufficient for higher taxa like genera or families, it is only sparsely sufficient to discriminate between closely related species (Mahmoud & Zaher 2015). Similarly, the ITS region is good for species differentiation in several genera (e. g. *Macalpinomyces* on *Eriachne* (Li et al. 2017b), *Malassezia* (Wang et al. 2014), *Tranzscheliella* (Li et al. 2017a)), but for some groups of closely related species its resolution is unsatisfactory (e. g. *Entyloma* on Asteraceae (Begerow et al. 2002), *Ustilago striiformis*, Savchenko et al. 2014a). In such groups there is a need to use multigene analysis, to produce reliable phylogenetic trees.

Rokas et al. (2003) have shown that usage of eight to ten different gene regions yields reliable phylogenetic trees. They recommended to use more than one locus, because dependence from only one phylogenetic information site could lead to wrong phylogenetic trees. Furthermore Hedtke et al. (2006) have shown that increasing taxon sampling could also improve the resulting tree.

1.5.1 Phylogenies of smut fungi

At present, only a small amount of different gene loci are used for smut fungi (Ustilaginomycotina). It is important that loci are located in the core genome or the mitochondrial genome. Furthermore protein coding genes are more conserved (Storch

et al. 2007) and are thus sufficient to distinguish between lineages, which could not be solved by using rDNA loci (Liu & Hall 2004). Point mutations regularly take place in non-coding DNA regions or the third codon position of protein coding genes.

Currently the most frequently used gene loci for solving phylogenetic questions within smut fungi are the ITS (internal transcribed spacer), SSU (small subunit) and LSU (large subunit) rDNA. The SSU region evolves relatively slowly, useful for studying distantly related organisms. The mitochondrial and RNA genes evolve faster and are especially helpful to distinguish within different orders or families. The ITS region evolves the fastest and may vary among species within a genus or even a population (Nillson et al. 2008, White et al. 1990). In GenBank at NCBI (National Center for Biotechnology Information) more than 3300 sequences of the ITS region, about 2000 sequences of LSU, and 270 for the SSU (Kruse et al. 2017a) are deposited. Most of the available smut phylogenies are based on one of these loci or a combination of them (e. g. Albu et al. 2015, Bauer et al. 1997, 2001a, b, Begerow et al. 1997, 2000, 2002a, b, 2004a, Boekhout et al. 2003, Castlebury et al. 2005, Li et al. 2017a,b, McTaggart et al. 2012, Nasr et al. 2014, Piątek et al. 2016, 2017, Stoll et al. 2003, 2005, Wang et al. 2015).

The ITS and LSU loci are also broadly used within fungal phylogenies in general and especially the ITS region has a broad use as a barcoding locus (Schoch et al. 2012). Due to its short length of only 600-800 base pairs and the availability in many copies in the genes, amplification and sequencing is easy.

The intended aim to solve phylogenetic relationships are multilocus analyses, as they give better resolution of the trees. For this instance, there are several different gene loci used within the smut fungi, which are still not popular (e.g Albu et al. 2015, Begerow et al. 2006, Lotze-Engelhard 2010, McTaggart et al. 2012, Wang et al. 2006), i.e. *atp*6, *tef*-1alpha, *ß-tubulin*, *rpb*1, *rpb*2, *cox*3, *gapdh*. Stored in GenBank, about 400 sequences for *tef*-1alpha, 234 for *rpb*2, 150 for *rpb*1 (most of the sequences from *rpb*1 and 2 coming from cultures), about 45 for *gapdh* and 35 for *atp*6 and *ß-tubulin* have been used in phylogenetic studies.

1.5.2 Morphology of smut fungi

A point sometimes overlooked is the importance of non-sequence data to support phylogenetic reconstructions (morphological, geographical and ecological data). Former mycologists had no choice beyond morphological and biological data. For that instance, they studied dried herbarium specimens. Today a combination of both strategies, phylogeny with morphology, is used and morphological data is mapped on phylogenetic trees. In a few papers, this approach was pursued (Bauer et al. 2001b, McTaggart et al. (2012). McTaggart et al. (2012) mapped attributes like absence of sterile cells or columella within the *Ustilago-Sporisorium-Macalpinomyces*-complex. The advantage of morphological data is that it could be used every time, whereas the success of DNA amplification decreases mostly with the age of the herbarium samples, because of the degradation of the DNA (Telle & Thines 2008).

1.5.3 Cryptic species and species definition

Dealing with different species and phylogenetic analysis will lead to another problem: cryptic species. Bickford et al. (2007) "*considered two or more species to be 'cryptic' if they are, or have been, classified as a single nominal species because they are at least superficially morphologically indistinguishable*." Sometimes also the term 'sibling species' occur in this context, as some authors equate this term with 'cryptic species' (Saez & Lozano 2005). Throughout the different fungal organism groups, cryptic species are determined by variation in nucleic acids of morphologically identical species (Beenken et al. 2012, Bauer et al. 2008, Göker et al. 2009, Le Gac et al. 2007, Liu & Hambleton 2013, Piątek et al. 2013, Ploch et al. 2011, Steenkamp et al. 2002, Voglmayr et al. 2014). Even within the smut fungi, several cryptic species were detected with molecular methods, which were mostly indistinguishable with the classical methods, using only spore morphology for species delimitation (Kruse et al. 2017b, 2018a,b, Piątek et al. 2013).

Another key point is the definition of a species. In Mayden (1997) different species concepts are surveyed and characterised as theoretical or operational. The operational ones can be used for species recognition. The evolutionary species concept is defined as "...*a single lineage of ancestor- descendent populations which maintains its identity from other such lineages and which has its own evolutionary*

tendencies and historical fate" (Wiley 1978). There are some other species concepts, which are more commonly used, but thought by Mayden (1997) to be less important than the evolutionary species concept: Morphological species concept, biological species concept and phylogenetic species concept. Most of the fungi worldwide are diagnosed by the morphological concept (Besl & Bresinsky 1997, Cai et al. 2011). But one weakness of this concept is, that when verifying it with either the biological or phylogenetical concept, further cryptic species were found.

In general, the biological species concept, which takes advantage of biological races and infection traits to identify different species or races, usually shows more species than the morphological concept. Furthermore, this concept has some weaknesses, because fungi without meiospores can't mate (asexual fungi) and gene flow in nature is unlikely the same as potential gene flow. The phylogenetic concept is broadly used by mycologists (Geiser et al. 1998. Kasuga et al.1999, Koufopanou et al. 1998, O'Donnell et al. 1998). To sum it up, Taylor et al. (2000) figured out that in many different studies dealing with different species concepts (e.g. Franzot et al. 1999, O'Donnell et al. 1998) the phylogenetic species concept revealed species that were not detected by the morphological concept and sometimes even not seen within the biological concept. However, the problem of characterising phylogenetic species still remains. Quaedvlieg et al. (2014) named the combination of these three species concepts/methods, based on the combination of morphological, ecological and molecular markers, as Consolidated Species Concept (CSC).

Indeed Maddison (1997) states while using more than one locus, different species will have different genealogies and between different species these genes should be concordant, but it still remains unclear, how many bases difference are needed to define a new species. When several loci show differences between two different species it is quite possible, that no recombination will take place.

Furthermore, two different species concepts exist, how to treat the diversity of certain organism: The broad species concept and the narrow species concept. Based on the older literature and species descriptions, many smut fungi specialists had the opinion that the majority of smut fungi are host specific to certain genera or even certain host plant species (e. g. Zillig 1921, Liro 1924). They based their interpretation mostly on morphological analysis as well as on ecological observations, like infection trials. Taking into account the possibility of phylogenetic analysis, most of the views

dealing with a narrow species complex have been supported by such analysis (Aime et al. 2006, Bauer et al. 2006, Begerow et al. 2006). Due to the fact, that smut fungi were known to have stable and reliable morphology, this allowed a confident identification of smut species, often supported by their narrow host range (Cai et al. 2011). With the help of phylogenetic methods, it is now possible to reveal synapomorphies and to interpret them with respect to evolutionary processes. For many other groups within fungi and fungal-like organisms, it was revealed, that the narrow species concept is mostly supported by the different phylogenetic analysis: For downy mildews (Choi et al. 2009, Cunnington 2006, García-Blázquez et al. 2008, Thines et al. 2009) or for *Microbotryum* (Le Gac et al. 2007, Lutz et al. 2005, 2008, Piątek et al. 2012, Refrégier et al. 2008), although *Microbotryum* on *Dianthus* shows some special characters, since *Microbotryum dianthorum* s. l. seems to be not strictly host specific, due to intermixture of different *Microbotryum* lineages on different *Dianthus*-hosts (Kemler et al. 2013). The broad species concept follows the opinion that a smut fungus could occur on several different host plants or even genera (Fischer & Shaw 1953, Vánky 2012, Zundel 1953) For some smut fungi a broad species concept was assumed, like for *Entyloma ranunculi-repentis*, *Ustilago striiformis*, *Macalpinomyces spermophorus* and several others. For most of the host-fungus-combinations within these complexes older names exist, which where thought to be synonymous in that case. For some powdery mildews, a broad species concept was imagined. *Golovinomyces orontii* is a typical example of a powdery mildew, which is polyphagous. It could infect many different host plants, even from different plant families (Braun & Cook 2012). For many smut fungi a narrow species concept is more realistic. Plants and their specific pathogens have coevolved for a long time. These findings are supported by several analyses, like Begerow et al. (2002), Kruse et al. (2018a,b), Savchenko et al. (2014) and Vánky & Lutz (2010).

The general problem is that borders were reached with the morphological criteria for species delimitation, due to the subtle charactersitics which could be used for determination. Mostly only the sori with the spores (shape, size, ornamentation, and colour) could be used for differentiation or species delimitation. And also the occurrence of several homoplasies during the whole system of smut fungi, renders the problem of morphological examination. Nowadays phylogenetic approaches are used as a tool to support morphological analysis and reveal evolutionary relationships, but

due to the limited gene regions which are used currently, homoplasies and the scarcity of clear cut differences, especially problems for closely related species occur.

1.6 Aim of the Study

Smut fungi are a diverse group with about 1.700 species over 115 different genera worldwide. Many of them are rare, one third of taxa was found only once. Furthermore, some smuts cause heavy infections of crops or have spread rapidly throughout exchange of planting material and seeds.

Different smut species mostly infect only a few susceptible hosts or even only one certain host plant species. A small number of taxa occur as polyphagous species on different host plant genera. Recent studies regarding phylogenies of smut fungi suggest the narrow species concept to reflect the more authentic situation. Even within the same host plant genus several different smut species could be observed. Thus smut fungi occuring on more than one single host may represent species complexes. It is common practice to use molecular phylogenetic methods to answer such taxonomic, systematic and evolutionary questions. The ITS region is widely used for identification and phylogeny in various fungal groups, and has been proposed as the barcoding locus for fungi. It is also important for identification and phylogenetic studies of smut fungi. In species complexes of various specialised plant pathogens, resolution of this standard barcoding locus, ITS, is mostly insufficient to resolve complexes of closely related species, e.g. in downy mildews, rusts and some lineages of smut fungi. A comparable situation is found within other loci, such as protein coding regions. Thus various smut samples could not be included in any phylogenetic studies. This suggests an improvement of the formerly used phylogenetic tools to get better phylogenies. Since smut fungi are very diverse it renders the task of designing different loci and primers, which work for all groups of smuts (Ustilaginomycotina) while at the same time excluding the amplification of all other fungal groups and host plants.

Furthermore it was the aim to improve the phylogeny of smut fungi in general, by the usage of new smut fungi which were not part of any investigations so far. For some smut genera no phylogenetic data of the barcoding locus ITS is deposited in any public database, for example the Doassansiaceae. But also the resolution of species complexes is really important and the linking of asexual smut specimens, to whom no

sexual species is known. With the investigation of the *Moesziomyces bullatus*, *Entyloma ranunculi-repentis*, *E. microsporum* and *Ustilago striiformis* complex first attempts were made to verify the narrow species concept for these groups.

Finally, another important aspect with the improvement of the phylogeny of smut fungi is the accurate description, naming and observing of the priority rules in the lines of the recent ICNafp, following the rule ‘one fungus one name’. This nowadays prohibits the dual naming of pleomorphic fungi. But especially within species complexes this causes problems and it shows once more the importance of type fungus-host combinations for the analysis and interpretation of data.

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2 General discussion, conclusions, side results and future prospects

2.1 Phylogeny of smut fungi

Smut fungi (Ustilaginomycotina) have a huge diversity with around 1.700 different species in over 115 genera. One third of them have only been found once, highlighting the rarity of this fungal group. Every year, new smut fungi are discovered and described as new species. Sometimes these species are cryptic or they are only known to occur as asexual yeasts in different environments.

Many smut species are parasites of crops and ornamental plants, causing damage and losses, for example *Ustilago nuda* (loose smut of barley)*, U. maydis* (corn smut) and *T. caries* (common bunt of wheat) (e. g. Aydoğdu et al. 2015, Diethart et al. 2017, Wilcoxson & Saari 1996). The majority of smut species are plant parasites that have a narrow host spectrum, similar to the downy mildews (Choi et al. 2015, Ojiambo et al. 2015) and rust fungi (Chen 2017, Singh et al. 2015). The smut fungi often infect only a certain plant genus or even plant species (e. g. Cai et al. 2011, Escudero 2015, Kruse et al. 2018a,b, McTaggart et al. 2012, Stoll et al. 2003, 2005). To better understand smut fungi a natural classification system based on evolutionary relationships is needed (Begerow et al. 1997, 2006, Wang et al. 2015).

The description of smut genera was predominantly based on ecology and microscopic attributes before the use of molecular methods. At the species rank, most taxa were described by host range and often subtle differences in spore morphology. These small differences were often inadequate to reliably differentiate similar species, which led to confusion in the taxonomic literature (Fischer 1943, 1953, Fischer & Holton 1943, Fischer & Shaw 1953, Vánky 2012, Zundel 1953). The absence of unambiguous morphological differences between different smut species makes it difficult to discriminate species based only on morphology and host range (Li et al. 2017a,b, Kruse et al. 2018a, Savchenko et al. 2014). Consequently a narrow species concept was not accepted by all authors (Fischer 1943, 1953, Fischer & Holton 1943, Fischer & Shaw 1953, Zundel 1953). A similar situation of host specific species with subtle morphological differences is found within Oomycota species (e. g. Choi et al. 2015, Göker et al. 2009, Voglmayr 2003). Species delineation is better served by combining molecular features, ecology and morphology, known as Consolidated Species

Concept (CSC) (Quaedvlieg et al. 2014). For fungi in general, and also for smut fungi, the ITS (internal transcribed region), together with one or two secondary gene regions, such as SSU (small subunit) and LSU (large subunit), are often adequate for systematic studies.

2.1.1 ITS (internal transcribed spacer)

Ribosomal DNA (rDNA) has been used to resolve phylogenies of different organisms, such as fungi. The LSU (large subunit) provides resolution at suprageneric splits, but is usually insufficient to discriminate closely related species (Mahmoud & Zaher 2015). The ITS (internal transcribed spacer) is adequate for species differentiation. It is characterized by two highly variable spacer regions and the highly conserved 5.8S region (White et al. 1990). This locus is ideal for analyses of smut fungi at species level, and is the official barcoding locus for fungi (Schoch et al. 2012, Stielow et al. 2015).

The ITS region of fungi is usually amplified and sequenced with the primers ITS4 and ITS1 (White et al. 1990). Design of these primers was a milestone for the identification and phylogenetic analysis of fungi. Meanwhile some variants of these primers had been published, specific for some special fungal groups. Gardes & Bruns (1993) developed two new primers, ITS4B and ITS1F, based on this primer set. The former one especially for the amplification of Basidiomycota and the latter one for Ascomycota.

DNA amplified from obligate pathogens is often contaminated by other fungi, such as yeasts and hyperparasites. Primers are best designed to broadly amplify all groups of smuts (Ustilaginomycotina) and exclude amplification of other fungal groups (for example contaminant fungi, hyperparasites etc.) and host plants. This means, the ITS primer for plant parasitic fungi has to be specific to the target, because a mixture of different DNAs is typical for extracting fungi from plants. Use of cultured smut fungi is another strategy to avoid ampflication of contaminating DNA. The more specific forward primer M-ITS1 (Stoll et al. 2003) was especially designed for smut fungi (Ustilaginales) and should reduce these problems.

During our preliminary work on molecular phylogeny of smut fungi, we found some genera could not be amplified with ITS primers, for example *Doassansia* Cornu,

Entyloma de Bary and *Heterodoassansia* Vánky. In these cases, the DNA of the host plant or some other contaminant fungi was amplified preferentially (Kruse et al. 2017a). Even with combinations of different ITS primers already published (ITS1F, ITS4B etc, see chapter 1.5.1) these difficulties still existed. We aimed to design new ITS primers that were more specific to smut fungi, to improve the tools for species identification and delimitation based on the barcoding locus for fungi (ITS).

In Kruse et al. (2017a) new smut specific primers for the ITS-region are presented – two reverse primers (smITS-R1, smITS-R2) and one forward primer (smITS-F). Although the amplification success of these newly designed primers was similar to the ITS4/M-ITS1 primer combination, which is usually used for smut fungi, certain significant differences were apparent. On a set of 205 samples of extracted smuts, only one quarter of all samples could successfully be amplified with the formerly used primer set (ITS4/M-ITS1), whereas the newly designed primers yielded the target DNA in three quarters of all samples. Most of the extracted DNA used in initial tests was not older than 20 years. The weakness of the primer combination ITS4/M-ITS1 is the unspecific reverse primer. It amplifies DNA of a diverse spectrum of organisms. By doing a short sequence BLAST (Altschul et al. 1990) of the ITS4 sequence (5'TCCTCCGCTTATTGATATGC3') results were obtained scattered over a broad set of taxonomic groups, i.e. plants, fungi, and sometimes even animals. Since both primers (M-ITS1/ITS4) are located in a conserved region on the gene, this could explain the high amplification result originating from plants, especially for DNA material extracted from herbarium specimens. The newly designed reverse primers are located in the beginning (within 200 bp) of the LSU region. Thus it is possible to amplify shorter fragments with the available primers (M-ITS1/ITS4) or even semi-nested or nested PCR to improve the results.

Although the results in Kruse et al. (2017a) showed the newly presented primers amplifed a similar amount of different genera, we were meanwhile able to get ITS sequences of several smut genera, which are not yet present in GenBank (https://www.ncbi.nlm.nih.gov/), like *Arcticomyces* Savile, *Burrillia* Setch., *Conidiosporomyces* Vánky, *Centrolepidosporium* R.G. Shivas & Vánky, *Doassansia* Cornu, *Heterodoassansia* Vánky, *Nannfeldtiomyces* Vánky and *Tracya* Syd. & P. Syd. Interestingly, the majority of these genera belong to the Exobasidiomycetes and herein especially to the family Doassansiaceae (data not shown). Actually ITS sequences for the whole family of Doassansiaceae are missing in GenBank and therefore missing in

molecular studies based on the internal transcribed spacer. The new ITS primer will improve the smut investigations based on ITS sequences, because it could amplify new genera and species of smut fungi, not deposited in GenBank yet.

2.1.2 Multilocus studies

Although the ITS locus is quite variable and sufficient to distinguish between different species, this region provides too few information when looking deeper into closely related species or species complexes. In such cases further loci are needed, to produce reliable trees. In some publications regarding smut fungi a combination of ITS and LSU is used (e. g. McTaggart et al. 2016, Savchenko et al. 2014a, see chapter 1.5.1). As mentioned in chapter 1.5.1, the LSU is mostly sufficient for the discrimination of suprageneric splits and the ITS for the differentiation between different species. Protein-coding loci show varying results and successes. Liu & Hall (2004) demonstrated that protein-coding genes resolve fungal lineages that could not be distinguished based on rDNA loci. Although some different protein-coding loci for smut fungi are available, most of them are not regularly used. Most commonly used in different studies are *atp*6, *tef*-1α, *ß-tubulin*, *rpb*1, *rpb*2, and *gadph* (e.g Albu et al. 2015, Begerow et al. 2006, Lotze-Engelhard 2010, McTaggart et al. 2012, Wang et al. 2006).

Sequences of *rpb*1 and *rpb*2 were mostly derived from culture material of smut fungi, without any other DNA present, e. g. from other fungi or plants. However, the available primers result in no or unsatisfactory amplification results for several smut fungi. In some cases, the amplified sequences of the different protein-coding genes showed a patchy distribution among the samples used (e. g. Begerow et al. 2006, McTaggart et al. 2012). It is still not fully resolved, which impact these incomplete datasets have on the calculation of phylogenetic trees and controversial opinions exist (e.g. Lemmon et al. 2009). Roure et al. (2013) pointed out, that the outcome strongly depends on the calculation method used. Furthermore, their analysis showed a decrease of the resolving power of the phylogenetic branches. They also highlighted, that there are problems with phylogenetic artefacts, if too many incomplete data (samples) are used. But in many cases it is difficult to produce an entire dataset. For smut fungi, most of the multilocus trees published in different journals rely on sequencing of two loci of rDNA, ITS and LSU (Begerow et al. 2002, Piątek et al. 2012,

2013, Stoll et al. 2005, Vánky & Lutz 2007, 2010) and these datasets are mostly complete. Rarely multilocus studies exist with more than two loci used, but always as incomplete data sets. In general, ribosomal loci were combined with protein coding loci to produce reliable trees (e. g. Begerow et al. 2006, McTaggart et al. 2012, Wang et al. 2015).

Lotze-Engelhard (2010) based his analysis of the genus *Urocystis* on three different loci (LSU, ITS, *tef*-1α), Begerow et al. (2006), McTaggart et al. (2012) and Kruse et al. (2018b) used four different loci for their analysis. Piątek et al. (2016) used five different loci and Wang et al. (2015) analysed seven different loci for their phylogenetic analysis. More than 80% of the species analysed by Wang et al. (2015) were collection samples available as pure cultures, simplifying the amplification of different loci due to the lack of other contaminant DNA which is present in herbarium material. For the first time, nine different loci were used by Kruse et al. (2018a) to produce phylogenetic trees for the Ustilaginomycotina (*Ustilago striiformis*).

Lotze-Engelhard (2010) analysed the *rpb*1/2-locus of *Urocystis*, which was used by Wang et al. (2015), but they could not amplify most of the samples. Our preliminary lab work yielded unsatisfactory results (weak bands, dual bands, regularly no amplification), too. As Lotze-Engelhard (2010) was working with herbarium samples, and our preliminary primer tests had problems in amplifying these regions for herbarium samples, it is hypothesized that cultures are more readily amplified than non-cultured fungi. It seems that this primer is not specific enough to amplify the target DNA in a mixture of plant and other fungal DNA. Thus, it only worked for pure cultures, where only the target organism is available.

The *atp*6 locus and the *ß-tubulin* locus used in Begerow et al. (2006) were not used again in any other smut phylogeny to date. Beside the difficulties to guess which primer pair they used (this information is missing in the paper as well as in the supplementary material), we assume that they used the *atp*6 primers published in Kretzer & Brunns (1999) and the *ß-tubulin* primers of van der Mewe et al. (2007). Lotze-Engelhard (2010) tested these loci for the genus *Urocystis* and did not get any amplification result. This is in line with our initial tests, which also achieved only negative amplification result (data not shown), and is assumed to be one reason for the rare usage of these two gene loci (less than 30 sequences each deposited in GenBank, https://www.ncbi.nlm.nih.gov/). Furthermore, the *ß-tubulin* primers were designed for the amplification of the Boletales, and the *atp*6 primers for the

amplification of rust fungi. It is likely that they were too specific to amplify smut fungi. All these preliminary tests and available publications regarding the usefulness of the above mentioned primers resulted in the attempt of designing new and more specific primers for multilocus approaches to reveal species boundaries and the phylogeny of smut fungi.

Most of the loci presented in Kruse et al. (2017c) were not yet used for phylogenetic analysis within the smut fungi. Only *atp* (ATP-Synthase) and *ß-tubulin* (beta-Tubulin) were used by Begerow et al. (2006), but with other primers and/or regions (*atp*2 vs. *atp*6). Since some genomes are published for several smut fungi (e. g. *Malassezia globosa* (Xu et al. 2007), *Melanopsichium pennsylvanicum* (Sharma et al. 2014), *Moesziomyces* [*Pseudozyma*] aphidis (Lorenz et al. 2014), *Sporisorium reilianum* (Schirawski et al. 2010), *Ustilago hordei* (Laurie et al. 2012) and *U. maydis* (Kämper et al. 2006)), there was an opportunity to design new primers instead of trying to improve already available ones. The analysis in Kruse et al. (2018a) shows, that these newly designed primers are suitable for distinguishing closely related species of *Ustilago*. This was a result of design based on smut genomes of the Ustilaginomycetes, published previously. Most of the lineages which were only poorly resolved in Savchenko et al. (2014a) are given here with medium to high support values and showing different lineages based on host plant genus or species.

For the Exobasidiomycetes, Kruse et al. (2018b) presented new primers for the gene regions shown in Kruse et al. (2017c). For the *map* and the *ssc*1-locus Kruse et al. (2018b) presented new primer combinations. The *atp*2 locus is a promising locus for amplifying a wide range of smut fungi (Ustilaginomycetes and Exobasidiomycetes). Own preliminary work has shown, that the newly designed primers are not working for every species of a genus, due to sequence differences between these species. For example, the *atp*2 primer gives good amplification results for *Entyloma* species, except of *Entyloma* on Apiaceae. Within this family only a small set of different species worked (data not shown). This aspect shows the diversity of smut fungi and that it is impossible to get all *atp*2 sequences with only one primer set. The primers given in Kruse et al. (2017c, 2018b) give the chance to further improve these primers and optimize them for specific species complexes and/or species. With the opportunity to use now nine different gene loci for smut phylogenies the work in Kruse et al. (2018a) allow to discriminate also closely related species and species complexes, which could not be solved previously (e. g. *Entyloma* on Asteraceae, Begerow et a. 2006).

2.1.2 Host adaption

Nowadays the most common opinion is that smut fungi adapted to a narrow host range (i. e. single genera or certain species) and that the evolution of obligate biotrophy of the dikaryon plays an important role by explaining the evolutionary success and also the high diversity of this group. The lack of clear morphological differences between several species and also the occurrence of several homoplasies within the system of smut fungi renders the task to improve the molecular tools, to solve these problems. In Kruse et al. (2017a,c) such an improvement was made for the primers used for amplification. It is important to have in mind, that a lack of morphological differences distinguishing species does not automatically refute the presence of different species (e.g. Göker et al. 2009, Thines et al. 2009).

Several studies have shown that different groups of plant parasitic microfungi often contain cryptic species or species complexes (e. g. Bauer et al. 2008, Beenken et al. 2012, Göker et al 2009, Liu & Hambleton 2013, Piątek et al. 2013, Ploch et al. 2011, Voglmayr et al. 2014, see chapter 1.5.3). For anther smuts (*Microbotryum*: Le Gac et al. 2007, Lutz et al. 2005, 2008, Refrégier et al. 2008, Piątek et al. 2012) and downy mildews (Choi et al. 2009, Cunnington 2006, García-Blázquez et al. 2008, Thines et al. 2009) a similar situation exists. Sometimes a differentiation of closely related species based only on morphology seems to be nearly impossible (e. g. Göker et al. 2009, Voglmayr 2003 for Oomycota, Piątek et al. 2013, Savchenko et al. 2014a, for smut fungi). This is often called morphological 'cryptic-ness', while these species are often evolutionary separated. Phylogenetically distinct species can co-occur on the same host species, for example *Entyloma corydalis* de Bary and *E. urocystoides* Bubák on *Corydalis* DC., *Entyloma ficariae* A.A. Fisch. Waldh. and *E. majewskii* Vánky & M. Lutz on *Ficaria verna* Huds. or *Entyloma dahliae* Syd. & P. Syd. and *E. doebbeleri* M. Piepenbr. on *Dahlia* Cav. (Begerow et al. 2002, Lutz & Piątek 2016, Vánky & Lutz 2010).

The splitting of species complexes will be substantial, due to the vast amount of highly host specific plant parasitic microfungi. They are adapted to a single host plant genus or host plant species. Since these cryptic species are often closely related, it is in many cases difficult to find morphological, ecological or other characteristics to distinguish them (see chapter 1.5.3). To solve this problem nowadays phylogenetic

approaches are used to form a phylogenetic species hypothesis. But it is still difficult to delimitate such closely related species.

Savchenko et al. (2014a) were mostly unable to distinguish cryptic species within *U. striiformis* based on LSU and ITS loci. Due to the high diversity of smut fungi, it was not possible to design a single primer set working for all Ustilaginomycotina. Thus Kruse et al. (2017d, 2018b) designed primers for each of the two classes, namely Ustilaginomycetes and Exobasidiomycetes. In Kruse et al. (2017d, 2018b) primers were designed for the Ustilaginomycetes, and in Kruse et al. (2018b) primers were designed for the Exobasidiomycetes.

Three different species complexes (*Entyloma ranunculi-repentis* complex, *Entyloma microsporum* complex, both on *Ranunculus* and *Ustilago striiformis complex* on Poaceae) could be analysed in detail by using the primers of Kruse et al. (2018a,b), resolving these closely related species complexes. As already mentioned in the introduction, it is desirable to base the multigene trees on eight to ten different loci, to produce a reliable phylogenetic tree. This was not possible for smut fungi, due to the limitations of suitable loci and primers.

The studies of Kruse et al. (2018a,b) clearly show that not only the amount of gene loci is important to improve the phylogenetic trees or the resolution within species complexes. Important is also the variability of the different gene loci within a certain species group. The different gene regions used in Kruse et al. (2018a,b) showed diverse results for their SNPS, which is shown by the diagnostic bases. While *Ustilago loliicola* Civ. shows only one diagnostic base within the *ssc*1 locus (and in none of the other nine loci used), *U. corcontica* (Bubák) Liro on *Calamagrostis villosa* (Chaix ex Vill.) J. F. Gmel. had only differences in the *atp*2 locus. This demonstrates that different loci have their resolution optima in different species or species groups. If the *atp*2 locus would not have been included in the multilocus analysis, the smut species on *Calamagrostis villosa* would not have been revealed as a species on its own.

Of course, it is difficult to find a clear definition of what constitutes a new species, subspecies or variety, based mainly on phylogenetic trees. As already illustrated in chapter 1.5.3, a clear definition for that purpose is missing. Indeed, it is explained, what a phylogenetic species is and which criteria have to be accomplished, but there is no literature outlining, how many genetic differences (for example bases) have to occur between two taxa, to consider them as two different species. Especially for cryptic species this is a critical point. Phylogenetic trees could be substantiated

with morphological analysis to highlight apomorphic differences. Since these differences are often small or not evident within cryptic species and the transitions are flowing, other characters have to be detected to define a species. Very usable are the above mentioned diagnostic bases. They characterize a species and are unique within this species complex or cryptic species. They separate a species from all other of this complex. But again there is no clear regulation of species delimitation based on diagnostic differences within the sequence.

The host plant is an important characteristic for many fungal species, because most of these species are assumed to have a narrow host range. Some studies have shown that most plant parasitic microfungi are closely connected with their host plant (Kruse et al. 2018a,b, Savchenko et al. 2014a,b), since they evolved together over a long time. Smut fungi are strongly host specific and often differing in the choice of their hosts (=ecology), clearly shown in Kruse et al. (2018a,b). Both studies presented in this work used the method for species delimitation based on the combination of morphological, ecological and molecular markers, known as Consolidated Species Concept (CSC) (Quaedvlieg et al. 2014).

However, the studies of Kruse et al. (2018a,b) pointed out further difficulties of species groups. In Kruse et al. (2018b), the phylogenetic tree was based on four different loci, but especially within the *Entyloma microsporum*-complex not all lineages could be resolved. Although most of the plant parasitic microfungi are host specific, the analysis revealed two different species within *Entyloma microsporum* occuring on *Ranunculus repens* L., the type host. On the one hand a generalist which could live on several different species of *Ranunculus* L. and on the other hand a specialist with its occurrence restricted to *R. repens*. Similarly this has been observed multiple times for white blister rusts (Oomycota). Thines et al. (2009) have shown that two different species of *Albugo* (Pers.) Roussel occur on the host plant *Arabidopsis thaliana* (L.) Heynh., a specialist which is mainly occurring on the leaves (*Albugo laibachii* Thines & Y.J. Choi) and a generalist which mostly infects the whole inflorescence (*Albugo candida* (Pers.) Roussel). The same with *Capsella bursa-pastoris* (L.) Med.: *Albugo koreana* Y.J. Choi, Thines & H.D. Shin is the specialist occuring mainly on the leaves of this host, and *Albugo candida* is the generalist infecting mostly the inflorescences (Choi et al. 2007).

The *Entyloma microsporum*-complex in Kruse et al. (2018b) is not distinguishable completely with usage of only four loci and further gene regions are

necessary. In Kruse et al. (2017c) another species complex *Moesziomyces bullatus* was investigated, only with the usage of the barcoding locus ITS. It shows, that for some species already the ITS locus is suitable to distinguish between closely related species. This was also shown by Savchenko et al. (2014b) for *Entyloma* on *Eryngium* L., by Li et al. (2017a) for *Tranzscheliella* Lavrov on Poaceae or by Li et al. (2017b) for *Macalpinomyces* Langdon & Full. on *Eriachne* Phil..

It depends on the smut species, genera or family, if the ITS barcoding locus is enough to distinguish intraspecifically, or if further loci have to be used in a multilocus approach. To produce reliable phylogenetic tree also the amount of samples/different fungal-host combination and the availability of the type specimen or alternatively the same fungal-host-combination compared to the type is necessary. This is shown by the work of Kruse et al. (2017c). *Moesziomyces* Vánky was assumed to be monotypic, until Wang et al. (2015) found three other yeasts of the genus *Pseudozyma* pro. temp. also belonging to this genus – mostly occuring as apathogenic yeasts in different habitats (Kruse et al. 2017c). Within the smut fungi there are several yeasts known (e. g. *Pseudozyma* pro. temp., *Tilletiopsis* Derx*., Entylomella* Höhn.) polyphyletic with the whole Ustilaginomycotina. These yeasts could occur nearly everywhere without a specific host. For some species the connection to the sexual stages could be linked based on multilocus approaches (Wang et al. 2015). Kruse et al. (2017c) pointed out that three further *Moesziomyces* species have to be included in this genus (*Moesziomyces eriocauli* (G.P. Clinton) Vánky, *M. verrucosus* (J. Schröt.) J. Kruse & Thines and *M. penicillariae* (Bref.) Vánky), whereas two of the former ones have to be excluded (*M. rugulosus* (Traquair, L.A. Shaw & Jarvis) Q.M. Wang, Begerow, F.Y. Bai & Boekhout and *M. aphidis* (Henninger & Windisch) Q.M. Wang, Begerow, F.Y. Bai & Boekhout) because of conspecifity to *Moesziomyces bullatus*, the type species of the genus. Wang et al. (2015) could not see this conspecifity, because they did not include the type fungal-host combination of *Moesziomyces bullatus* in their studies, but the sister species *M. penicillariae*. Recently, a conspecifity was shown between *Pseudozyma tsukubaensis* (Onishi) Boekhout (one of five remaining species of the genus *Pseudozyma* which have to be connected to their sexual stage) and *Ustilago spermophora* Berk. & M.A. Curtis, a parasite in fruits of *Eragrostis* Host. species (Tanaka & Honda 2017). Unfortunately, they did not include the host of the type species of *Ustilago spermophora*, thus it is impossible to verify the conspecifity between these two species. This highlights how important it is to work with type

species or comparable identical fungal-host-combinations of smuts, because many smut fungi are highly host specific (see chapter 1.5.3). This is sometimes very difficult, because many smut fungi have been found only once worldwide and some collections are quite old (>100 years). To get DNA and an intact sequence out of these samples is still a challenge. Some authors already tested different kits, taqs and extraction methods (e.g. Telle & Thines 2008). Our preliminary worked yielded best results by using the Analytik Jena Kit (Qiagen, Hilden) and an increasing of the amount of utilized DNA.

Since most smut fungi are assumed to have a narrow host range, it sounds obvious that all smut fungi occuring on a couple a different host plant genera or host plant species could represent a species group. Smut species with a broad host range are currently: *Cintractia axicola* (Berk.) Cornu, *C. limitata* G.P. Clinton, *Entyloma fergussonii* (Berk. & Broome) Plowr., *Farysia thuemenii* (A.A. Fisch. Waldh.) Nannf., *Jamesdicksonia dactylidis* (Pass.) R. Bauer, Begerow, A. Nagler & Oberw., *Macalpinomyces spermophorus*, *Schizonella melanogramma* (DC.) J. Schröt., *Sporisorium andropogonis* (Opiz) Vánky, *Sp. consanguineum* (Ellis & Everh.) Vánky, *Thecaphora saponariae* (F. Rudolphi) Vánky, *Th. thlaspeos* (Beck) Vánky, *Tilletia bromi* (Brockm.) Nannf., *T. contraversa* J.G. Kühn, *T. fusca* Ellis & Everh., *Tranzscheliella hypodytes*, *T. williamsii, Urocystis agropyri (Preuss)* A.A. Fisch. Waldh., *U. ranunculi* (Lib.) Moesz, *Ustilago bromivora* (Tul. & C. Tul.) A.A. Fisch. Waldh., *U. avenae* (Pers.) Rostr., *U. hordei* (Pers.) Lagerh., *U. scitaminea* Syd., *U. trichophora* (Link) Kunze and *U. tritici* (Pers.) Rostr. (e. g. Vánky 2012). All these species occur on several different host plant genera. They will harbour a lot of new species and should be investigated in detail with the usage of the new tools presented in this work.

Looking into species complexes implies also deep systematic and taxonomic work. According to the priority rules the oldest epithet has to be found, to reveal the valid species name (McNeil et. al. 2012). Mostly species complexes have many different names deposed in the synonymy-list. When splitting a complex in several host specific lineages, these synonymies have to be considered. Sometimes several problems occur while searching for the correct type specimen. Especially the abolishment of the dual naming of pleomorphic fungi renders the task to find the valid names for smut fungi, since the names of these asexual stages have also taken to account. Many pleomorphic fungi are having more than one legitimate name recently.

Rossman et al. (2016) pointed out, that this has influenced many plant associated fungi, many of which have more than one name. They highlighted that many mistakes are based on wrong assignment. Mostly the oldest epithet was not placed in the oldest or preferred genus. This was also the case for '*Entyloma ranunculi-repentis*' - Rossman et al. (2016) pointed out, that the oldest valid name which has priority is *Ramularia gibba* Fuckel. Nobody realized that the type species of *Entyloma gibbum* (Syn. *Ramularia gibba*) is a chimera of two different smut fungi (*Entyloma ranunculi-repentis* and *Entyloma microsporum*), both on the herbarium sample and within the species description of the type collection. To prevent confusion, Kruse & Thines (2017) proposed the name *Entyloma gibbum* [*Ramularia gibba*] for rejection and thus pointed out that *Entyloma eburneum* (J. Schröt.) J. Kruse, M. Lutz, Piątek & Thines would then be the next valid published name.

The studying of the *Ustilago striiformis* complex in Kruse et al. (2018a) revealed a related problem. During the investigation of synonymies of this species complex, *U. salweyi* Berk. & Broome was found out to be the valid name for the stripe smut on *Holcus lanatus* L.. Stevenson (1946) flagged the name *Ustilago salweyi* as a "nomen ambiguum", but no action was taken to reject the name. Following the ICN (McNeill et al. 2012), the name *U. salweyi* has priority over *Uredo striiformis* Westend. as it was published two years earlier (Berkeley & Broome 1850).

2.2 Difficulties in obtaining multilocus studies

Making good and reliable trees is not only dependent on the gene or locus used, but also on the number of species in the tree or different host-fungus-combinations. Which tool and method should be used is based on the research question. Since many smut fungi are very rare (Vánky 2012), it is often impossible to work with fresh material or recently collected material (not older than 20 years). One third of all smuts was only found once worldwide. And also the availability of cultures is limited and in many cases there is only one fungus-host combination available, usually the type species.

Often it is necessary to work with material from different herbaria. Due to many huge collections of plant parasitic microfungi in different public herbaria, there is the opportunity to use a large amount of different smut species. Unfortunately, many important collections are quite old (e.g. Fuckel 1863, Jaap 1906, 1908, 1909, 1911, 1913, 1915, 1916, 1918, Sydow 1912, Vestergren 1900) and especially many type

species of smut fungi are often more than 100 years old. Due to the problem of DNA degradation correlating with the age, it is a big challenge to get the DNA in a sufficient quality (Telle & Thines 2008). Sampling of DNA is crucial for the outcome of the PCR. If DNA is degraded it is no longer possible to get long fragments. In that case it is possible to amplify smaller fragments. Several different primers are available for such purpose, but as most of them are often unspecific, the DNA of other fungal groups or even plants is also amplified (Vilgalys & Hester 1990). Our preliminary research on amplifying DNA from type material of smut specimens older than 20 years often yielded in unsatisfying results. Several different extraction protocols and modifications were tested with smut samples of different age. The Analytik Jena Kit (Qiagen, Hilden) with addition of PTB (N-phenacylthiazolin-bromide) in the extraction step revealed the best results. The newly designed ITS primers (Kruse et al. 2017a) helped in amplifying the DNA, also in combination with ITS1 or ITS2 primers to get a smaller fragment. But the success for the different smut fungi sometimes differs between different genera or species, which could be reasoned by various specific primers.

A negative sequencing result can have various reasons. It is not only the case of possible degradation of DNA because of the age. The quality of the DNA depends on many circumstances. Mostly the storing conditions of the herbarium samples are not known, or the treatment with different insecticides or other substances. And also the regular freezing applied to keep insects away could have a negative impact on the quality of DNA (Redchenko et al. 2012, Kruse et al. 2017c).

The following observations could be made during our previous experiments (data not published). Herbarium contamination with other fungi and/or coamplification of other fungi during the PCR. Miranda et al. (2010) pointed out, that the non-specific amplification results have a serious impact on the phylogenetic results.
Especially when doing nested or semi-nested PCR which is highly sensitive, other fungi frequently contaminated results (data not shown). For fungi a contamination of samples is especially reported for clinical samples (e.g. Czurda et al. 2016, Loeffler et al. 1999). There are several possible options, why the sequencing of a smut fungus yields in a sequence of other fungi or plants.

1. Contamination with other smut fungi. Amplifying smuts with spores embedded in the host tissue (for example *Entyloma*) sometimes yields in amplification results of spore mass producing genera like *Sporisorium* Ehrenb. ex Link*, Ustilago* (Pers.) Roussel or *Urocystis* Rabenh.. As the formerly-known primers are best working

for Ustilaginomycetes they will preferably amplify species belonging to that group, even if there are only few DNA traces in the sample. When collecting and drying smut samples by different collectors the same newspaper for pressing the species could have been used for several different smuts. Or on the post way from one herbaria to another with the specimens in their paper capsules. Spores can likely be transferred from one herbaria sheet to another during the shaking in the post car.

2. Amplification result of Ascomycota, like *Alternaria* Nees, or *Penicillium* Link. These fungi are so called ubiquists, as they could occur nearly everywhere. Especially in maturity these fungi coexist with smuts and other fungi on the sori. Since Ascomycota are amplifying preferably, some sequencing results could be explained by this.

3. Spores of various fungi occuring nearby the smut fungi could land on the leaves, like *Trametes* Fr., *Boletus* L., *Phallus* Junius ex L. etc. by wind or even insects. Similarities during own preliminary observations were revealed between different smut fungi in cultivation trials. Fresh material of *Urocystis agropyri* (Preuss) A.A. Fisch. Waldh. was collected nearby a maize field and tried to be cultivated. Even after thorough surface sterilization of the sample the resulting yeast belonged to *Ustilago maydis* (DC.) Corda, which occurred on some corncobs in the field nearby and has contaminated various surfaces in the surrounding due to massive spores production of this species (data not shown).

4. Amplification of endophytes of the plant (Chiang et al. 2001, Gazis & Chaverri 2010, Petrini 1991, Ploch & Thines 2011). Many plants have endophytic fungi which live within the plant tissue, but growing symptomless without causing injury to the host plant (Maheshwari 2006, Neto et al. 2004).

Still, much work is left in understanding the species boundaries and phylogeny of smut fungi. The findings outlined in the thesis present tools for phylogenetic analysis of smut fungi, many species complexes of smut fungi could be solved in future, based on ITS or multilocus studies. It was confirmed with *Ustilago striiformis* and *Entyloma ranunculi-repentis* that smut fungi are mainly host specific, supporting the narrow species complex and augments the necessity to revaluate some smut species with broad host ranges. And also phylogenies of smut fungi in general could be advanced with the usage of the new primers, unless species could be sequenced, which were not part of any investigation. Using different loci will enhance the backbone of smut

phylogenies, even on genus or family level, revealing a splitting of huge genera in several small genera or not. But also other approaches for obtaining insights in population genetics are feasible. Since already some genomes of smut fungi are published, specific microsatellites could be designed for a detailed investigation of population genetics. This will give insights to diversity, speciation and specialisation of some smut fungi. Our future work will focus on the investigation of several species complexes, like *Ustilago avenae-bromivora-hordei* and the goal is to create an improved phylogeny of the whole Ustilaginomycotina with a broader sampling and the gene loci developed in this work.

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Appendices

3 New smut-specific primers for the ITS barcoding of Ustilaginomycotina. Mycological Progress 16: 213–221.

Julia Kruse & Young-Joon Choi & Marco Thines

Statement of Joint Authorship

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What did the PhD student or the Co-Authors contributed to this work?

(1) Development and planning

PhD student JK: 50 %

Co-Author Y-JC: 10 %

Co-Author MT: 40 %

(2) Performance of the individual investigations and experiments

PhD student JK: 80%, collection trips, primer design (smut specific ITS primer), primer testing (laboratory experiments, molecular work)

Co-Author Y-JC: 15%, helped with the primer designing

Co-Author MT: 5%, helped with the primer designing

(3) Preparation of the data collection and figures

PhD student JK: 90%, create different excel files for different primer combinations, pictures with power point, data compilation

Co-Author MT: 10%, helped with the designing of figures and tables

(4) Analyse and interpretation of data

PhD student JK: 50%, literature search, phylogenetic trees, general interpretation

Co-Author Y-JC: 10%, helped with phylogenetic trees

Co-Author MT: 40%, important feedback on several points and new ideas for interpretation

(5) Writing the manuscript

PhD student JK: 55%

Co-Author Y-JC: 5%

Co-Author MT: 40%

Date/Place: _____

Signature PhD. student: _____

Verification of the statements above

Signature Supervisor (=Corresponding author):

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ORIGINAL ARTICLE

New smut-specific primers for the ITS barcoding of Ustilaginomycotina

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Abstract The smut fungi (Ustilaginomycotina) are a highly diverse group, containing about 115 genera and 1700 species, most of which are biotrophic plant pathogens. As for other fungal groups, the ITS rDNA region is widely used to determine smut fungi at species level due to its high discriminatory power and for phylogenetic reconstructions within genera. So far, two primer sets, ITS1/ITS4 and M-ITS1/ITS4, were generally used to amplify smut fungi, but these often co-amplify host plants or contaminant fungi and do not yield satisfactory amplification for a variety of smut fungi. In the present study, based on a selection of genera that include more than 90% of the species of smut fungi (more than half of the genera of smut fungi), three new primers, smITS-F, smITS-R1 and smITS-R2, situated in the SSU or LSU region, were designed to avoid the amplification of host plants and to extend the coverage of PCR amplification for as many smut genera as possible.

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Introduction

Smut fungi (subphylum Ustilaginomycotina) contain about 115 genera and more than 1700 species (Begerow et al. 2014). Most of them parasitise angiosperm plants, in particular within Poaceae and Cyperaceae, but some species are associated with other tracheophytes, like *Melaniella* on spikemosses (*Selaginella*), *Exoteliospora* and *Violaceomyces* on ferns and *Uleiella* on conifers (Vánky 2012; Albul et al. 2015). For many smut fungi yeast-like anamorphs are known, e.g. species of the genera *Rhodotorula* and *Pseudozyma* (Begerow et al. 2006; Wang et al. 2015). Wang et al. (2015) recently transferred most anamorphic species of the genus *Pseudozyma* to a corresponding teleomorph genus.

There are two classes of smut fungi within the Ustilaginomycotina. The Ustilaginomycetes mostly share the characteristic that they develop a brownish spore mass of thick-walled teliospores at various parts of the plants (Vánky 2012). The human-pathogenic Malasseziales have either been included in this class or been referred to as their own class (Wang et al. 2014). Recent phylogenomic studies (Sharma et al. 2015; Mishra et al. unpublished data) inferred an early divergence for *Ceraceosorus bombacis*, being sister to a group formed by *Malassezia* and all other smut fungi with sequenced genomes. This means that the monophyly of the Exobasidiomycetes is not yet fully resolved and the higher-level classification of the smut fungi is still in flux. Thus, we here refer to the Exobasidiomycetes in the circumscription given in Begerow et al. (2006). The Exobasidiomycetes are typically non-teliosporic plant parasites (Bauer et al. 2001; Begerow et al. 2006), even though some genera, such as

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Jamesdicksonia and *Tilletia* (Begerow et al. 2006; Vánky 2012), form blackish teliospores. If this is the result of parallel convergent evolution or if the non-teliosporic smut fungi have lost the ability to produce teliospores is currently unclear. The class Microbotryomycetes, well known as anther smuts parasitizing various plant families, especially the Caryophyllaceae, was formerly considered to belong to the Ustilaginomycotina, but is now classified under the rust fungi (Pucciniomycotina) (Bauer et al. 1997). Similarly, the class Entorrhizomycetes, which was long believed to belong to the Ustilaginomycotina (Vánky 2012), has been proposed to constitute a subphylum of its own (Bauer et al. 2015), which is either the sister-group to the smut fungi or even the sister-group to all Dikarya.

Smut fungi include several well-known genera, such as *Ustilago*, *Tilletia* and *Entyloma*. Some species cause global economic losses, e.g. loose smut of barley (*Ustilago nuda*), maize smut (*Ustilago maydis*), and wheat bunt (*Tilletia tritici*). *Ustilago maydis* has also become a model organism for dissecting plant–pathogen interactions (Kämper et al. 2006). Several phylogenetic studies for smut fungi have been published during the past two decades, mostly based on ITS or LSU rDNA loci (Albul et al. 2015; Begerow et al. 1997, 2000; Boekhout et al. 2003; Castlebury et al. 2005; Cunnington et al. 2005; McTaggart et al. 2012; Nasr et al. 2014; Piątek et al. 2016; Savchenko et al. 2014; Stoll et al. 2003, 2005). The nrLSU provides a better resolution of the suprageneric splits but has insufficient resolution to discriminate between closely related species (Mahmoud and Zaher 2015). Similar to other fungal groups (Schoch et al. 2012), the ITS region has demonstrated its usefulness for species differentiation in several genera (e.g. *Malassezia*; Wang et al. 2014), while some groups of very closely related species are still not fully resolved based on ITS sequences (*Ustilago striiformis*; Savchenko et al. 2014). Due to the highly divergent groups within Ustilaginomycotina, it is difficult to design primer pairs that can be used for all groups of smut fungi, while at the same time being specific enough to minimize unspecific amplification, e.g. of host plants and other fungal groups.

Variants of the commonly used primer pair ITS1 and ITS4 (White et al. 1990) have been developed for more specific amplification of fungi. These include the reverse primer ITS4-B (Gardes and Bruns 1993), modified for amplification of Basidiomycetes. The forward primer ITS1-F has been modified for amplification of fungi, in particular Ascomycota (Gardes and Bruns 1993), and the forward primer M-ITS1 (Stoll et al. 2003) was designed for the amplification of Ustilaginaceae, but has also proven useful for ITS amplification of other groups of Ustilaginomycetes. However, for some groups of smut fungi, none of the available primer combination yielded satisfactory results in preliminary amplification tests and an amplification of host DNA or contaminant fungi was often observed. Thus, it was the aim of the current study to design smut-specific primers to improve PCR amplification for this diverse group of fungi and ideally cover some genera for which amplification is difficult with the existing primer pairs.

Materials and methods

Fungal specimens

The specimens used in this study are listed in Table 1. The nomenclature of the hosts is derived from The International Plant Names Index (http://www.ipni.org), for all genera covered in that publication. For the names and classification of the smut fungi, we followed Vánky (2012) and complemented the dataset using the indexfungorum (http://www.indexfungorum.org/) database for recent genus descriptions and changes in nomenclature.

Primer design

A representative set of sequences of the large subunit (LSU) and the small subunit (SSU) were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) covering plants, non-target fungi (Basidiomycota and Ascomycota) and Ustilaginomycotina. Alignments for each locus (LSU and SSU) were carried out using mafft (Katoh and Standley 2013) v.7, employing the Q-INS-I algorithm. The alignment was then inspected using Geneious 6.0.6 (Biomatters, New Zealand) to identify potential primer positions. Subsequently, primers were designed manually considering their specificity, GC-content (40–65%) and internal structure (no inverse matches of more than 6 bp at the 3′ end). Three forward primers (smITS-F, 18S-S1F, 18S-S2FM) were designed from the SSU region, of which smITS-F performed best in initial tests and was thus used for further experiments, and three reverse primers, smITS-R1, smITS-R2, and ITS-S3 R, were designed from the LSU region, of which smITS-R1 and R2 performed best in initial tests and were thus used for further experiments. We furthermore slightly modified the existing M-ITS1 primer (named M-ITS1M) and used it in some initial tests (Table 2; Fig. 1).

DNA extraction, PCR and sequencing

About 2–20 mg of infected plant tissue was taken from each dried herbarium specimen. Tissue samples were disrupted in a mixer mill (MM200; Retsch, Germany), using two iron beads of 3 mm and five to eight iron beads of 1 mm diameter per sample and shaking at 25 Hz for 5–10 min. Genomic DNA was extracted using the E.Z.N.A. Plant DNA Kit short protocol (Omega, Norcross, GA, USA). PCR amplification of the ITS-rDNA of the specimens was performed using four forward primers, M-ITS1 (Stoll et al. 2003), smITS-F (designed

Table 1 Smut species used for primer testing

Species	Host	Hostfamily	Collection details			ITS GenBank Acc. No.
			Location	Year	Collector	Voucher /strain
Ahmadiago euphorbiae	*Euphorbia dracunculoides*	Euphorbiaceae	India	Unknown	H. Chauduri	BPI 160414
***Anomalomyces* sp.**	*Panicum trachyrhachis*	Poaceae	Australia	2006	M. J. Ryley	VPRI 40639a
Antherospora hortensis	*Muscari armeniacum*	Hyacinthaceae	Germany	2013	J. Kruse	GLM-F105770
Antherospora tourneuxii	*Bellevalia dubia*	Liliaceae	Italy	2014	J. Kruse	GLM-F105791
Anthracocystis themedae-arguentis	*Themeda quadrivalvis*	Poaceae	Australia	1996	A. A. Mitchell, C. & K. Vánky	BRIP Vánky Ust. exs. 972
Anthracoidea arenaria	*Carex brizoides*	Cyperaceae	Germany	2011	J. Kruse	GLM-F105771
Anthracoidea echinospora	*Carex acuta*	Cyperaceae	Germany	2013	J. Kruse	GLM-F105772
Anthracoidea sempervirentis*	*Carex sempervirens*	Cyperaceae	Germany	2012	J. Kruse	GLM-F105803
Arcticomyces warmingii	*Saxifraga rotundifolia*	Saxifragaceae	Germany	2014	J. Kruse	GLM-F105799
Bambusiomyces shiraianus	*Arundinaria faberi*	Poaceae	Ceylon (Sri Lanka)	1974	K. Vánky	BRIP Vánky Ust. exs. 171
Botryoconis saccardoi	*Oreodaphne* sp.	Lauraceae	Brasilia	1906	Rick	HBG 113/2301
Burrillia ajrekari	*Monochoria vaginalis*	Pontederiaceae	China	1985	L. Guo & K. Vánky	BRIP Vánky Ust. exs. 504
Ceraceosorus bombacis	pure culture			2016		ATCC 22867
Cintractia axicola	*Fimbristylis dichotoma*	Cyperaceae	India	1995	N. D. Sharma et al.	BRIP Vánky Ust. exs. 951
Cladosterigma fusisporum	Myrtaceae	Myrtaceae	Brasilia	1898	F. Noack	HBG 111/2301
Clintamra nolinae	*Nolina microcarpa*	Asparagaceae	USA	1969	R. Duran	WSP 58501
Conidiosporomyces ayresii	*Panicum maximum*	Poaceae	Argentinia	1999	C. & K. Vánky	HUV 19.314
Dermatosorus cyperi	*Cyperus* aff. *celluloso-reticulatus*	Cyperaceae	Venezuela	1993	R. Berndt, C. & K. Vánky	WSP 70652
Doassansia limosellae	*Limosella aquatica*	Plantaginaceae	Germany	2000	H. & U. Richter	GLM-F105787
Doassansia sagittariae	*Sagittaria sagittata*	Alismataceae	Germany	2000	H. & U. Richter	GLM-F105788
Doassansiopsis hydrophila	*Potamogeton polygonifolius*	Potamogetonaceae	Germany	2009	V. Kummer	GLM-F105789
Doassinga callitrichis	*Callitriche stagnalis*	Plantaginaceae	Germany	1986	F. Oberwinkler & K. Vánky	BRIP Vánky Ust. exs. 560
Drepanoconis brasiliensis	*Nectandra reticulata*	Lauraceae	Brasilia	1889	E. Ule?	HBG 99/2301
Eballistra lineata	*Zizania aquatica*	Poaceae	Canada	1989	B., B. & J. Nielsen	BRIP Vánky Ust. exs. 741
Entorrhiza aschersoniana*	*Juncus bufonius*	Juncaceae	Germany	2011	J. Kruse	GLM-F105804
Entorrhiza casparyana	*Juncus articulatus*	Juncaceae	Germany	2012	H. Jage et al.	GLM-F105790
Entyloma calendulae*	*Calendula officinalis*	Asteraceae	Germany	2010	J. Kruse	GLM-F105805
Entyloma hieracii	*Hieracium murorum* s. l.	Asteraceae	Germany	2013	J. Kruse	GLM-F105773
Entyloma magnusii s. l.	*Helichrysum arenarium*	Asteraceae	Germany	2014	J. Kruse	GLM-F105795
Exobasidium japonicum	*Rhododendron kiusianum*	Ericaceae	Germany	2014	J. Kruse	GLM-F105792
Exobasidium uvae-ursi*	*Arctostaphylos uva-ursi*	Ericaceae	Italy	2013	J. Kruse	GLM-F105774
Exoteliospora osmundae	*Osmunda regalis*	Osmundaceae	Unknown	Unknown	Unknown	UZH 234 (ex TUB)
Farysia thuemenii	*Carex riparia*	Cyperaceae	Germany	2004	H. John	GLM-F064758
Farysporium endotrichum	*Gahnia xanthocarpa*	Cyperaceae	New Zealand	1990	E., U. & K. Vánky	BRIP Vánky Ust. exs. 828
Fereydounia khargensis	pure culture			2016		CBS 13305
Floromyces anemarrhenae						

Table 1 (continued)

Species	Host	Hostfamily	Collection details			ITS GenBank Acc. No.
			Location	Year	Collector	Voucher /strain
	Anemarrhena asphodeloides	Asparagaceae	China	2007	T. - Z. Liu	M-0216090
Franzpetrakia microstegii	*Phacelurus latifolius*	Poaceae	China	2002	R. G. Shivas & H. E. C. Evans	HUV 20.192
Graphiola phoenicis	*Phoenix canariensis*	Arecaceae	New Zealand	2005	B. Rhode	PDD 85521
Heterodoassansia hottoniae	*Hottonia palustris*	Primulaceae	Germany	2000	S. Rätzel	GLM-F047407
Heterodoassansia hygrophilae	*Hygrophila auriculata*	Acanthaceae	India	1992	N. D. Sharma et al.	BRIP Vánky Ust. exs. 815
Heterodoassansia ranunculina	*Ranunculus peltatus*	Ranunculaceae	Germany	2005	H. Jage	GLM-F076002
Heterotolyposporium lepidospermatis	*Lepidosperma ensiforme*	Cyperaceae	Australia	1996	C. & K. Vánky	BRIP Vánky Ust. exs. 957
Jamesdicksonia brizae	*Briza minor*	Poaceae	New Zealand	2008	E. H. C. McKenzie	PDD 95170
Laurobasidium lauri	*Laurus azorica*	Lauraceae	Spain	2002	S. Bräutigam	GLM-F046197
Leucocintractia leucoderma	*Rhynchospora corymbosa*	Cyperaceae	Costa Rica	1991	T. & K. Vánky	BRIP Vánky Ust. exs. 810
Macalpinomyces neglectus	*Setaria pumila*	Poaceae	Germany	2012	J. Kruse	GLM-F105775
Melanopsichium pennsylvanicum	pure culture			2016		ex Mycotheca Graecensis 285
Melanotaenium endogenum	*Galium album*	Rubiaceae	Germany	2014		GLM-F105796
Melanustilospora ari	*Arum maculatum*	Araceae	Germany	2014	J. Kruse	GLM-F105800
Microstroma juglandis	*Juglans regia*	Junglandaceae	Germany	2013	J. Kruse	GLM-F105776
Moesziomyces bullatus	*Echinochloa crus-galli*	Poaceae	Germany	2011	J. Kruse	GLM-F105777
Moreaua mauritiana	*Fimbristylis ovata*	Cyperaceae	India	1995	N. D. Sharma, C. & K. Vánky	M-0040282
Mundkurella mossii	*Aralia nudicaulis*	Araceae	Canada	1991	Y. & E. Hiratsuka	BRIP Vánky Ust. exs. 834
Mycosyrinx cissi	*Cissus verticillata*	Vitaceae	Costa Rica	1991	T. & K. Vánky	BRIP Vánky Ust. exs. 835
Nannfeldtiomyces sparganii	*Sparganium erectum*	Sparganiaceae	Germany	2004	H. Jage	GLM-F074570
Neovossia moliniae	*Molinia caerulea*	Poaceae	Germany	2002	H. Jage	GLM-F063830
Orphanomyces arcticus	*Carex davalliana*	Cyperaceae	Austria	2012	J. Kruse	GLM-F105778
Pericladium tiliacearum	*Grewia villosa*	Malvaceae	Namibia	2005	S. Neser	WSP 71988
Pseudodermatosorus sagittariae	*Sagittaria planitiana*	Alismataceae	Venezuela	1993	R. Berndt, C. & K. Vánky	WSP 70317
Pseudodoassansia hydrocleidis	*Hydrocleis nymphoides*	Alismataceae	Argentinia	Unknown	C. & K. Vánky	PDD 74716
Restiosporium baloskionis	*Baloskion tetraphyllum*	Restionaceae	Australia	2001	R. G. Shivas & K. Vánky	BRIP Vánky Ust. exs. 1292
Rhamphospora nymphaeae	*Nymphaea*-Hybrid	Nymphaceae	Germany	2014	J. Kruse	GLM-F105801
Rhamphospora nymphaeae*	*Nymphaea alba*	Nymphaceae	Germany	2011	J. Kruse	GLM-F105806
Schizonella elynae	*Elyna myosoroides*	Cyperaceae	Italy	2013	J. Kruse	GLM-F105779
Sporisorium andropogonis	*Bothriochloa ischaemum*	Poaceae	Germany	2014	J. Kruse	GLM-F105797
Sporisorium vanderstytii	*Hyparrhenia hirta*	Poaceae	Italy	2014	J. Kruse	GLM-F105793
Stegocintractia capitata	*Juncus capitatus*	Juncaceae	Germany	2007	U. Schlüter et al.	HUV 21.504
Thecaphora saponariae	*Saponaria officinalis*	Caryophyllaceae	Germany	2013	J. Kruse	GLM-F105780
Tilletia controversa	*Alopecurus aequalis*	Poaceae	Germany	2001	S. Bräutigam	GLM-F044677
Tilletia olida	*Brachypodium pinnatum*	Poaceae	Germany	2013	J. Kruse	GLM-F105781
Tilletia olida*		Poaceae	Germany	2012	J. Kruse	GLM-F105807

Table 1 (continued)

Species	Host	Hostfamily	Collection details			ITS GenBank Acc. No.
			Location	Year	Collector	Voucher /strain
	Brachypodium pinnatum					
***Tilletiopsis* sp.**	*Dianthus* sp.	Caryophyllaceae	Australia	1999	G. Kalc-Wright	VPRI 22019
Tracya hydrocharidis	*Hydrocharis morsus-ranae*	Hydrocharitaceae	Germany	2014	J. Kruse	GLM-F105802
Tranzscheliella hypodytes	*Bromus erectus*	Poaceae	Germany	2013	J. Kruse	GLM-F105782
Trichocintractia utriculicola	*Rhynchospora corymbosa*	Cyperaceae	Argentinia	1999	C. & K. Vánky	HUV 19.316
Uleiella chilensis	pure culture			2016		TUB 020322
Urocystis agropyri	*Elymus repens*	Poaceae	Germany	2013	J. Kruse	GLM-F105783
Urocystis agropyri*	*Elymus repens*	Poaceae	Germany	2012	J. Kruse	GLM-F105808
Urocystis colchici	*Colchicum autumnale*	Colchicaceae	Germany	2013	J. Kruse	GLM-F105784
Ustacystis waldsteiniae	*Waldsteinia geoides*	Rosaceae	Hungaria	1987	J. Gönczöl, U. & K. Vánky	BRIP Vánky Ust. exs. 629
Ustanciosporium montagnei	*Rhynchospora alba*	Cyperaceae	Germany	2009	K. Wöldecke	GLM-F105785
Ustilago avenae	*Avena barbata*	Poaceae	Italy	2014	J. Kruse	GLM-F105794
Ustilago avenae*	*Arrhenatherum elatius*	Poaceae	Germany	2011	J. Kruse	GLM-F105809
Ustilago esculenta	pure culture			2016		Us_es_01
Ustilago serpens	*Elymus repens*	Poaceae	Germany	2014	J. Kruse	GLM-F105798
Vankya ornithogali	*Gagea lutea*	Liliaceae	Germany	2007	J. Kruse	GLM-F105786
Websdanea lyginiae	*Lyginia barbata*	Restionaceae	Australia	1994	K. Websdane	HUV 16.361

[a] Samples used in the initial set of tests

in this study), ITS1 (White et al. 1990) and ITS1-F (Gardes and Bruns 1993), and three reverse primers, ITS4 (White et al. 1990), smITS-R1, and smITS-R2 (designed in this study). All primers used in this study are listed in Table 2. PCR was performed in an Eppendorf MasterCycler 96, equipped with a vapo protect lid (Eppendorf, Hamburg, Germany) using the following conditions. For the first set of amplification on representative genera of the Ustilaginomycotina (indicated in Table 1), 18S-S1F, 18S-S2FM, smITS-F, M-ITS1M, ITS1-F and M-ITS1 were each combined with smITS-R1, smITS-R2, ITS-S3R, NL1R (O'Donnell 1993), ITS4, or 5.8S (Vilgalys and Hester 1990). PCRs were performed with an initial denaturation at 95 °C for 4 min, and 36 cycles of denaturation at 95 °C for 40 s., annealing at 55 °C for 40 s. and elongation at 72 °C for 1 min, followed by a final elongation at 72 °C for 4 min. The best primer combinations, smITS-F with smITS-R1, M-ITS1 with smITS-R1, and M-ITS1 with smITS-R2 were subsequently tested using gradient PCR (49–62 °C).

Table 2 Primers tested for the amplification of the ITS rDNA region

Primer name	Sequence	Source
M-ITS1	GGTGAACCTGCAGATGGATC	Stoll et al. (2003)
ITS1	TCCGTAGGTGAACCTGCGG	White et al. (1990)
ITS1-F	CTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns (1993)
ITS4	TCCTCCGCTTATTGATATGC	White et al. (1990)
smITS-R1	AGATGGCATTTACCACCCATTTTGM	This study
smITS-R2	AGATGGMATTTACCACCCAT	This study
ITS-S3R	TATTTAGCYTTAGATGGC	This study
smITS-F	CAAACTYGGTCATTTAGAGGAAGTAA	This study
18S-S1F	ACGCGCGCTACACTGACAGAG	This study
18S-S2FM	ACTACCGATTGAATGGCTYARTGAG	This study
M-ITS1M	GGTGAACCTGCRGAWGGATC	This study (modified M-ITS1)
NL1R	CTTTTCCTCCGCTTATTGATATGC	O'Donnell (1993)
5.8S	CGCTGCGTTCTTCATCG	Vilgalys and Hester (1990)

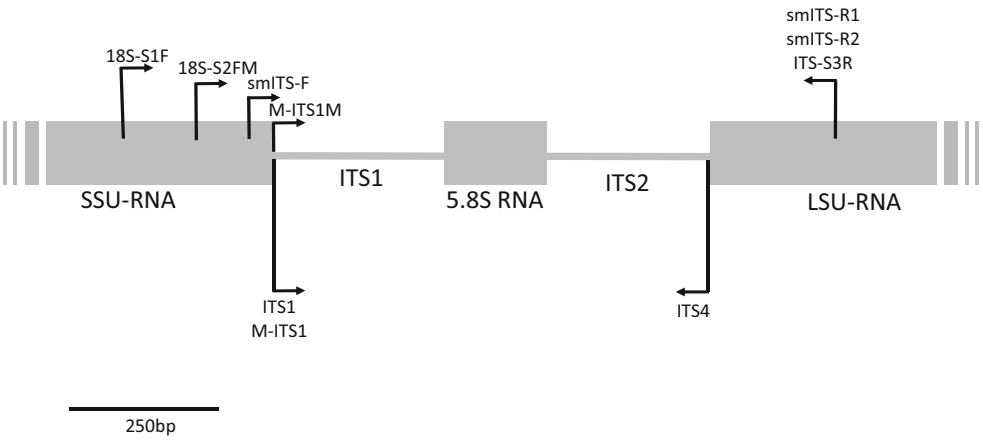

Fig. 1 Primers for amplification of Internal transcribed spacers (ITS-rDNA) region. Primer names located in the *upper* part of the figure are the ones designed in this study; the ones in the *lower* part represent primers already published

The optimum annealing temperature for all combinations was in the range from 54 to 56 °C. After these initial tests, the best five primer combinations were tested on a set of 79 ustilaginomycete species belonging to 68 different genera (Table 1). PCR products were sequenced at the sequencing facility of the Biodiversity and Climate Research Centre (BiK-F, Frankfurt, Germany) using the ITS4 primer. The resulting sequences were deposited in GenBank (Table 1).

Results

Initial amplifications using the primer combination M-ITS1/ITS4 often led to negative results for herbarium specimens. A selection of 205 samples from 39 genera which were negative in a first round of amplification (no PCR result or multiple bands) were evaluated with respect to a second and third amplification on increased amounts of DNA with M-ITS1/ITS4 and with one of the newly described reverse primers M-ITS1/smITS-R2 (first attempt) or with the newly designed primers smITS-F/smITS-R2 (second attempt). The correct sequence was obtained for 59 of the 205 samples amplified with M-ITS1/ITS4, whereas 164 of the 205 samples were successfully sequenced using the new reverse primer smITS-R2. It is noteworthy that the ITS region of plants was not amplified, and the amplification of multiple fragments was reduced by 72%. The complete corresponding results are given in Table S1, and a summary of the results is presented in Table 3.

PCR efficiency for five different primer combinations was subsequently evaluated on a set of DNA samples comprising more than half of the known genera of the Ustilaginomycotina, which represent more than 90% of the known species in this subphylum (Tables 4, S2). For the tests, two well-known primer combinations (ITS1/ITS4, M-ITS1/ITS4) and three primer combinations with newly designed primers (smITS-F/smITS-R1, M-ITS1/smITS-R1, M-ITS1/smITS-R2) were used. The unspecific ITS1/ITS4 combination was indicative of the presence of amplifiable DNA, as this primer combination will yield amplification for both fungi and plants.

The amplification and sequencing success of the Ustilaginomycotina-specific primer combinations was similar when well-amplifiable specimens were used (Tables 4, S2). Collectively, the new primers successfully amplified eight samples that could not be amplified by M-ITS1/ITS4. Considering all specific primer combinations used, 52 of the 79 samples could be successfully amplified and sequenced. The discriminatory power of the new primers hinges on the ustilaginomycete-specific reverse primers designed in this study. Samples that were difficult to amplify because of age or paucity of material could be successfully sequenced (Tables 3, S2).

Discussion

The ITS region is widely used for identification and phylogeny in various fungal groups, and has been proposed as the barcoding locus for fungi (Schoch et al. 2012; Stielow et al. 2015). It is also important for the identification and phylogenetic studies of smut fungi (e.g. Begerow et al. 1997; Stoll

Table 3 Summary of sequencing results of 205 samples of smut fungi for two primer combinations

	Primer pair	
	M-ITS1/ITS4	M-ITS1/smITS-R2
Correct	59	164
Contaminant fungi	5	2
Plant	14	0
Multiple bands	94	26
No amplification	33	13

For a full representation of the results, see Table S1

Table 4 Summary of the amplification and sequencing success of the five different primer combinations on the order and family level using well-amplifiable DNA

	Primer combinations				
	ITS4, ITS1	M-ITS1, ITS4	smITS-F, smITS-R1	M-ITS1, smITS-R1	M-ITS1, smITS-R2
Order					
Ceraceosorales	0 [0] (0)	0 (0)	0 [0] (0)	0 [0] (0)	0 [0] (0)
Doassansiales	0 [0] (0)	6 (2)	6 [2] (2)	6 [0] (0)	2 [0] (0)
Entorrhizales	1 [0] (0)	1 (0)	0 [0] (0)	1 [0] (0)	1 [0] (0)
Entylomatales	0 [0] (0)	2 (0)	1 [0] (0)	2 [0] (0)	2 [0] (0)
Exobasidiales	0 [0] (0)	2 (0)	4 [2] (0)	3 [1] (0)	4 [2] (0)
Georgefischeriales	0 [0] (0)	1 (0)	0 [0] (0)	1 [0] (0)	1 [0] (0)
Microstromatales	0 [0] (0)	1 (0)	0 [0] (0)	1 [0] (0)	1 [0] (0)
Tilletiales	0 [0] (0)	2 (0)	3 [1] (0)	3 [1] (0)	3 [1] (0)
Urocystidales	1 [0] (0)	10 (2)	8 [0] (0)	8 [0] (0)	8 [0] (0)
Ustilaginales	3 [0] (0)	17 (1)	19 [4] (0)	17 [4] (2)	17 [3] (0)
Total	5 [0] (0)	42 (5)	41 [9] (2)	42 [6] (2)	39 [6] (0)
Family					
Anthracoideaceae	0 [0] (0)	6 (1)	6 [1] (0)	6 [2] (1)	4 [1] (0)
Ceraceosoraceae	0 [0] (0)	0 (0)	0 [0] (0)	0 [0] (0)	0 [0] (0)
Clintamraceae	0 [0] (0)	0 (0)	0 [0] (0)	0 [0] (0)	0 [0] (0)
Cryptobasidiaceae	0 [0] (0)	0 (0)	0 [0] (0)	0 [0] (0)	0 [0] (0)
Doassansiaceae	0 [0] (0)	5 (2)	5 [2] (2)	3 [0] (0)	1 [0] (0)
Doassansiopsidaceae	0 [0] (0)	1 (0)	1 [0] (0)	1 [0] (0)	1 [0] (0)
Eballistraceae	0 [0] (0)	0 (0)	0 [0] (0)	0 [0] (0)	0 [0] (0)
Entorrhizaceae	1 [0] (0)	1 (0)	0 [0] (0)	1 [0] (0)	1 [0] (0)
Entylomataceae	0 [0] (0)	2 (0)	1 [0] (0)	2 [0] (0)	2 [0] (0)
Exobasidiaceae	0 [0] (0)	1 (0)	3 [2] (0)	2 [1] (0)	3 [2] (0)
Fereydouniaceae	0 [0] (0)	1 (0)	1 [0] (0)	1 [0] (0)	1 [0] (0)
Floromycetaceae	1 [0] (0)	3 (0)	3 [0] (0)	3 [0] (0)	3 [0] (0)
Georgefischeriaceae	0 [0] (0)	1 (0)	0 [0] (0)	1 [0] (0)	1 [0] (0)
Glomosporiaceae	0 [0] (0)	1 (0)	1 [0] (0)	1 [0] (0)	1 [0] (0)
Graphiolaceae	0 [0] (0)	1 (0)	1 [0] (0)	1 [0] (0)	1 [0] (0)
Incertae sedis	0 [0] (0)	0 (0)	0 [0] (0)	0 [0] (0)	0 [0] (0)
Melanotaeniaceae	0 [0] (0)	1 (0)	1 [0] (0)	1 [0] (0)	1 [0] (0)
Microstromataceae	0 [0] (0)	1 (0)	0 [0] (0)	1 [0] (0)	1 [0] (0)
Mycosyringaceae	0 [0] (0)	0 (0)	0 [0] (0)	0 [0] (0)	0 [0] (0)
Pericladiaceae	0 [0] (0)	0 (0)	0 [0] (0)	1 [1] (1)	0 [0] (0)
Rhamphosporaceae	0 [0] (0)	1 (0)	1 [0] (0)	1 [0] (0)	1 [0] (0)
Tilletiaceae	0 [0] (0)	2 (0)	3 [1] (0)	3 [1] (0)	3 [1] (0)
Tilletiariaceae	0 [0] (0)	0 (0)	0 [0] (0)	0 [0] (0)	0 [0] (0)
Uleiellaceae	0 [0] (0)	1 (0)	1 [0] (0)	1 [0] (0)	1 [0] (0)
Urocystidaceae	1 [0] (0)	5 (2)	3 [0] (0)	3 [0] (0)	3 [0] (0)
Ustilaginaceae	2 [0] (0)	8 (0)	10 [2] (0)	9 [1] (0)	10 [2] (0)
Websdaneaceae	0 [0] (0)	0 (0)	0 [1] (0)	0 [0] (0)	0 [0] (0)
Total	5 [0] (0)	42 (5)	41 [9] (2)	42 [6] (2)	39 [6] (0)

Numbers in square brackets indicate samples not amplified by the M-ITS1/ITS4 combination; the number in parentheses indicates the amount of private amplifications

et al. 2003). Smut fungi are mostly parasitic on plants (Begerow et al. 2006) and provide a challenge to amplify with general ITS primers, which tend to amplify host DNA or contaminant fungi. So far, only two primer sets were commonly

used to amplify the ITS-rDNA region of smut fungi: the unspecific combination ITS1/ITS4 and the combination M-ITS1/ITS4, in which M-ITS1 provides a higher specificity towards smut fungi. However, an amplification of host plants and contaminant fungi cannot be fully avoided. In particular, contaminant Ascomycota and basal Basidiomycota are often problematic, as they tend to be amplified preferentially, especially in case of older herbarium specimens. With the primers developed in this study, almost no non-target ITS was amplified, greatly enhancing amplification success from herbarium samples. A downside of the new primers designed in this study is that the amplicon length typically varies between 1200 and 1400 bp, an amplicon length that can be assumed to be more difficult to amplify from older herbarium specimens (Telle and Thines 2008). Amplicons can be sequenced using the ITS4 primer, but the sequencing of the full-length amplicons offers the opportunity to obtain parts of the more conserved flanking regions, which might be helpful to resolve deeper splits than possible with ITS sequences alone.

It is noteworthy that the amplification success was variable for some genera or even the same samples. This could be due to the following reasons. First, the quality of the DNA is dependent on the age and treatment of the sample. Herbaria treat their samples differently, some frost the samples regularly (especially before sending them to other institutions) or they befog the samples with a variety of insecticides. Both methods have a negative impact on the quality of the DNA (Redchenko et al. 2012). In addition, the sampling from herbarium specimens is a crucial factor determining the outcome of PCRs. For those cases in which the same sample could not be amplified from separate DNA extractions, it is conceivable that the fragment used in the current study was further degraded, e.g. by further freeze/thaw cycles, or the fragment taken did not contain the same amount of pathogen as in the previous sampling. In addition to these variables and the potential impact of the DNA extraction method, the choice of polymerase can also modify the outcome of PCR (Telle and Thines 2008).

Due to the high discriminatory power and wide applicability of the primers developed in this study, we are confident that various samples which previously could not be included in phylogenetic studies can now potentially be amplified and sequenced. However, the huge divergence that is between major groups of smut fungi renders the task of designing one primer pair that would include all the diversity of the Ustilaginomycotina—while at the same time excluding all other fungal groups and host plants—difficult. Thus, it seems possible that, for some groups of smut fungi, especially in the highly divergent Exobasidiomycetes, dedicated primers will need to be developed in the future, based on genome sequences of various representatives of this class.

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Supplementary Material

Table S1. Amplification and sequencing success of various DNA samples that could not be amplified in a first round of PCR using M-ITS1/ITS4

		primer pair				
		M-ITS1/ITS4			M-ITS1/smITS-R2	smITS-F/smITS-R1
DNA code	Genus	1st trial	2nd trial	3rd trial	M-ITS1/smITS-R2	smITS-F/smITS-R1
2716	*Urocystis*	non-target fungi			correct	
2709	*Urocystis*	correct (D)			correct	
2708	*Sporisorium*	correct (D)			correct	
2707	*Sporisorium*	non-target fungi	correct (D)	correct (D)	correct	
2706	*Sporisorium*	correct			correct	
2705	*Sporisorium*	correct			correct	
2701	*Tilletia*	correct	no amplification		no amplification	
2692	*Entyloma*	correct			correct	
2691	*Entyloma*	plant			correct	
2690	*Antherospora*	no amplification	correct	correct	correct	
2393	*Tilletia*	correct (D)			correct	
2392	*Thecaphora*	correct			correct	
2390	*Thecaphora*	correct (D)			correct	
2377	*Thecaphora*	correct			correct	
2375	*Tubisorus*	correct (D)			correct (D)	
2374	*Triodiomyces*	multiple bands			correct	
2373	*Trichocintractia*	multiple bands			correct	
2371	*Testicularia*	correct			correct	correct
2367	*Phragmotaenium*	multiple bands			correct (D)	
2363	*Mycosyrinx*	multiple bands			multiple bands	multiple bands
2355	*Langdonia*	multiple bands			correct	
2323	*Exobasidium*	multiple bands			correct	
2321	*Erratomyces*	plant (D)			correct	
2319	*Erratomyces*	correct			correct	
2318	*Entyloma*	multiple bands			correct	
2317	*Entyloma*	correct			correct	
2315	*Drepanoconis*	no amplification			no amplification	
2314	*Conidiosporomyces*	multiple bands			correct	
2313	*Conidiosporomyces*	correct			correct	
2312	*Conidiosporomyces*	correct (D)			correct (D)	
2247	*Moreaua*	correct			correct	
2245	*Floromyces*	no amplification	no amplification	correct	correct	
2228	*Entyloma*	no amplification			correct	
2226	*Entyloma*	no amplification			correct	
2027	*Macalpinomyces*	correct			correct	
2021	*Entyloma*	plant			correct	
2017	*Entyloma*	multiple bands	no amplification		correct (D)	

Table S1. Amplification and sequencing success of various DNA samples that could not be amplified in a first round of PCR using M-ITS1/ITS4

		primer pair				
		M-ITS1/ITS4			M-ITS1/smITS-R2	smITS-F/smITS-R1
DNA code	Genus	1st trial	2nd trial	3rd trial	M-ITS1/smITS-R2	smITS-F/smITS-R1
2016	*Entyloma*	plant (D)	no amplification	multiple bands	correct (D)	
2015	*Entyloma*	plant (D)	no amplification	multiple bands	multiple bands	
1895	*Urocystis*	no amplification			no amplification	correct
1894	*Urocystis*	no amplification			no amplification	correct
1892	*Urocystis*	no amplification			correct	
1885	*Urocystis*	correct			correct	
1883	*Urocystis*	multiple bands			correct	
1867	*Tranzscheliella*	multiple bands			correct	
1864	*Tranzscheliella*	multiple bands			correct	
1844	*Thecaphora*	multiple bands			multiple bands	
1832	*Thecaphora*	correct			correct	
1830	*Thecaphora*	multiple bands			multiple bands	
1828	*Thecaphora*	multiple bands			correct	
1827	*Thecaphora*	multiple bands			correct	
1824	*Sporisorium*	correct			correct	
1819	*Schizonella*	no amplification			no amplification	correct
1817	*Neovossia*	multiple bands			correct	
1816	*Neovossia*	correct			correct	
1815	*Neovossia*	correct			correct	
1811	*Neovossia*	multiple bands	no amplification		multiple bands	correct
1810	*Neovossia*	correct	no amplification	multiple bands	multiple bands	
1805	*Neovossia*	correct	no amplification	multiple bands	correct	
1803	*Nannfeldtiomyces*	no amplification			correct	
1802	*Moesziomyces*	multiple bands			correct	
1795	*Melanustilospora*	multiple bands			correct	
1792	*Melanustilospora*	multiple bands			multiple bands	
1790	*Heterodoassansia*	correct (D)			no amplification	correct
1789	*Heterodoassansia*	no amplification	plant		no amplification	correct
1788	*Heterodoassansia*	no amplification	plant		correct	
1785	*Farysia*	no amplification			no amplification	correct
1783	*Farysia*	no amplification	no amplification	non-target fungi	correct	
1779	*Entyloma*	plant			correct	
1778	*Entyloma*	multiple bands			correct	
1776	*Entyloma*	multiple bands			correct	
1775	*Entyloma*	multiple bands			correct	
1774	*Entyloma*	multiple bands			correct	
1773	*Entyloma*	multiple bands			multiple bands	
1771	*Entyloma*	multiple bands			correct	
1770	*Entyloma*	multiple bands			correct	
1768	*Entyloma*	multiple bands			correct	

Table S1. Amplification and sequencing success of various DNA samples that could not be amplified in a first round of PCR using M-ITS1/ITS4

		primer pair				
		M-ITS1/ITS4			M-ITS1/smITS-R2	smITS-F/smITS-R1
DNA code	Genus	1st trial	2nd trial	3rd trial	M-ITS1/smITS-R2	smITS-F/smITS-R1
1767	*Entyloma*	plant			multiple bands	
1766	*Entyloma*	plant			multiple bands	
1765	*Entyloma*	multiple bands			correct	
1759	*Entyloma*	correct (D)			correct	
1757	*Entyloma*	multiple bands			correct	
1755	*Entyloma*	plant			correct	
1753	*Entyloma*	multiple bands			multiple bands	
1743	*Entyloma*	multiple bands			correct	
1740	*Entyloma*	multiple bands			correct	
1736	*Entyloma*	D (plant)			correct	
1735	*Entyloma*	multiple bands			correct (D)	
1731	*Entyloma*	multiple bands			correct	
1729	*Entyloma*	multiple bands			correct	
1727	*Entyloma*	multiple bands			multiple bands	
1720	*Entyloma*	multiple bands			correct	
1719	*Entyloma*	no amplification	no amplification	correct	multiple bands	
1711	*Entyloma*	multiple bands			correct	
1708	*Entyloma*	correct			correct	
1704	*Entorrhiza*	no amplification			correct	
1701	*Entorrhiza*	multiple bands			multiple bands	
1699	*Doassansia*	correct	correct		correct	
1697	*Doassansia*	no amplification			correct	
1669	*Doassansia*	multiple bands			correct	
1693	*Doassansia*	no amplification			correct	
1645	*Thecaphora*	multiple bands			correct	
1641	*Microstroma*	multiple bands			correct	
1638	*Microstroma*	multiple bands			correct	
1636	*Microstroma*	multiple bands			correct	
1632	*Entyloma*	multiple bands			correct	
1630	*Entyloma*	multiple bands			no amplification	
1627	*Entyloma*	multiple bands			correct	
1625	*Vankya*	correct			correct	
1577	*Urocystis*	multiple bands			correct (D)	
1569	*Ustilago*	correct	correct	correct	correct	
1567	*Ustilago*	multiple bands			correct	
1566	*Entyloma*	multiple bands			no amplification	multiple bands
1561	*Sporisorium*	correct			correct	
1556	*Tranzscheliella*	multiple bands			correct	
1549	*Thecaphora*	multiple bands			correct	
1544	*Sporisorium*	no amplification			correct	

Table S1. Amplification and sequencing success of various DNA samples that could not be amplified in a first round of PCR using M-ITS1/ITS4

		primer pair				
		M-ITS1/ITS4			M-ITS1/smITS-R2	smITS-F/smITS-R1
DNA code	Genus	1st trial	2nd trial	3rd trial	M-ITS1/smITS-R2	smITS-F/smITS-R1
1542	*Sporisorium*	multiple bands			correct	
1534	*Anthracocystis*	no amplification			multiple bands	
1524	*Schizonella*	multiple bands			correct	correct
1514	*Macalpinomyces*	correct			correct (D)	
1509	*Farysia*	multiple bands			non-target fungi	correct
1505	*Exobasidium*	no amplification			correct	
1496	*Entyloma*	multiple bands			multiple bands	correct
1494	*Entyloma*	multiple bands			multiple bands	
1492	*Entyloma*	multiple bands			correct	
1489	*Entyloma*	no amplification	correct		multiple bands	multiple bands
1488	*Entyloma*	no amplification			correct	
1484	*Entyloma*	multiple bands			non-target fungi	
1483	*Entyloma*	multiple bands			correct	
1472	*Entyloma*	plant			correct	
1471	*Entyloma*	no amplification			correct	
1466	*Entorrhiza*	correct			correct	
1464	*Entorrhiza*	no amplification			correct	
1460	*Doassansia*	multiple bands			correct	
1456	*Doassansia*	no amplification	multiple bands		correct (D)	
1373f	*Entyloma*	multiple bands			correct	
1373c	*Entyloma*	multiple bands	multiple bands		correct	
1373b	*Entyloma*	multiple bands	multiple bands		correct	
1373a	*Entyloma*	multiple bands	multiple bands		correct	
1370	*Vankya*	multiple bands	multiple bands		correct (D)	correct (D)
1369	*Vankya*	correct			correct	
1367	*Vankya*	correct			multiple bands	
1366	*Triodiomyces*	correct	multiple bands		correct	
1365	*Moesziomyces*	correct			correct	
1364	*Ustilago*	multiple bands	multiple bands	multiple bands	multiple bands	correct
1362	*Ustilago*	correct			correct	
1361	*Ustilago*	correct			correct	
1360	*Ustilago*	non-target fungi			correct	
1359	*Ustilago*	correct			correct	
1357	*Ustilago*	multiple bands	multiple bands	multiple bands	correct	correct
1356	*Ustilago*	correct			correct	
1355	*Ustilago*	correct			correct	
1354	*Ustilago*	correct			correct	
1346	*Urocystis*	multiple bands			correct	
1343	*Urocystis*	multiple bands			correct	
1339	*Trichocintractia*	multiple bands	multiple bands	correct	correct	

Table S1. Amplification and sequencing success of various DNA samples that could not be amplified in a first round of PCR using M-ITS1/ITS4

		primer pair				
		M-ITS1/ITS4			M-ITS1/smITS-R2	smITS-F/smITS-R1
DNA code	Genus	1st trial	2nd trial	3rd trial	M-ITS1/smITS-R2	smITS-F/smITS-R1
1336	*Tranzscheliella*	correct (D)			correct (D)	
1332	*Tranzscheliella*	multiple bands			multiple bands	
1330	*Tranzscheliella*	multiple bands	multiple bands		multiple bands	correct
1326	*Tranzscheliella*	non-target fungi			correct	
1317	*Tilletia*	correct			correct	
1316	*Tilletia*	correct			correct	
1310	*Thecaphora*	multiple bands			multiple bands	
1307	*Sporisorium*	multiple bands	multiple bands		correct	correct
1306	*Sporisorium*	correct			correct	
1305	*Sporisorium*	correct			correct	
1300	*Sporisorium*	multiple bands			correct	
1299	*Sporisorium*	multiple bands			correct	
1272	*Mycosyrinx*	multiple bands	no amplification		no amplification	
1271	*Mundkurella*	no amplification	plant		correct	
1261	*Moreaua*	non-target fungi			correct	
1260	*Moreaua*	no amplification			correct	
1250	*Macalpinomyces*	multiple bands	multiple bands		multiple bands	
1249	*Leucocintractia*	multiple bands	multiple bands	multiple bands	correct	
1248	Heterotolyposporium	no amplification			correct	
1246	Heterotolyposporium	multiple bands	multiple bands		multiple bands	multiple bands
1241	Farysia	no amplification			correct	
1232	Farysia	no amplification	multiple bands		correct	
1227	Entyloma	multiple bands			correct	
1226	Entyloma	multiple bands			correct	
1222	Entyloma	multiple bands			correct	
1221	Entyloma	multiple bands			correct	
1220	Entyloma	multiple bands			correct	
1219	Entyloma	correct			correct	
1218	Entyloma	plant			no amplification	multiple bands
1216	Entorrhiza	no amplification			correct	
1215	Doassansiopsis	correct			correct	
1214	Conidiosporomyces	correct	correct	multiple bands	correct	
1213	Conidiosporomyces	correct			correct	
1212	Cintractia	correct			no amplification	
1211	Cintractia	correct			correct	
1198	Anthracocystis	correct			correct	
798	Farysia	multiple bands			correct	correct
791	Entyloma	plant			correct	correct
790	Entyloma	no amplification			correct	correct
789	Entyloma	plant			correct	correct

Table S1. Amplification and sequencing success of various DNA samples that could not be amplified in a first round of PCR using M-ITS1/ITS4

		primer pair				
		M-ITS1/ITS4			M-ITS1/smITS-R2	smITS-F/smITS-R1
DNA code	Genus	1st trial	2nd trial	3rd trial	M-ITS1/smITS-R2	smITS-F/smITS-R1
786	Entyloma	multiple bands			correct	correct
784	Entyloma	multiple bands			correct	
782	Entyloma	correct			correct	
549	Ustilago	multiple bands			correct	
509	Ustilago	multiple bands			correct	
484	Ustilago	multiple bands			correct	
462	Ustilago	correct			multiple bands	
460	Ustilago	correct			correct	

(D) = additional bands present.

Table S2. Amplification and sequencing success for well-amplifiable DNA samples

primer combinations					Species details (Fungi)							
ITS4, ITS1	M-ITS1, ITS4	smITS-F, smITS-R1	M-ITS1, smITS-R1	M-ITS1, smITS-R2	species	class	order	family	host	year	Herbar No.	ITS GenBank Acc. No.
D	D	o	o	o	*Ahmadiago euphorbiae*	Ustilaginomycetes	Ustilaginales	incertae sedis	*Euphorbia dracunculoides*	unknown	BPI 160414	-
F	D	D	o	o	*Anomalomyces* sp.	Ustilaginomycetes	Ustilaginales	incertae sedis	*Panicum trachyrhachis*	2006	VPRI 40639a	-
F	x	x	x	x	*Antherospora hortensis*	Ustilaginomycetes	Urocystidales	Floromycetaceae	*Muscari armeniacum*	2013	GLM-F105770	KY424462
x	x	x	x	x	*Antherospora tourneuxii*	Ustilaginomycetes	Ustilaginales	incertae sedis	*Bellevallia dubia*	2014	GLM-F105791	KY424463
D	x	x	x	x	*Anthracocystis themedae-arguentis*	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	*Themeda quadrivalvis*	1996	HUV 972	KY424448
P	D	o	o	o	*Anthracoidea arenaria*	Ustilaginomycetes	Ustilaginales	Anthracoideaceae	*Carex brizoides*	2011	GLM-F105771	-
D	D	D	D	D	*Anthracoidea echinospora*	Ustilaginomycetes	Ustilaginales	Anthracoideaceae	*Carex acuta*	2013	GLM-F105772	-
P	o	x	x	x	*Arcticomyces warmingii*	Exobasidiomycetes	Exobasidiales	Exobasidiaceae	*Saxifraga rotundifolia*	2014	GLM-F105799	KY424481
F	o	o	o	o	*Bambusiomyces shiraianus*	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	*Arundinaria faberi*	1974	HUV 171	-
F	o	o	o	o	*Botryoconis saccardoi*	Exobasidiomycetes	Exobasidiales	Cryptobasidiaceae	*Oreodaphne* sp.	1906	HBG 113/2301	-
F	x	x	x	o	*Burrillia ajrekari*	Exobasidiomycetes	Doassansiales	Doassansiaceae	*Monochoria vaginalis*	1985	HUV 504	KY424472
D	D	o	D	D	*Ceraceosorus bombacis*	Exobasidiomycetes	Ceraceosorales	Ceraceosoraceae	pure culture	2016	ATCC 22867	-
D	x	x	x	o	*Cintractia axicola*	Ustilaginomycetes	Ustilaginales	Anthracoideaceae	*Fimbristylis dichotoma*	1995	HUV 951	KY424452
F	o	o	o	o	*Cladosterigma fusisporum*	Exobasidiomycetes	Exobasidiales	incertae sedis	Myrtaceae	1898	HBG 111/2301	-
D	o	o	D	o	*Clintamra nolinae*	Ustilaginomycetes	Ustilaginales	Clintamraceae	*Nolina microcarpa*	1969	WSP 58501	-
P	x	x	x	x	*Conidiosporomyces ayresii*	Exobasidiomycetes	Tilletiales	Tilletiaceae	*Panicum maximum*	1999	HUV 19.314	KY424465
D	D	x	x	x	*Dermatosorus cyperi*	Ustilaginomycetes	Ustilaginales	Anthracoideaceae	*Cyperus* aff. *celluloso-reticulatus*	1993	WSP 70652	KY424456
D	D	o	o	o	*Doassansia limosellae*	Exobasidiomycetes	Doassansiales	Doassansiaceae	*Limosella aquatica*	2000	GLM-F105787	-
P	P	o	o	o	*Doassansia sagittariae*	Exobasidiomycetes	Doassansiales	Doassansiaceae	*Sagittaria sagittata*	2000	GLM-F105788	-
P	x	x	x	x	*Doassansiopsis hydrophila*	Ustilaginomycetes	Urocystidales	Doassansiopsidaceae	*Potamogeton polygonifolius*	2009	GLM-F105789	KY424467
P	o	o	o	o	*Doassinga callitrichis*	Exobasidiomycetes	Doassansiales	Doassansiaceae	*Callitriche stagnalis*	1986	HUV 560	-
o	o	o	o	o	*Drepanoconis brasiliensis*	Exobasidiomycetes	Exobasidiales	Cryptobasidiaceae	*Nectandra reticulata*	1889	HBG 99/2301	-
P	P	o	o	o	*Eballistra lineata*	Exobasidiomycetes	Georgefischeriales	Eballistraceae	*Zizania aquatica*	1989	HUV 741	-
x	x	D	x	x	*Entorrhiza casparyana*	Entorrhizomycetes	Entorrhizales	Entorrhizaceae	*Juncus articulatus*	2012	GLM-F105790	KY424495
P	x	D	x	x	*Entyloma hieracii*	Exobasidiomycetes	Entylomatales	Entylomataceae	*Hieracium murorum* s.l.	2013	GLM-F105773	KY424469

Table S2. Amplification and sequencing success for well-amplifiable DNA samples

primer combinations					Species details (Fungi)							
ITS4, ITS1	M-ITS1, ITS4	smITS-F, smITS-R1	M-ITS1, smITS-R1	M-ITS1, smITS-R2	species	class	order	family	host	year	Herbar No.	ITS GenBank Acc. No.
F	x	x	x	x	*Entyloma magnusii*	Exobasidiomycetes	Entylomatales	Entylomataceae	*Helichrysum arenarium*	2014	GLM-F105795	KY424471
F	D	x	D	x	*Exobasidium japonicum*	Exobasidiomycetes	Exobasidiales	Exobasidiaceae	*Rhododendron kiusianum*	2014	GLM-F105792	KY424480
F	x	x	x	x	*Exobasidium uvae-ursi*	Exobasidiomycetes	Exobasidiales	Exobasidiaceae	*Arctostaphylos uva-ursi*	2013	GLM-F105774	KY424482
F	F	o	o	o	*Exoteliospora osmundae*	Ustilaginomycetes	Ustilaginales	Melanotaeniaceae	*Osmunda regalis*	unknown	UZH 234	-
F	o	F	D	F	*Farysia thuemenii*	Ustilaginomycetes	Ustilaginales	Anthracoideaceae	*Carex riparia*	2004	GLM-F064758	-
F	o	o	o	o	*Farysporium endotrichum*	Ustilaginomycetes	Ustilaginales	Anthracoideaceae	*Gahnia xanthocarpa*	1990	HUV 828	-
D	x	x	x	x	*Fereydounia khargensis*	Ustilaginomycetes	Urocystidales	Fereydouniaceae	pure culture	2016	CBS 13305	KY424466
D	x	x	x	x	*Floromyces anemarrhenae*	Ustilaginomycetes	Urocystidales	Floromycetaceae	*Anemarrhena asphodeloides*	2007	M-0216090	KY424464
D	o	o	D	o	*Franzpetrakia microstegii*	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	*Phacelurus latifolius*	2002	HUV 20.192	-
F	x	x	x	x	*Graphiola phoenicis*	Exobasidiomycetes	Exobasidiales	Graphiolaceae	*Phoenix canariensis*	2005	PDD 85521	KY424483
D	D	x	D	D	*Heterodoassansia hottoniae*	Exobasidiomycetes	Doassansiales	Doassansiaceae	*Hottonia palustris*	2000	GLM-F047407	KY424476
P	x	x	x	o	*Heterodoassansia hygrophilae*	Exobasidiomycetes	Doassansiales	Doassansiaceae	*Hygrophila auriculata*	1992	HUV 815	KY424473
P	x	o	D	o	*Heterodoassansia ranunculina*	Exobasidiomycetes	Doassansiales	Doassansiaceae	*Ranunculus peltatus*	2005	GLM-F076002	KY424475
D	F	o	x	D	*Heterotolyposporium lepidospermatis*	Ustilaginomycetes	Ustilaginales	Anthracoideaceae	*Lepidosperma ensiforme*	1996	HUV 957	KY424474
P	x	o	x	x	*Jamesdicksonia brizae*	Exobasidiomycetes	Georgefischeriales	Georgefischeriaceae	*Briza minor*	2008	PDD 95170	KY424494
F	o	o	o	o	*Laurobasidium lauri*	Exobasidiomycetes	Exobasidiales	Exobasidiaceae	*Laurus azorica*	2002	GLM-F046197	-
F	o	D	o	o	*Leucocintractia leucoderma*	Ustilaginomycetes	Ustilaginales	Anthracoideaceae	*Rhynchospora corymbosa*	1991	HUV 810	-
D	D	x	D	x	*Macalpinomyces neglectus*	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	*Setaria pumila*	2012	GLM-F105775	KY424442
D	x	x	x	x	*Melanopsichium pennsylvanicum*	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	pure culture	2016	ex Mycotheca Graecensis 285	KY424444
P	x	x	x	x	*Melanotaenium endogenum*	Ustilaginomycetes	Ustilaginales	Melanotaeniaceae	*Galium album*	2014	GLM-F105796	KY424493
F	x	x	x	x	*Melanustilospora ari*	Ustilaginomycetes	Urocystidales	Urocystidaceae	*Arum maculatum*	2014	GLM-F105800	KY424461
P	x	D	x	x	*Microstroma juglandis*	Exobasidiomycetes	Microstromatales	Microstromataceae	*Juglans regia*	2013	GLM-F105776	KY424479
x	x	x	x	x	*Moesziomyces bullatus*	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	*Echinochloa crus-galli*	2011	GLM-F105777	KY424447
D	x	x	x	x	*Moreaua mauritiana*	Ustilaginomycetes	Ustilaginales	Anthracoideaceae	*Fimbristylis ovata*	1995	M-0040282	KY424491

Table S2. Amplification and sequencing success for well-amplifiable DNA samples

primer combinations					Species details (Fungi)							
ITS4, ITS1	M-ITS1, ITS4	smITS-F, smITS-R1	M-ITS1, smITS-R1	M-ITS1, smITS-R2	species	class	order	family	host	year	Herbar No.	ITS GenBank Acc. No.
P	x	o	o	o	*Mundkurella mossii*	Ustilaginomycetes	Urocystidales	Urocystidaceae	*Aralia nudicaulis*	1991	HUV 834	KY424468
P	D	o	o	o	*Mycosyrinx cissi*	Ustilaginomycetes	Urocystidales	Mycosyringaceae	*Cissus verticillata*	1991	HUV 835	-
P	D	o	o	o	*Nannfeldtiomyces sparganii*	Exobasidiomycetes	Doassansiales	Doassansiaceae	*Sparganium erectum*	2004	GLM-F074570	-
F	x	x	x	x	*Neovossia moliniae*	Exobasidiomycetes	Tilletiales	Tilletiaceae	*Molinia caerulea*	2002	GLM-F063830	KY424489
P	x	x	x	x	*Orphanomyces arcticus*	Ustilaginomycetes	Ustilaginales	Anthracoideaceae	*Carex davalliana*	2012	GLM-F105778	KY424454
P	o	D	x	D	*Pericladium tiliacearum*	Ustilaginomycetes	Ustilaginales	Pericladiaceae	*Grewia villosa*	2005	WSP 71988	KY424497
D	x	o	o	o	*Pseudodermatosorus sagittariae*	Exobasidiomycetes	Doassansiales	Doassansiaceae	*Sagittaria planitiana*	1993	WSP 70317	KY424477
P	o	x	D	D	*Pseudodoassansia hydrocleidis*	Exobasidiomycetes	Doassansiales	Doassansiaceae	*Hydrocleis nymphoides*	unknown	PDD 74716	KY424478
P	o	o	D	o	*Restiosporium baloskionis*	Ustilaginomycetes	Ustilaginales	Websdaneaceae	*Baloskion tetraphyllum*	2001	HUV 1292	-
D	x	x	x	x	*Rhamphospora nymphaeae*	Exobasidiomycetes	Doassansiales	Rhamphosporaceae	*Nymphaea*-Hybride.	2014	GLM-F105801	KY424485
P	x	x	x	x	*Schizonella elynae*	Ustilaginomycetes	Ustilaginales	Anthracoideaceae	*Elyna myosoroides*	2013	GLM-F105779	KY424455
D	x	x	x	x	*Sporisorium andropogonis*	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	*Bothriochloa ischaemum*	2014	GLM-F105797	KY424450
F	D	x	x	x	*Sporisorium vanderstytii*	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	*Hyparrhenia hirta*	2014	GLM-F105793	KY424449
D	o	o	o	o	*Stegocintractia capitata*	Ustilaginomycetes	Ustilaginales	Anthracoideaceae	*Juncus capitatus*	2007	HUV 21.504	-
F	x	x	x	x	*Thecaphora saponariae*	Ustilaginomycetes	Urocystidales	Glomosporiaceae	*Saponaria officinalis*	2013	GLM-F105780	KY424492
F	F	F	o	o	*Tilletia controversa*	Exobasidiomycetes	Tilletiales	Tilletiaceae	*Alopecurus aequalis*	2001	GLM-F044677	-
P	D	x	x	x	*Tilletia olida*	Exobasidiomycetes	Tilletiales	Tilletiaceae	*Brachypodium pinnatum*	2013	GLM-F105781	KY424487
D	D	o	D	o	*Tilletiopsis* sp.	Exobasidiomycetes	Georgefischeriales	Tilletiariaceae	*Dianthus* sp.	1999	VPRI 22019	-
D	x	x	x	x	*Tracya hydrocharidis*	Exobasidiomycetes	Doassansiales	Doassansiaceae	*Hydrocharis morsus-ranae*	2014	GLM-F105802	-
P	x	x	x	x	*Tranzscheliella hypodytes*	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	*Bromus erectus*	2013	GLM-F105782	KY424451
D	x	x	D	o	*Trichocintractia utriculicola*	Ustilaginomycetes	Ustilaginales	Anthracoideaceae	*Rhynchospora corymbosa*	1999	HUV 19.316	KY424453
F	x	x	x	x	*Uleiella chilensis*	Ustilaginomycetes	Ustilaginales	Uleiellaceae	pure culture	2016	ex TUB 020322	KY424484
F	x	x	x	x	*Urocystis agropyri*	Ustilaginomycetes	Urocystidales	Urocystidaceae	*Elymus repens*	2012	GLM-F105783	KY424458
x	x	x	x	x	*Urocystis colchici*	Ustilaginomycetes	Urocystidales	Urocystidaceae	*Colchicum autumnale*	2013	GLM-F105784	KY424459
P	P	D	o	o	*Ustacystis waldsteiniae*	Ustilaginomycetes	Urocystidales	Urocystidaceae	*Waldsteinia geoides*	1987	HUV 629	-

Table S2. Amplification and sequencing success for well-amplifiable DNA samples

primer combinations					Species details (Fungi)							
ITS4, ITS1	M-ITS1, ITS4	smITS-F, smITS-R1	M-ITS1, smITS-R1	M-ITS1, smITS-R2	species	class	order	family	host	year	Herbar No.	ITS GenBank Acc. No.
P	x	o	o	o	*Ustanciosporium montagnei*	Ustilaginomycetes	Ustilaginales	Anthracoideaceae	*Rhynchospora alba*	2009	GLM-F105785	KY424490
F	x	x	x	x	*Ustilago avenae*	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	*Avena barbarta*	2014	GLM-F105794	KY424445
x	x	x	x	x	*Ustilago esculenta*	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	pure culture	2016	Us_es_01	KY424441
D	x	x	x	x	*Ustilago serpens*	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	*Elymus repens*	2014	GLM-F105798	KY424443
D	x	o	o	o	*Vankya ornithogali*	Ustilaginomycetes	Urocystidales	Urocystidaceae	*Gagea lutea*	2007	GLM-F105786	KY424460
D	o	o	o	o	*Websdanea lyginiae*	Ustilaginomycetes	Ustilaginales	Websdaneaceae	*Lyginia barbata*	1994	HUV 16.361	-
5 [0] (0)	**42 (5)**	**41 [9] (2)**	**42 [6] (2)**	**39 [6] (0)**								

Green (x) = correct sequencing result, red (o) = no amplification, grey (D) = multiple bands presemt, orange (F) = contaminant fungi, blue (P) = plant. Numbers in square brackets in the summary indicate samples not amplified by the M-ITS1/ITS4 combination, the number in round brackets indicates the amount of private amplifications.

4 New smut-specific primers for multilocus genotyping and phylogenetics of Ustilaginaceae. Mycological Progress 16: 917–925.

Julia Kruse, Bagdevi Mishra, Young-Joon Choi, Rahul Sharma & Marco Thines

Statement of Joint Authorship

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What did the PhD student or the Co-Authors contributed to this work?

(1) Development and planning

PhD student JK: 50 %

Co-Author BM: 1 %

Co-Author Y-JC: 1 %

Co-Author RS: 1%

Co-Author MT: 47 %

(2) Performance of the individual investigations and experiments

PhD student JK: 44%, extraction of smut DNA for tests, primer testing (laboratory experiments, molecular work), improvement of primer

Co-Author BM: 35%, designing of smut specific primers based on smut genomes, helped with depositing sequences in GenBank

Co-Author Y-JC: 15%, helped with primer designing

Co-Author RS: 4%, helped with primer designing

Co-Author MT: 2%, helped with primer designing

(3) Preparation of the data collection and figures

PhD student JK: 95%, create different excel files for different primer combinations (data compilation), pictures with power point

Co-Author MT: 5%, helped with the designing of figures and tables

(4) Analyse and interpretation of data

PhD student JK: 50%, literature search, calculating of genetic distances, general interpretation

Co-Author BM & Y-JC: each 5 %, helped with interpretation of usage of different loci/multilocus approach

Co-Author MT: 40%, important feedback on several points and new ideas for interpretation

(5) Writing the manuscript

PhD student JK: 55%

Co-Author BM: 10 %

Co-Author Y-JC & RS: each 5%

Co-Author MT: 25%

Date/Place: _____

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Verification of the statements above

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_____ Date/Place: _____

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ORIGINAL ARTICLE

New smut-specific primers for multilocus genotyping and phylogenetics of Ustilaginaceae

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Abstract The Ustilaginomycotina, often collectively referred to as smut fungi, represent one of the three subphyla of the Basidiomycota. Smut fungi predominantly parasitize Angiosperms, are globally distributed, and contain several economically important pathogens. The most species-rich family of the smut fungi is the Ustilaginaceae. To investigate the molecular phylogeny of smut fungi, most studies rely on nrDNA loci, such as ITS and LSU. Protein coding genes, like *rpb*1, *rpb*2, *TeF*1*a*, *atp*6, and *ß-tubulin*, have been used in some studies. However, because of the huge diversity of smut fungi and the lack of dedicated primers, amplification of these loci has proven difficult for several groups. Thus, it was the aim of the current study to develop primers for new loci for the smut fungi with the focus on the largest family, the Ustilaginaceae. Here, the development and testing of new primers for nine loci based on protein-coding genes is reported (*myosin*, *map*, *rpl3*, *tif*2, *ssc*1, *ß-tubulin*, *sdh*1, *rpl*4A and *atp*2). A list of various primer combinations for the amplification of the new loci is given, with the corresponding PCR conditions and the best combinations for several genera of the Ustilaginaceae and some other Ustilaginomycetes. We hope that the primers presented in this study will be useful in overcoming the limitations of currently-used loci in terms of phylogenetic resolution, especially with respect to resolving species complexes and providing a better resolution of the higher-level phylogenetic relationships of smut fungi.

Keywords Molecular phylogeny · Multilocus · New primers, primer development · Protein coding genes · Smut fungi · Ustilaginaceae · Ustilaginales

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Introduction

The phylum Basidiomycota contains three subphyla (Agaricomycotina, Pucciniomycotina and Ustilaginomycotina). Ustilaginomycotina (smut fungi in a phylogenetic sense) are a diverse group in which more than 120 genera are currently recognised (Vánky 2012; Begerow et al. 2014). The majority of the c.1700 species in the Ustilaginomycotina are parasitic to various angiosperm hosts, where they cause diverse symptoms, e.g. blackish or brownish stripes or galls in different plant organs, tiny white spots of aggregated basidia on lower leaf surfaces, or light-brown spore masses in flowers (Vánky 2012; Begerow et al. 2014). A large fraction of smut fungi are not known to produce teliospores, e.g. the orders Exobasidiales and Microstromatales (Begerow et al. 1997). Phylogenetic investigations have revealed that many broad-host-range pathogens actually represent species complexes of host-specific

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species, e.g. *Ustilago striiformis* (Savchenko et al. 2014), and *Antherospora vaillantii* (Piątek et al. 2013). Thus, it can be assumed that the diversity of smut fungi is significantly higher than currently known. Most of the Ustilaginomycotina are dimorphic, with a free-living monokaryotic yeast stage and a parasitic dikaryotic hyphal stage. Phylogenetic investigations have revealed that evolution of smut symptoms on host plants has occurred independently several times (Begerow et al. 1997). As an example, the Microbotryales are members of the rust fungi (Pucciniomycotina) but share both the dimorphic lifestyle and high organ specificity with members of the Ustilaginomycetes. At present, four different classes are recognised in the Ustilaginomycotina, namely Ustilaginomycetes, Moniliellomycetes, Malasseziomycetes (Wang et al. 2014), and Exobasidiomycetes (Begerow et al. 2014).

The Ustilaginaceae is the largest family within the smut fungi (Vánky 2012). At present, 4–15 different genera are included in this family. Many investigations have taken place in the past year within the Ustilaginaceae to solve phylogenetic relationships of the different genera (e.g. McTaggart et al. 2012, 2016), linking teleomorphs and anamorphs for some anamorphic yeast species (Wang et al. 2015), or to solve species complexes (e.g. Savchenko et al. 2014).

As for other fungal groups, the most frequently used locus for barcoding and phylogeny of smut fungi are the nrITS regions, with more than 3000 sequences deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/, accessed November 2016). In addition, nrLSU has been widely used for analysing relationships within and between genera, with about 2000 deposited sequences (https://www.ncbi.nlm.nih.gov/genbank/, accessed November 2016). For investigating higher-level relationships, nrSSU has been used far less frequently in smut fungi, reflected by less than 300 sequences deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/accessed November 2016). Most of the available smut phylogenies are based on one of these loci or a combination of them (e.g. Albu et al. 2015; Bauer et al. 1997; 2001a, b; Begerow et al. 1997, 2000, 2002a, b, 2004a; Boekhout et al. 2003; Stoll et al. 2003, 2005; Castlebury et al. 2005; McTaggart et al. 2012; Nasr et al. 2014; Wang et al. 2015; Piątek et al. 2016). However, focussing just on nuclear ribosomal loci might not be optimal, due to the multi-copy nature and poorly understood constraints with respect to concerted evolution of the ribosomal cistrons (Kijpornyongpan and Aime 2016).

Storch et al. (2007) concluded that protein-coded genes are generally more conserved and can be more reliably aligned. Due to codon redundancy, there are often few constraints with respect to mutations in the third codon position. In line with this, Liu and Hall (2004) showed that some protein-coding genes offered enough phylogenetic resolution to resolve some lineages which were indistinguishable based on rDNA loci.

In an effort to increase phylogenetic resolution, some protein-coding loci used in other fungal groups have sometimes been employed in phylogenetic investigations in smut fungi with varying success, including *atp*6, *tef-α*, *ß-tubulin*, *rpb*1, *rpb*2, and *gadph* (e.g. Albu et al. 2015; Begerow et al. (2004b); McTaggart et al. 2012; Lotze-Engelhard 2010; Savchenko et al. 2017; Wang et al. 2006). Currently, about 450 sequences for *tef-α*, 250 for *rpb*2, 150 for *rpb*1, and less than 50 for *gadph*, *atp*6, and *ß-tubulin* have been deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/ accessed November 2016). Many of these sequences have been derived from cultures, reflecting the difficulties in selective amplification of the target species in the presence of host tissue and mycelia of contaminant fungi. Currently available primers have proven to result in no or unsatisfactory amplification for some species or species complexes of the Ustilaginaceae, the family which harbours the vast majority of known species (Vánky 2012). This conclusion can be deduced from the patchy distribution of respective sequences among samples used (e.g. in McTaggart et al. 2012), but Lotze-Engelhard (2010) also documented that *atp*6 and *ß-tubulin* loci could not be amplified from the genus *Urocystis* using published primers.

As a consequence of these obstacles, there are currently no phylogenetic studies with both a broad taxon sampling and a comprehensive sequence set for several protein-coding regions for the Ustilaginaceae and most species complexes are incompletely resolved (e.g. Savchenko et al. 2014). To overcome these limitations, it was the aim of this study to provide new smut-specific primers for protein-coding loci, especially for the Ustilaginaceae, on the basis of published genomes of smut fungi.

Materials and methods

Fungal specimens

The specimens used in this study are listed in Tables 1 and 3 (see below). All DNA used was derived from dried herbarium material (containing both pathogen and host). The nomenclature of the hosts is derived from The International Plant Names Index (www.ipni.org); for smut fungi, the nomenclature of Vánky (2012) was used as a reference, with some additions from Thines (2016).

DNA extraction

About 2–20 mg of infected plant tissue was taken from each dried herbarium specimen. Tissue samples were disrupted in a mixer mill (MM200; Retsch, Germany), using two iron beads of 3 mm and five to eight iron beads of 1 mm diameter per sample, and shaking at 25 Hz for 5–10 min. Genomic DNA was extracted using a phenol–chloroform protocol adapted and modified from McKinney et al. (1995). The extraction

Table 1 Smut species used for primer testing

Species	Host	Host family	Collection details				
			Country	Year	Collector	Herbarium no.	GenBank Acc. no. for various loci
Anthracoidea sempervirentis[a]	*Carex sempervirens*	*Cyperaceae*	Austria	2012	J. Kruse	GLM-F105803	ssc1: MF151810; ß-tubulin: MF151782
Entorrhiza aschersoniana[a]	*Juncus bufonius*	*Juncaceae*	Germany	2011	J. Kruse	GLM-F105804	
Entyloma calendulae[a]	*Calendula officinalis*	*Asteraceae*	Germany	2010	J. Kruse	GLM-F105805	sdh1: MF151805; atp2: MF151778, MF151779, MF151780
Exobasidium uvae-ursi[a]	*Arctostaphylos uva-ursi*	*Ericaceae*	Italy	2013	J. Kruse	GLM-F105774	ssc1: MF151811; rpl4A: MF151799
Rhamphospora nymphaeae[a]	*Nymphaea alba*	*Nymphaceae*	Germany	2011	J. Kruse	GLM-F105806	atp2: MF151771
Schizonella elynae	*Elyna myosoroides*	*Cyperaceae*	Italy	2013	J. Kruse	GLM-F105779	rpl3: MF151794; tif2: MF151817, MF151818; ssc1: MF151812; ß-tubulin: MF151783; atp2: MF151772, MF151773, MF151774
Sporisorium vanderystii	*Hyparrhenia hirta*	*Poaceae*	Spain	2012	J. Kruse	GLM-F105810	myosin: MF151790; rpl3: MF151795; tif2: MF151819; ssc1: MF151813; ß-tubulin: MF151784; sdh1: MF151806; atp2: MF151768
Thecaphora saponariae	*Saponaria officinalis*	*Caryophyllaceae*	Germany	2013	J. Kruse	GLM-F105780	myosin: MF151791; rpl3: MF151796; tif2: MF151820; ß-tubulin: MF151785; rpl4A: MF151800; atp2: MF151781
Tilletia olida[a]	*Brachypodium pinnatum*	*Poaceae*	Germany	2012	J. Kruse	GLM-F105807	tif2: MF151821; ssc1: MF151814; rpl4A: MF151801; atp2: MF151775
Tranzscheliella hypodytes	*Bromus erectus*	*Poaceae*	Germany	2013	J. Kruse	GLM-F105811	rpl3: MF151797; rpl4A: MF151802[b], atp2: MF151769
Urocystis agropyri[a]	*Elymus repens*	*Poaceae*	Germany	2013	J. Kruse	GLM-F105808	myosin: MF151792; map: MF151788; tif2: MF151822; ssc1: MF151815; ß-tubulin: MF151786; sdh1: MF151807; atp2: MF151770
Ustilago perennans[a]	*Arrhenatherum elatius*	*Poaceae*	Germany	2011	J. Kruse	GLM-F105809	myosin: MF151793; map: MF151789; rpl3: MF151798; tif2: MF151823, MF151824; ssc1: MF151816; ß-tubulin: MF151787; sdh1: MF151808, MF151809; rpl4A: MF151803, MF151804; atp2: MF151767, MF151776, MF151777

[a] Samples used in the first set of tests

[b] Sequence from another comparable sample (same host–fungus combination)

buffer (100 ml stock) contained 2.5 ml of 2 M Tris pH 8.0, 4 ml of 5 M NaCl, 40 μl of 0.5 M EDTA, 5 ml of 10% SDS and 100 mg/ml Proteinase K. Of this buffer, 100 μl were added per sample to every tube, followed by an incubation at 37 °C for at least 30 min after mixing the sample. Subsequently, 200 μl of buffered, saturated phenol was added to each sample, tubes were gently mixed and then centrifuged at 12,000 *g* for 2 min. After collecting the supernatant, 200 μl of chloroform/isoamyl alcohol (24:1) was added, followed by centrifugation and supernatant collection. Subsequently, 18 μl of 3 M sodium acetate were added to the supernatant and nucleic acids were precipitated by adding 400 μl of 100% ethanol and centrifugation for 10 min at 8000 *g*. The resulting pellet was rinsed with an aqueous 70% ethanol solution and dried. Subsequently, the pellet was resuspended in 100 μl of double-distilled water. DNA quantity was measured with the Implen Nano Photometer (Munich, Germany) and the DNA concentration of all samples was adjusted to the same amount (5–10 ng/μl) by dilution with double-distilled water.

Primer design and selection of markers

The protein-encoding genes from the genomes of *Malassezia globosa* (Xu et al. 2007), *Ustilago hordei* (Laurie et al. 2012), *Sporisorium reilianum* (Schirawski et al. 2010), *Ustilago maydis* (Kämper et al. 2006) and *Melanopsichium pennsylvanicum* (Sharma et al. 2014) were used for orthology analyses. OrthoMCL v.2.0.3 (Li et al. 2003) was used for predicting the orthologs among the five genomes with the percentage identity cutoff of 50% and an e-value cutoff of e^{-5}. From the output of OrthoMCL, 1:1 orthologs among the five genomes were extracted.

The 1:1 ortholog list was used to fetch the corresponding genes from the five genomes along with 200-bp upstream and downstream sequences. Multiple sequence alignments of each gene group were carried out using mafft (Katoh et al. 2002) v.6.953b with default parameters. The *cons* module from EMBOSS (Rice et al. 2000) v.6.4.0.0 was used to generate a consensus sequence, and sequence chunks were selected from the multiple sequence alignments that were at least 50 bases long and showed a conservation greater than 60%. Such conserved sequence chunks were paired if in PCR they could result in amplicons of 400–1200 bp. Using a sliding window approach, 18- to 24-nt-long consensus sequences in pairs were selected with a GC content of 40–55%. These potential primers from the consensus pairs were further filtered applying the following criteria. No self-folding with four base complement with two or more bases in between; forward and reverse primers not to be reverse complement to each other with six bases or with four + four bases; the last base G or C; second last base T or A; the same base not to be occurring more than three times consecutively; the same type of base not to be present more than five times consecutively; and the primer not to have more than three degenerate bases.

The potential primers were mapped onto all the five genomes, and those matching to multiple locations in the same genome were excluded, keeping primers supposedly amplifying a unique location in the genome.

Primer testing

Forty different primer combinations were tested for the 14 different loci on 12 different genera of smut fungi (Table 1) with PCR conditions as follows. Initial denaturation for 240 s at 95 °C, 36 cycles of denaturation for 40 s at 95 °C, annealing for 60 s at 53 °C, and elongation for 60 s at 72 °C, followed by a final elongation for 240 s at 72 °C. The resulting amplicons were sequenced at the sequencing facility of the Biodiversity and Climate Research Centre (BiK-F) and their identity confirmed by comparison to sequences deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) by BLAST searches (Altschul et al. 1990). After these initial tests, the 22 primer combinations with the best amplification performance in terms of ability to amplify different samples without unspecific amplification were tested for further evaluation of the best gene loci. Two loci were excluded from further investigations due to unstable amplification performance. The 22 best primer combinations for 10 different loci were tested on 8 different smut genera (Table 1, indicated by superscript 'a') with a gradient PCR (±10 °C from the original annealing temperature) to identify optimum amplification temperatures. If this seemed necessary due to unsatisfactory results, some primers were slightly modified with Geneious 6.0.6 (Biomatters, New Zealand) based on newly obtained sequences and tested again. Based on the gradient PCR, the best primers for the different loci were chosen, with respect to the amplification strength and the absence of multiple bands. The best primer combinations are listed in Table 2 with their suggested range of suitable annealing temperatures (different smut genera sometimes had different temperature optima for best amplification results). Although the primers were designed based on mainly Ustilaginomycetes genomes, they were also tested for other smut genera and even Exobasidiomycetes, to obtain hints regarding the potential amplification of other groups of smut fungi. In a second test round, the newly designed primers were tested on a selection of different genera and species of the Ustilaginaceae (18 different smut species) to investigate their usefulness for phylogenetic approaches. For that approach the Kimura-2-model-based genetic distance for each locus apart from the ß-tubulin locus that did not give amplification for some species, was calculated using Mega 6.06 (Tamura et al. 2013) based on single alignments of the sequences of the 18 different smut species.

Table 2 Summary of the amplification for nine protein-coding loci with the newly designed primer tested on 12 different genera of smut fungi

Gene name	Gene description	Name	primer sequence	Name	primer sequence	°C	fragment length	*Entorrhiza*	*Tilletia*	*Exobasidium*	*Entyloma*	*Rhamphospora*	*Urocystis*	*Anthracoidea*	*Schizonella*	*Thecaphora*	*Tranzscheliella*	*Sporisorium*	*Ustilago*
myosin	Myosin-I	myosin_F1	RTACTTGATCTGRTGMAG	myosin_R1	TGAMAACAACTCGTTCGARCAR	53-55	637						x			x		x	x
	Myosin I	myosin_F3	CAGACCSARRTACTTGATC	myosin_R0.5	ATGCTSKCSTCSAACCCKCACTT	55	NA						x					x	x
	Myosin I	myosin_F3	CAGACCSARRTACTTGATC	myosin_R3	ATCGAYGACCAYGCCGAC	55	1095						x					x	x
map	methionine aminopeptidase	map_F1	TTCCACCAYTCCATCATCTC	map_R3	AGWSSATCATCATCATGCAG	60	616						x						x
	methionine aminopeptidase	map_F1	TTCCACCAYTCCATCATCTC	map_R5	GARTACAAYACYGCCAAG	53	1052						x						x
	methionine aminopeptidase	map_F2	CGAGTTCTGCTGCATGATG	map_R6	TCCGHGACAAGACCCGHGTC	56	353						x						x
rpl3	RPL3-60s_ribosomal_protein_L3	rpl3_F1	AGAACTGGTACCGHWSCAAG	rpl3_R1	GCACCRATACAGGCGAYCTTAC	53	406								x	x	x*	x	x
tif2	TIF2 initial translation factor eIF4A	tif2_F1	CCRATRCGGTGRATGTAG	tif2_R1	CTCAARATGTTCTGYCTYG	50	557						x			x		x	x
	TIF2 initial translation factor eIF4A	tif2_F3	TCRAGRCAGAACATYTTGAG	tif2_R3	TSCGTGGTGTCTWCGCYTAC	50-53	412		x				x		x	x		x	x
ssc1	SSC1 mitochondrial HSP70 member	ssc1_F1	ASGTCATYATGGTYGGTG	ssc1_R1	CTTGTTSGTKGCCTTGTC	52	479			(x)			x	(x)				x	x
	SSC1 mitochondrial HSP70 member	ssc1_F2	CATYATGGTYGGTGGTATGAG	ssc1_R1	CTTGTTSGTKGCCTTGTC	51-53	475		(x)		x		x		(x)			x	x
ß-tubulin	tubulin beta chain	ß-tubulin_F1	YCAGTGTGGTAACCARGTC	ß-tubulin_R1	AASGTCTCGTCSGAGTTC	52	570						x					x	x
	tubulin beta chain	ß-tubulin_F3	AGACCGGYCAGTGTGGTAAC	ß-tubulin_R1	AASGTCTCGTCSGAGTTC	55	577						x	x				x	x
	tubulin beta chain	ß-tubulin_F4	TKCAGACCGGYCAGTGTG	ß-tubulin_R1	AASGTCTCGTCSGAGTTC	55	580						x		x	(x)		x	x
	tubulin beta chain	ß-tubulin_F2	ACCGGYCAGTGTGGTAAC	ß-tubulin_R2	GAAGCAGATGTYRTAVAG	51	612						x		x			x	x
sdh1	SDH1-succinate dehdrogenase ubiquinone flavoprotein	sdh1_F1	AGGABGGCAAGATYTACCAG	sdh1_R1	TATCGCTCCATRAAYCGCTCMC	54	499						x						x
	SDH1-succinate dehdrogenase ubiquinone flavoprotein	sdh1_F2	GABGGCAAGATYTACCAG	sdh1_R3	TCGCTCCATRAAYCGCTC	53-56	495				x		x					x	x
rpl4A	RPL4A-ribosomal protein L4-A	rpl4A_F1	YCCGTGGTGGTCACATGTTC	rpl4A _R1	GGATYTCRTCMGAGTTGATG	50	588		x							x	x		x
	RPL4A-ribosomal protein L4-A	rpl4A _F2	TAACATGGYCCGTGGTGGTCAC	rpl4A _R1	GGATYTCRTCMGAGTTGATG	51	596									x	x		
	RPL4A-ribosomal protein L4-A	rpl4A _F3	TAACATGGYCCGTGGTGGTC	rpl4A _R1	GGATYTCRTCMGAGTTGATG	51	596		x							x			x
	RPL4A-ribosomal protein L4-A	rpl4A _F4	CCGTGGTGGTCACATGTTC	rpl4A _R1	GGATYTCRTCMGAGTTGATG	53	587			x						x			x
atp2	ATP2-F1F0-ATPase complex F1 beta subunit	atp2_F3	CGTATCWTGAACGTCATTGG	atp2_R3	CGACTTGTARTCCTGGAG	55	870						x					x	x
	ATP2-F1F0-ATPase complex F1 beta subunit	atp2_F6	CGTGGTGGTAAGATTGGTCT	atp2_R4	AGCGACTTGTARTCCTGGAG	56	707				x	x	x		x	x	x	x	x
	ATP2-F1F0-ATPase complex F1 beta subunit	atp2_F8	ACCACGARATGATYGAGAC	atp2_R4	AGCGACTTGTARTCCTGGAG	52-57	559		(x)		x	x	x			x	x	x	x
	ATP2-F1F0-ATPase complex F1 beta subunit	atp2_F6	CGTGGTGGTAAGATTGGTCT	atp2_R3	CGACTTGTARTCCTGGAG	56	705				x		x		x	x	x	x	x

*only successful with a comparable DNA from a comparable sample (same host/fungus combination), x, shading in green: positive sequencing result, (x), shading in yellow: resulting sequence of bad quality (e.g. due to unspecific amplification)

Results

Altogether, 22 forward and 17 reverse primers were designed for several loci (*myosin*, *map*, *rpl3*, *tif*2, *ssc*1, *ß-tubulin*, *sdh*1, *rpl*4A, and *atp*2) applicable in 25 different combinations for one or more genera of the smut fungi in a strict sense (Ustilaginomycetes). Table 2 shows the amplification success for the different genera of smut fungi tested in this study. Almost any combination resulted in successful amplification for the genus *Ustilago* (24 of 25), representing all nine different loci that could be reliably amplified. For the genus *Urocystis*, seven of nine loci could be successfully applied (*rpl*4A and *rpl*3 failed). The orders Urocystidales and Ustilaginales (both belonging to the Ustilaginomycetes) revealed best amplification success, as compared to the Exobasidiomycetes. Within the Ustilaginales, only the genus *Anthracoidea* proved to be difficult to amplify and sequence, with only one of the loci providing reliable amplification and sequencing results. Loci for the outgroup Entorrhizomycotina (Bauer et al. 2015), represented by the genus *Entorrhiza*, could not be amplified by the primer combinations used in the current study. The best amplification and sequencing results for the different smut genera was achieved for the *atp*2 locus, for which seven to eight different genera could be successfully amplified. For *tif*2 (F3/R3), six genera were still successfully amplified of (Table 2). For five loci, amplification success was achieved also for the *Exobasidiales*, sometimes with primer

Table 3 Smut specimens used for calculating genetic distances

DNA-no.	Species	Host	Location	Date	Collector	Herbar. no.	ITS	LSU	*atp*2	*ssc*1	*ß-tubulin*	*map*	*myosin*	*rpl*4A
222	*Moesziomyces bullatus*	*Echinochloa crus-galli*	Germany, Schleswig-Holstein	19.09.2011	J. Kruse	GLM-F105777	KY424435	MF668628	MF678352	MF678367	-	MF678362	MF678363	MF678365
445	*Ustilago calamagrostidis*	*Calamagrostis epigejos*	Germany, Baden-Württemberg	20.07.2013	J. Kruse	GLM-F105819	KY929538	MF668623	KY930144	KY929981	MF678358	KY929698	KY929628	KY929838
455	*Ustilago* aff. *filiformis*	*Glyceria fluitans*	Germany, Bavaria	10.05.2013	J. Kruse	GLM-F105824	KY929543	MF668619	KY930149	KY929986	MF678357	KY929703	KY929633	KY929843
463	*Ustilago nuda*	*Hordeum vulgare*	Germany, Bavaria	12.05.2012	J. Kruse	GLM-F105826	KY929545	MF668621	KY930151	KY929988	-	KY929705	KY929635	KY929845
498	*Ustilago bromina*	*Bromus inermis*	Germany, Saxony-Anhalt	04.06.2011	J. Kruse	GLM-F105843	KY929562	MF668627	KY930168	KY930005	MF678356	KY929715	KY929645	KY929855
506	*Ustilago neocopinata*	*Dactylis glomerata*	Germany, Lower Saxony	19.05.2011	J. Kruse	GLM-F105848	KY929567	MF668626	KY930173	KY930010	MF678355	KY929717	KY929647	KY929857
524	*Ustilago salveii*	*Holcus lanatus*	Germany, Lower Saxony	22.05.2010	J. Kruse	GLM-F107416	KY929573	MF668625	KY930179	KY930016	MF678354	KY929721	KY929651	KY929861
553	*Ustilago trichophora*	*Echinochloa crus-galli*	Germany, North Rhine-Westphalia	04.10.2010	J. Kruse	GLM-F107424	KY929581	MF668620	KY930187	KY930024	MF678353	KY929728	KY929658	KY929868
884	*Sporisorium* aff. *occidentale*	*Andropogon gerardii*	USA	30.07.1989	not known	TUB s.n. (ex HUV)	KY929614	MF668618	KY930220	KY930057	-	KY929755	KY929685	KY929895
1305	*Ustilago* (*Sporisorium*) aff. *sorghi*	*Sorghum plumosum*	Australia	20.02.1996	A. A. Mitchell, C. & K. Vánky	HUV No 970 (TUB)	KY929617	MF668616	KY930223	KY930060	-	KY929757	KY929687	KY929897
1359	*Ustilago* aff. *schroeteriana* (*Sporisorium*)	*Paspalum virgatum*	Costa Rica	15.03.1991	T. & K. Vánky	HUV No 888 (TUB)	KY929616	MF668631	KY930222	KY930059	-	KY929756	KY929686	KY929896
1610	*Ustilago scrobiculata*	*Calamagrostis epigejos*	Germany, Brandenburg	24.06.2007	V. Kummer	GLM-F107433	KY929590	MF668629	KY930196	KY930033	-	KY929735	KY929665	KY929875
1617	*Ustilago* aff. *syntherismae*	*Digitaria sanguinalis*	Germany, Brandenburg	11.08.2001	V. Kummer	GLM-F107436	KY929593	MF668633	KY930199	KY930036	MF678361	KY929737	KY929667	KY929877
1822	*Ustilago* (*Sporisorium*) aff. *andropogonis*	*Bothriochloa ischaemum*	Germany, Saxony-Anhalt	25.07.2004	H. Jage & H. John	GLM-F062665	KY929533	MF668617	KY930139	KY929976	-	KY929694	KY929624	KY929834
1825	*Ustilago* (*Sporisorium*) *cruenta*	*Sorghum bicolor*	Greece	11.05.2006	H-W, Otto	GLM-F078871	KY929535	MF668630	KY930141	KY929978	-	KY929696	KY929626	KY929836
2354	*Sporisorium* (*Langdonia*) aff. *inopiatum*	*Aristida adscensionis*	Zambia	12.04.2001	C., T. & K. Vánky	M-0215944	KY929612	MF668632	KY930218	KY930055	-	KY929754	KY929684	KY929894
2398	*Ustilago brizae*	*Briza media*	Austria, Tirol	21.07.2014	J. Kruse	GLM-F107442	KY929599	MF668624	KY930205	KY930042	MF678360	KY929742	KY929672	KY929882
3370	*Ustilago* aff. *bromivora*	*Bromus sterilis*	Spain, Andalusia	02.05.2015	J. Kruse	GLM-F107449	KY929606	MF668622	KY930212	KY930049	MF678359	KY929748	KY929678	KY929888

Table 4 Average genetic distances (Kimura-2 model) for 18 different members of the Ustilaginaceae (from Table 3)

Locus	Average genetic distance
ITS	0.094
LSU	0.024
atp2	0.051
map	0.067
myosin	0.132
rpl3	0.064
rpl4A	0.086
sdh1	0.059
ssc1	0.087
tif2	0.076

combinations different from those successful in *Ustilaginomycetes* (Tables 2). Since the best amplification and sequencing results were observed for members of the Ustilaginaceae, further tests were conducted on several species of the Ustilaginaceae. Based on 18 different species (Table 3) representing the diversity of the family, genetic distances were calculated. For comparison, the highly variable barcoding locus ITS, which has often been used in phylogenies below the genus level, and the less-variable locus from the LSU region used in several phylogenetic studies (mostly suitable to distinguishing between genera) were used. For the species included, the ITS locus revealed an average genetic distance value of 0.094, while the LSU locus showed only 0.024. The average genetic distance of the loci presented in this study ranged from 0.05 to 0.13 (Table 4), suggesting that they are informative enough for phylogenetic analysis.

Discussion

Smut fungi are a species-rich group occurring worldwide (Vánky 2012). Thus, it is common practice to use molecular phylogenetic methods to assist taxonomic, systematic and evolutionary questions. For this purpose, nuclear rDNA loci are easily accessible and widely used. A reason for this is the general lack of dedicated primers for other loci for smut fungi. Thus, host plant or contaminant fungus amplification is frequent, especially when dealing with herbarium specimens (Kruse et al. 2017). This is reflected by the fact that for the loci *rpb*1 and *rpb*2, sequences are mostly derived from pure cultures (e.g. Wang et al. 2015). Also, Lotze-Engelhard (2010) was unsuccessful in obtaining smut amplification for the genus *Urocystis* extracted from plant material. As for most species of smut fungi cultures are not available, phylogenetic investigations rely on herbarium specimens. Thus, we based our tests on herbarium samples.

As Liu and Hall (2004) demonstrated that protein-coding genes can be sufficient to resolve relationships between fungal lineages that could not be distinguished based on rDNA loci, new primers for protein-coding genes suitable for multigene analyses in smut fungi were developed in the current study. Because the smut fungi are a highly diverse group, and there is a lack of comprehensive genome sampling across all classes of smut fungi, the primer design in this study was focussed on the Ustilaginomycetes, by far the most species-rich class of the Ustilaginomycotina. As a consequence, most of the primers developed in the present study provided good amplification and sequencing success for *Urocystis* and Ustilaginaceae (comprising about two-thirds of the species of smut fungi), but some primers also amplified other groups well. The analysis of the genetic distances of the single loci revealed that they might be suitable and phylogenetically informative enough to resolve relationships between smut genera of the Ustilaginaceae as well as closely related species complexes, if applied collectively. Preliminary testing using the closely related species *Ustilago perrennans* on *Arrhenatherum* and *Ustilago avenae* on *Avena* sp., which are often regarded as conspecific, revealed 16 SNPs when all loci presented in this study were investigated, while in ITS only 2 SNPs could be observed (unpublished data). We thus hope that the newly developed set of primers will prove to be a useful tool for gaining deeper insights into the evolution and phylogeny of smut fungi. The number of loci needed to resolve species complexes will inevitably vary depending on the degree of genetic differentiation in the complex, but we recommend to always use as many of the loci introduced here as possible to achieve the best results in terms of resolution and phylogenetic robustness.

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5 Asexual and sexual morphs of *Moesziomyces* revisited. IMA Fungus 8: 117–129.

Julia Kruse, Gunther Doehlemann, Eric Kemen & Marco Thines

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(1) Development and planning

PhD student JK: 60 %

Co-Author GD & EK: each 2 %

Co-Author MT: 36 %

(2) Performance of the individual investigations and experiments

PhD student JK: 80 %, extraction of smut DNA for tests (PCR, sequencing), yeast cultivation, morphological investigations (molecular work)

Co-Author GD & EK: each 2 %, extraction of DNA of *Pseudozyma* ex *Arabidopsis* infected with *Albugo*

Co-Author MT: 16 %, helped with experiments and yeast cultivation

(3) Preparation of the data collection and figures

PhD student JK: 95%, create measurement table, pictures with power point and phylogenetic tree

Co-Author MT: 5%, helped with the designing of figures and tables

(4) Analyse and interpretation of data

PhD student JK: 60%, literature search, phylogenetic tree, morphological comparison, asexual smuts

Co-Author GD & EK: each 7 %, *Pseudozyma* ex *Arabidopsis* infected with *Albugo* (ideas), asexual smuts

Co-Author MT: 26%, important feedback on several points and new ideas for interpretation

(5) Writing the manuscript

PhD student JK: 70%

Co-Author GD & EK: each 5 %

Co-Author MT: 20%

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ARTICLE

Asexual and sexual morphs of *Moesziomyces* revisited

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Abstract: Yeasts of the now unused asexually typified genus *Pseudozyma* belong to the smut fungi (*Ustilaginales*) and are mostly believed to be apathogenic asexual yeasts derived from smut fungi that have lost pathogenicity on plants. However, phylogenetic studies have shown that most *Pseudozyma* species are phylogenetically close to smut fungi parasitic to plants, suggesting that some of the species might represent adventitious isolations of the yeast morph of otherwise plant pathogenic smut fungi. However, there are some species, such as *Moesziomyces aphidis* (syn. *Pseudozyma aphidis*) that are isolated throughout the world and sometimes are also found in clinical samples and do not have a known plant pathogenic sexual morph. In this study, it is revealed by phylogenetic investigations that isolates of the biocontrol agent *Moesziomyces aphidis* are interspersed with *M. bullatus* sexual lineages, suggesting conspecificity. This raises doubts regarding the apathogenic nature of asexual morphs previously placed in *Pseudozyma*, but suggests that there might also be pathogenic sexual morph counterparts for those species known only from asexual morphs. The finding that several additional species currently only known from their yeast morphs are embedded within the genus *Moesziomyces*, suggests that the yeast morph might play a more dominant role in this genus as compared to other genera of *Ustilaginaceae*. In addition, phylogenetic reconstructions demonstrated that *Moesziomyces bullatus* has a narrow host range and that some previously described but not widely used species names should be applied for *Moesziomyces* on other host genera than *Echinochloa*.

Key words:
ecology
evolution
phylogeny
plant pathogens
pleomorphic fungi
Ustilaginomycotina
yeast

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INTRODUCTION

Ustilaginales is the largest order within the smut fungi (*Ustilaginomycetes*), including species forming a blackish to brownish powdery spore mass in different organs of monocotyledonous, and exceptionally dicotyledonous plants (Vánky 2012, Begerow *et al.* 2006). The order includes nine families, encompassing 54 genera. The *Anthracoideaceae* with the occurrence on *Cyperaceae* and *Juncaceae*, and *Ustilaginaceae*, with few exceptions parasitic to the *Poaceae*, are the largest families within the order. The latter contains the three largest smut genera, *Anthracocystis*, *Sporisorium,* and *Ustilago*. The difference between these three closely related genera is the almost complete lack of a plant-derived columella within sori formed by *Ustilago* species (Vánky 2012, McTaggart *et al.* 2012). Within *Ustilago* some species are economically important pathogens, like corn smut (*Ustilago maydis*) or wheat smut (*Ustilago nuda*). *Ustilago maydis* is a species for which one of the first fungal genomes was sequenced (Kämper *et al.* 2006). Smut fungi of the *Ustilaginales* usually feature both an asexual yeast morph and a sexual morph infecting host plants in a biotrophic manner. In rare cases yeasts of the *Ustilaginales* could also be found to be affecting humans (McNeil & Palazzi 2012, Teo & Tay 2006). The earliest case of an invasive infection with an *Ustilago* species, possibly *U. maydis*, was reported in 1946 (Moore *et al.* 1946). But spores of the *Ustilaginales* potentially also cause pneumonias, allergic reactions, or asthma (Valverde *et al.* 1995).

There are several studies dealing with the phylogeny of *Ustilaginomycotina*, mostly based on the LSU or ITS locus and some of them include asexual morphs as well (e.g. Begerow *et al.* 2000, 2006, Stoll *et al.* 2005, Wang *et al.* 2006, 2015, Boekhout 2011). Wang *et al.* (2015) link many asexual yeasts to their corresponding sexual morphs, an important step within the naming of pleomorphic fungi, as dual naming of sexual and asexual morphs is now discontinued (Hawksworth *et al.* 2011).

Pseudozyma has been used for species of ustilaginomycetous yeasts belonging to *Ustilaginales* which are mostly

believed to be apathogenic (Begerow *et al.* 2000, 2006). The genus was described in 1985, by Bandoni (1985), and later refined by Boekhout (1995). After Sampaio (2004) and finally Wang *et al.* (2015) established that the type species *Pseudozyma prolifica* was a synonym of *Ustilago maydis*, the name *Pseudozyma* should no longer be used. However, the phylogenetic position of some species referred to *Pseudozyma* is still unclear. Wang *et al.* (2015) suggested using the name *Pseudozyma* now with the addition "*pro tempore*" for five *Pseudozyma* species with an unclear phylogenetic position. In the current study we give *Pseudozyma* names with reference to the new combinations recently made, where possible.

To date, about 20 *Pseudozyma* species names were validly published (www.indexfungroum.org). Fifteen are now linked to a corresponding sexually typified genus (Wang *et al.* 2015). Of these, *Pseudozyma antarctica*, *P. aphidis*, *P. parantarctica*, and *P. rugulosa* were transferred to *Moesziomyces* (Wang *et al.* 2015).

Ustilaginalean yeasts are isolated from diverse habitats, for *Ustilaginales*, but mostly from grasses (Boekhout 1995, Avis *et al.* 2001). Some were isolated from flowers, leaves or fruits of other plants, but it is also possible to isolate them from soil or even human blood or secretory fluid (Sugita *et al.* 2003, Arendrup *et al.* 2014). Apart from the few clinical cases of *Ustilago maydis* infestation, it was not known until 2003 that species referred to *Pseudozyma* could also infect humans (Sugita *et al.* 2003). However, such species can cause invasive infections, especially in immunosuppressed individuals (Arendrup *et al.* 2014). The infection risk, according to Prakash *et al.* (2014), is the same as being colonised by non-albicans *Candida* infections. Furthermore, Avis *et al.* (2001) and Gafni *et al.* (2015) reported antifungal properties of some pseudozyma-like yeast species, including *Moesziomyces aphidis,* and some strains have been reported to be natural antagonists of powdery mildews (*Erysiphales*). Colonization of leaf surfaces by these yeasts provides a natural source of protection against some plant pathogenic fungi (Gafni *et al.* 2015).

Of the ustilaginalean yeasts, especially *Moesziomyces antarcticus* and *M. aphidis*, formerly treated as *Pseudozyma* species, have been frequently isolated from various substrates. *Moesziomyces antarcticus* was isolated from plants and soil, but also from blood of humans (Boekhout 2011). *Moesziomyces aphidis* was described in 1995 and first isolated from the secretion of an aphid, but it has later been isolated from water (Boekhout 2011) and various other sources, including soil and human blood. Wang *et al.* (2015) showed that these pseudozyma-like species, together with *M. parantarcticus* and *M. rugulosus*, belong to the genus *Moesziomyces*, which before had been generally regarded as monotypic (Vánky 2005).

Moesziomyces mainly differs from other smut fungi in having remnants of ruptured sterile cells (Vánky 1977). Vánky (1977) initially included four species in the genus *Moesziomyces*. Three of them, occurring on different genera of grasses (*Leersia, Paspalum, Pennisetum*) were later considered to be conspecific and united under the oldest name, *M. bullatus* (Vánky 1986, 2005). The other species, *M. eriocauli* (Vánky 1986), was transferred to a new genus *Eriomoeszia*, because of a thin cortex of sterile cells, which surrounds the spore balls (Vánky 2005). After the transfer of four pseudozyma-like species *(M. antarcticus, M. aphidis, M. parantarcticus* and *M. rugulosus*) to this genus, it now contains five species (Wang *et al.* 2015).

Given the high host specificity observed for most *Ustilaginales* species (McTaggart *et al.* 2012, Escudero 2015, Li *et al.* 2017), it seemed doubtful that *Moesziomyces bullatus* was parasitic to seven not closely related genera, suggesting that more species might be present in the genus, some of which might be conspecific with smuts in the past named in *Pseudozyma*. It was the aim of the current study to clarify the relationships of asexual and sexual morphs in the genus *Moesziomyces*.

MATERIAL AND METHODS

Fungal material

The fungal material used in this study is listed in Tables 1 and 2. The nomenclature of the hosts is derived from the latest version of The International Plant Names Index (www.ipni.org), the nomenclature of the fungi follows Vánky (2012) and MycoBank (www.mycobank.org).

Yeast cultivation

Fresh material of *Moesziomyces bullatus* collected in 2015 (GLM-F105817) was used for yeast cultivation. A suspension of spores in 2 mL water was prepared. Three tubes with 200 µL spore suspension were exposed to three different conditions: (1) heating of the suspension on a thermomixer at 45 °C for 5 min (Shetty & Safeeulla 1979); (2) chilling the suspension overnight on ice; and (3) incubation for 5 min at room temperature (*ca.* 20 °C). From each tube 20 µL suspension was each plated on two plates of SAM (Thines lab Standard Agar Medium, consisting of 20 g agar, 20 g PDB, 10 g yeast extract, 10 g malt extract, 40 mL clarified vegetable juice, 960 mL water) with the addition of 75 mg Rifampicin/L. One set of plates was incubated at 30 °C, the other set at room temperature. After 3 d on every plate abundant yeast growth was recognized. Pure cultures were produced by picking and transferring individual single colonies to the SAM medium (isolate A1–A10). To isolate pseudozyma-morphs associated with *Albugo laibachii* on *Arabidopsis thaliana*, *A. laibachii* spores were suspended in water and treated with antibiotics to remove bacteria. Subsequently, the suspension was plated on PDA at 20 °C and colonies were singled out after 7 d.

DNA extraction, PCR and sequencing

In total 5–20 mg of infected plant tissue from herbarium specimens and yeast colonies were disrupted in a mixer mill (MM2, Retsch), using two iron beads of 3 mm and 5–8 iron beads of 1 mm diam per sample and shaking at 25 Hz for 5–10 min. Genomic DNA was extracted using the BioSprint 96 DNA Plant Kit (Qiagen, Hilden) on a KingFisher Flex robot (Thermo Scientific, Dreieich). PCR amplification of the complete ITS nrDNA was performed using the M-ITS1 forward primer (Stoll *et al.* 2003) and the ITS4 (White *et al.* 1990) or smITS-R1 reverse primer (Kruse *et al.* 2017).

Table 1. List of *Moesziomyces* specimens used in the present study.

Species	Host	Host family	Location	Year	Collector	Fungarium no.	ITS GenBank acc. no.
Moesziomyces aphidis	*Arabidopsis thaliana* infected with *Albugo laibachii*	*Brassicaceae*	UK, Norwich	2007	E. Kemen	GLM-F107578	KY930224
Moesziomyces bullatus	*Echinochloa crus-galli*	*Poaceae*	Germany, Bavaria	2013	J. Kruse	GLM-F105812	KY424439
	Echinochloa crus-galli	*Poaceae*	Germany, Bavaria	2013	J. Kruse	GLM-F105813	KY424428
	Echinochloa crus-galli	*Poaceae*	Germany, Bavaria	2013	J. Kruse	GLM-F105814	KY424429
	Echinochloa muricata	*Poaceae*	Germany, Saxony	2000	H. Jage	GLM-F047045	KY424430
	Echinochloa muricata	*Poaceae*	Germany, Saxony	2000	D. Schulz, B. Huber & F. Klenke	GLM-F047047	KY424431
	Echinochloa muricata	*Poaceae*	Germany, Saxony-Anhalt	2003	H. Jage	GLM-F065276	KY424432
	Echinochloa muricata	*Poaceae*	Germany, Saxony-Anhalt	2005	H. Jage	GLM-F076000	KY424433
	Echinochloa crus-galli	*Poaceae*	Germany, North Rhine-Westphalia	2010	J. Kruse	GLM-F105815	KY424434
	Echinochloa crus-galli	*Poaceae*	Germany, Schleswig-Holstein	2011	J. Kruse	GLM-F105777	KY424435
	Echinochloa crus-galli	*Poaceae*	Poland	1979	K. Vánky	HUV No. 283, ex TUB	KY424436
	Echinochloa crus-galli	*Poaceae*	Germany, Hesse	2015	J. Kruse	ex-GLM-F105817 culture No. A1 GLM-F107575	KY424437
	Echinochloa crus-galli	*Poaceae*	Germany, Hesse	2015	J. Kruse	ex-GLM-F105817 culture No. A3 GLM-F107576	KY424427
	Echinochloa crus-galli	*Poaceae*	Germany, Hesse	2015	J. Kruse	ex-GLM-F105817 culture No. A10 GLM-F107577	KY424438
Moesziomyces penicillariae	*Pennisetum glaucum*	*Poaceae*	Westafrica, Gambia	1973	K. Vánky	HUV No. 154, ex TUB	KY424440

Table 2. List of additional sequences used in the phylogenetic tree, downloaded from GenBank.

Species	Source	ITS GenBank acc. no.	Citation
Eriomoeszia eriocauli	*Eriocaulon cinereum*	AY740041	Stoll *et al.* (2005)
Macalpinomyces eriachnes	*Eriachne helmsii*	AY740038	Stoll *et al.* (2005)
Moesziomyces bullatus	*Paspalum distichum*	AY74015)3	Stoll *et al.* (2005)
	human preterm low birth weight infant	KF926673	Okolo *et al.* (2015)
	-	DQ831013	Matheny *et al.* (2006)
	human preterm low birth weight infant	KF926673	Okolo *et al.* (2015)
	-	DQ831013	Matheny *et al.* (2006)
Pseudozyma antarctica	-	JX094775	Gujjari *et al.* (unpublished)
	-	JN942669	An (unpublished)
	unpolished Japanese rice	AB089360	Sugita *et al.* (2003)
	Antarctica sediment	AF294698	Avis *et al.* (2001)
	Albizia julibrissin flower	AY6415)57	Wei *et al.* (2005)
	lake sediment	AB089358	Sugita *et al.* (2003)
Pseudozyma aphidis	Japanese pear fruit	AB204896	Yasuda *et al.* (2007)
	human pulmonary infection	Q743064	Parahym *et al.* (2013)
	Saccharum officinarum	AB704889	Morita *et al.* (2012)
	Leucaena glauca	HQ662536	Wei *et al.* (2011)
	human	EU105)207	Lin *et al.* (2008)
	human blood	AB089362	Sugita *et al.* (2003)
	human	HQ848933	Xie *et al.* (unpublished)
	Fallopia japonica	KC282385	Wang & Liu (unpublished)
	blood culture from hospitalized patient	KM610219	Bosco-Borgeat & Taverna (unpublished)
	Leucaena glauca	HQ647299	Wei *et al.* (2011)
	Saccharum officinarum	AB704890	Morita *et al.* (2012)
	poplar leaf	KM268868	Sun & Yan (unpublished)
	Forcipomia taiwana	KM555221	Chen (unpublished)
	seaweeds	KP269028	Wang *et al.* (unpublished)
	aphid secretion	**AF294699**	Avis *et al.* (2001)
	Neoreglia cruenta	FN424100	Garcia *et al.* (unpublished)
	Saccharum officinarum	AB704878	Morita *et al.* (2012)
	giant panda secrete	KF973199	Li *et al.* (unpublished)
	Camellia sinensis foliar lesions	HQ832804	Li *et al.* (unpublished)
	Echinochloa crus-galli	GU390690	Hamayun & Ahmad (unpublished)
	aphid secretion on *Solanum pseudocapsicum*	JN942666	An (unpublished)
	mulberry leaf	KF443199	Qiu *et al.* (unpublished)
	Citrus leaf	JQ425372	Soliman (unpublished)
	-	JN942667	An (unpublished)
Pseudozyma hubeiensis	*Magnolia denudata* wilting leaf	DQ008954	Wang *et al.* (2006)
Pseudozyma parantarctica	-	JN544036	Chen (unpublished)
	yam tuber steep water	KF619567	Babajide *et al.* (2015)
	-	KP132543	Irinyi *et al.* (2015)
	human blood	AB089356	Sugita *et al.* (2003)
	-	**NR 130693**	An (unpublished)
Pseudozyma rugulosa	mouldy *Zea mays* leaf	AB089370	Sugita *et al.* (2003)
	ex-leaf of corn	**AF294697**	Avis *et al.* (2001)
	plant leaf	HE650886	Han *et al.* (2002)
Pseudozyma sp.	*Hyoscyamus muticus*	AB500693	Abdel-Motaal & Itu (unpublished)

Table 2. (Continued).

Species	Source	ITS GenBank acc. no.	Citation
	Coffea arabica	EU002890	Vega *et al.* (unpublished)
	Hyoscyamus muticus	AB500690	Abdel-Motaal & Itu (unpublished)
	Coffea arabica	DQ778919	Vega *et al.* (2008)
	Saccharum officinarum leaves	LC05)3989	Surussawadee & Limtong (unpublished)
	shoot of tip pepper	GU975792	Sim *et al.* (unpublished)
	marine environment	DQ178645	Chang *et al.* (2008)
	Helicoverpa armigera caterpilla gut	AM160637	Molnar & Prillinger (unpublished)
	marine sediment	KC834821	Qu *et al.* (unpublished)
	-	KR047769	Wang *et al.* (unpublished)
	pharmaceutical effluent	KF922220	Selvi & Das (unpublished)
	barley kernels and leaf	HG532070	Korhola *et al.* (2014
Uncultured fungus	Ericaceae roots	HQ260042	Walker *et al.* (2011)
	cleaned rice	AB235999	Ikeda *et al.* (2007)
Uncultured fungus clone	*Axonopus compressus* soil	HQ436080	Kee & Chia (unpublished)
Uncultured *Ustilago*	tomato rhizosphere	KF493994	Johnston-Monje *et al.* (unpublished)

* type collections are highlighted in bold

The reaction was performed in a thermocycler (Eppendorf Mastercycler 96 vapo protect, Eppendorf, Hamburg) with an initial denaturation at 95 °C for 4 min, 36 PCR cycles of denaturation at 95 °C for 40 s, annealing at 56 °C for 40 s and elongation at 72° C for 60 s, followed by a final elongation at 72° C for 4 min. The resulting amplicons were sequenced at the Biodiversity and Climate Research Centre (BiK-F) laboratory using the PCR primers. Sequences were deposited in GenBank (NCBI, Table 1).

Phylogenetic reconstructions

The dataset included sequences of *Moesziomyces* species sexual and asexual morphs, both newly sequenced (Table 1) and downloaded from GenBank (Table 2). First all available sequences were extracted from NCBI on the basis of sequence similarity. Subsequently sequences were removed that were: (1) highly redundant with already-included ITS genotypes; or (2) of doubtful sequence quality, i.e. with mutations at positions highly conserved or with nucleotide changes only towards one end of the sequences.

Macalpinomyces eriachnes was selected as an outgroup, based on the phylogenetic tree in Shivas *et al.* (2013). Alignments were made using mafft v. 7 (Katoh & Standley 2013) employing the Q-INS-I algorithm and removing leading and trailing gaps. The resulting total alignment length was 576 bp. For phylogenetic analyses, Minimum Evolution (ME) analysis was done with Mega v. 6.06 (Tamura *et al.* 2013), using the Tamura-Nei substitution model, assuming partial deletion at a cut-off of 80 % and 1000 bootstrap replicates. Maximum Likelihood (ML) analysis was done using RAxML on the webserver TrEase (www.thines-lab.senckenberg.de/trease) with all parameters were set to default values. For Bayesian analysis also the webserver TrEase was used for calculating 10 M tree generations on four incrementally heated MC chains. The first 30 % of the trees obtained this way were discarded to ensure sampling of the stationary phase. All other parameters were set to default.

Morphological examination

For light microscopy, the herbarium specimens GLM-F105814 and GLM-F105812 were transferred to distilled water on a slide. Morphological examination was carried out using a Zeiss Imager M2 AX10 microscope (Carl Zeiss, Göttingen). Measurements of the spore balls and spores were performed at ×400. Measurements are reported as maxima and minima in parentheses, and the mean plus and minus the standard deviation of a number of measurements given in parenthesis; the means are given in italics (Table 3).

RESULTS

The isolated yeasts from fresh *Moesziomyces bullatus* samples from *Echinochloa crus-galli* and *E. muricatus* were fast-growing on SAM medium. The colour of the yeasts was cream to light reddish, and the shape of the colonies was regular and roundish.

A phylogenetic hypothesis for the sampled *Moesziomyces* species and the cultivated yeast asexual morphs is given in Fig. 1. The results of the Minimum Evolution, Maximum Likelihood and Bayesian Analyses were congruent. The clade comprising the type of *M. aphidis* also includes *M. bullatus s. str.* from *Echinochloa crus-galli* and *E. muricata*, as well as the sequence of the type of *M. rugulosa*, an isolate of *Moesziomyces* from *Albugo laibachii* on *Arabidopsis thaliana* and many other isolates not determined to the species level from various sources. While visual inspection of the alignments revealed that there was some sequence variation within the *Moesziomyces bullatus* clade, the relationships of the four subgroups was not resolved apart from the clustering

Table 3. Measurements from 25 spore balls and 100 teliospores for collections of *Moesziomyces bullatus* on *Echinochloa crus-galli* from two different clades.

	***Moesziomyces bullatus* ex *Echinochloa crus-galli*, GLM-F105814**						***Moesziomyces bullatus* ex *Echinochloa crus-galli*, GLM-F105812**					
	sporeballs			**spores**			**sporeballs**			**spores**		
No.	length	breadth	l/b	length	breadth	l/b	length	breadth	l/b	length	breadth	l/b
1	148,5	100	1,49	7,5	7,5	1	120,5	76,5	1,58	8,5	7	1,21
2	111	91	1,22	8	7,5	1,07	81	68,5	1,18	8	7,5	1,07
3	79	65	1,22	7	7	1	64,5	55,5	1,16	7,5	7	1,07
4	118,5	97,5	1,22	8,5	7	1,21	58,5	53	1,1	8	7	1,14
5	58,5	58,5	1	8	6	1,33	97	53,5	1,81	7,5	7,5	1
6	100,5	83,5	1,2	8	7,5	1,07	76	61,5	1,24	8	6	1,33
7	125	113	1,11	7,5	7	1,07	94	87	1,08	7,5	6,5	1,15
8	93	89,5	1,04	7,5	7	1,07	61	44	1,39	7,5	6,5	1,15
9	95,5	59	1,62	8	6	1,33	101,5	69	1,47	7,5	6,5	1,15
10	82	61	1,34	7	7	1	101	74	1,36	8,5	6	1,42
11	102	69,5	1,47	9	7	1,29	144,5	73,5	1,97	8	6,5	1,23
12	53	41,5	1,28	9	7	1,29	84	66	1,27	9	7	1,29
13	95	78	1,22	8,5	6,5	1,31	90	69,5	1,29	8	6,5	1,23
14	53	41,5	1,28	7,5	6,5	1,15	83,5	61	1,37	8,5	6,5	1,31
15	138,5	96,5	1,44	8,5	6,5	1,31	96,5	51,5	1,87	8,5	7,5	1,13
16	93	71,5	1,3	7	7	1	111,5	78,5	1,42	7,5	6,5	1,15
17	115	91	1,26	7	6,5	1,08	91,5	68,5	1,34	7	6,5	1,08
18	71	49,5	1,43	7,5	6,5	1,15	68,5	49,5	1,38	7	5,5	1,27
19	111,5	104	1,07	7,5	7	1,07	96	82,5	1,16	8,5	7	1,21
20	117,5	95,5	1,23	7,5	7,5	1	84,5	78,5	1,08	7,5	7	1,07
21	92,5	75,5	1,23	7,5	7	1,07	86,5	62	1,4	7,5	6,5	1,15
22	80,5	68	1,18	8	7	1,14	122,5	86	1,42	8,5	7,5	1,13
23	156,5	97,5	1,61	8	7	1,14	113,5	78,5	1,45	7,5	5,5	1,36
24	58,5	53,5	1,09	8,5	7	1,21	106	105	1,01	8	6	1,33
25	52,5	52	1,01	8,5	6	1,42	105,5	84,5	1,25	7	6	1,17
26				7	6,5	1,08				7	7	1
27				7,5	7,5	1				6,5	6,5	1
28				8,5	8	1,06				6,5	5,5	1,18
29				6,5	6	1,08				8,5	7,5	1,13
30				7	6	1,17				8	6,5	1,23
31				7,5	6	1,25				8	6	1,33
32				8	7,5	1,07				7,5	6,5	1,15
33				7	6,5	1,08				7	6,5	1,08
34				7	6,5	1,08				8,5	8	1,06
35				8,5	7,5	1,13				8	7,5	1,07
36				7	6	1,17				8	7,5	1,07
37				7,5	6	1,25				8,5	7	1,21
38				7	6	1,17				8	7	1,14
39				7,5	6,5	1,15				8	6,5	1,23
40				8	6,5	1,23				6,5	6	1,08
41				6,5	6	1,08				7	7	1
42				6,5	6	1,08				8,5	6	1,42
43				8,5	6,5	1,31				7	6,5	1,08
44				8,5	7,5	1,13				7	7	1
45				7,5	6,5	1,15				8	6,5	1,23
46				8,5	7	1,21				8,5	7	1,21

Table 3. (Continued).

	Moesziomyces bullatus ex *Echinochloa crus-galli*, GLM-F105814						*Moesziomyces bullatus* ex *Echinochloa crus-galli*, GLM-F105812					
	sporeballs			spores			sporeballs			spores		
No.	length	breadth	l/b	length	breadth	l/b	length	breadth	l/b	length	breadth	l/b
47				7	7	1				7,5	6,5	1,15
48				7	6,5	1,08				8	7,5	1,07
49				6,5	6,5	1				8	6	1,33
50				9	7	1,29				7,5	7	1,07
51				8	6	1,33				6	5,5	1,09
52				8	6	1,33				7,5	6	1,25
53				7,5	7	1,07				7	7	1
54				7	6,5	1,08				8,5	6,5	1,31
55				7	7	1				8	8	1
56				9	7,5	1,2				8	7	1,14
57				7,5	6,5	1,15				7	6,5	1,08
58				7,5	6,5	1,15				7	7	1
59				8	6,5	1,23				7,5	6,5	1,15
60				8	6,5	1,23				7,5	7	1,07
61				7,5	6,5	1,15				7,5	6,5	1,15
62				8,5	7	1,21				8,5	7	1,21
63				8	8	1				8,5	7,5	1,13
64				8	7,5	1,07				8	6,5	1,23
65				7,5	7,5	1				9	7	1,29
66				7,5	7,5	1				9	7,5	1,2
67				7,5	7	1,07				7,5	7	1,07
68				7	5,5	1,27				7,5	5,5	1,36
69				8,5	7	1,21				7,5	6	1,25
70				7,5	7	1,07				7,5	6,5	1,15
71				8	7,5	1,07				8,5	6,5	1,31
72				8	7	1,14				8,5	6,5	1,31
73				8	6,5	1,23				9,5	7	1,36
74				7,5	7	1,07				8,5	6,5	1,31
75				7,5	7	1,07				8,5	7	1,21
76				8	7	1,14				9,5	6,5	1,46
77				7,5	7	1,07				9	7	1,29
78				8	6,5	1,23				7,5	6,5	1,15
79				8,5	6,5	1,31				9	7	1,29
80				8	6	1,33				7,5	7	1,07
81				8	5,5	1,45				9	7	1,29
82				8	5,5	1,45				8	6,5	1,23
83				7	6,5	1,08				8,5	6,5	1,31
84				7	6,5	1,08				7,5	6,5	1,15
85				8,5	7	1,21				7,5	7	1,07
86				7,5	5,5	1,36				9	7	1,29
87				9	6,5	1,38				8	8	1
88				7,5	6,5	1,15				8,5	7	1,21
89				7	6	1,17				8,5	7	1,21
90				8	8	1				7,5	7,5	1
91				9	6,5	1,38				7,5	6	1,25
92				8	7,5	1,07				8,5	6,5	1,31

Table 3. (Continued).

	***Moesziomyces bullatus* ex *Echinochloa crus-galli*, GLM-F105814**						***Moesziomyces bullatus* ex *Echinochloa crus-galli*, GLM-F105812**					
	sporeballs			**spores**			**sporeballs**			**spores**		
No.	length	breadth	l/b	length	breadth	l/b	length	breadth	l/b	length	breadth	l/b
93				8	7,5	1,07				9	6,5	1,38
94				7,5	7,5	1				7,5	6	1,25
95				7,5	6,5	1,15				6,5	6	1,08
96				8	7,5	1,07				7,5	7	1,07
97				7,5	7	1,07				8,5	7,5	1,13
98				8,5	7	1,21				7,5	6	1,25
99				10	7	1,43				7,5	6,5	1,15
100				7,5	7,5	1				8	7	1,14

of *Pseudozyma aphidis* and the majority of *M. bullatus* isolates with the clade containing the type of *P. rugulosa*. Collections from *E. crus-galli* with smut symptoms were present in two different clades. The morphological investigation of a sexual morph from each clade (GLM-F105812 and GLM-F105814) revealed no morphological differences. *Moesziomyces bullatus* clustering within the majority of *M. aphidis* had the following spore characteristics: sporeballs variable in shape and size, 52.5–156.5 x 41.5–113 µm, spores ovoid, globose, often irregular, pale yellow-brown, (6.5–)7–*7.8*–8.5(–10) × (5.5–)6–*6.8*–7.5(–8) µm, a length/breadth ratio of 1.01–1.15–1.39 (*n* = 100) (Fig. 2). In comparison, the collection of *M. bullatus* clustering together with the sequence of the type species of *M. rugulosus* showed the following spore characteristics: sporeballs variable in shape and size, 58.5–144.5 x 44–105 µm, spores ovoid, globose, often irregular, pale yellow-brown, (6–)7–*7.9*–8.5(–9.5) × (5.5–)6–*6.7*–7.5(–8) µm, a length/breadth ratio of 1.01–1.19–1.38 (*n* = 100) (Fig. 2). The sister group to *M. bullatus* was formed by *M. antarcticus*. The four lineages of *M. bullatus* formed an isolated clade with high to maximum support in all analyses together with samples classified as *M. antarcticus*.

Apart from the groups mentioned above, four additional distinct groups were revealed. Two of these corresponded to lineages formed by sexual smuts of the genus *Moesziomyces* isolated from plants with smut disease symptoms. One of these corresponded to *M. bullatus s. lat.* on *Paspalum distichum*, and the other to *Eriomoeszia eriocauli*. The remaining two lineages formed a monophyletic clade with high support in Minimum Evolution Analysis. One lineage included sequences of yeasts classified as *M. parantarcticus,* the other a sexual morph of a plant pathogenic fungus of the genus *Moesziomyces* from *Pennisetum glaucum*, as well asexual morphs isolated from symptom-free barley and a preterm infant.

DISCUSSION

Moesziomyces is a morphologically well-defined genus in the smut fungi, mainly characterised by ruptured sterile cells in the sori around the spores. The genus was believed to be monotypic by Vánky (2012), but phylogenetic investigations of the past decade have shown that several species previously assigned to the asexually typified yeast genus *Pseudozyma,* were closely related to *Moesziomyces bullatus* (Begerow *et al.* 2000, 2006, Wang *et al.* 2006, 2015). In the latest edition of the *International Code of Nomenclature for algae, fungi and plants* (ICN) it is ruled that the dual naming for asexual and sexual morphs of fungi has been discontinued (McNeill *et al.* 2012). Consequently, Wang *et al.* (2015) attempted to resolve the names of species placed in the genus *Pseudozyma* as far as possible, and combined those related to *Moesziomyces bullatus s.lat.* into *Moesziomyces*.

The yeast asexual morphs were, for example, found to live epiphytically on different hosts (Boekhout 1995), but also to occur on a variety of other substrates. Due to their asexual reproduction with pullulating and division, it is possible for them to colonize suitable habitats in a short period of time. Of these yeasts, *Pseudozyma aphidis* is often considered as a biocontrol agent for plant pathogenic fungi (Avis *et al.* 2001, Buxdorf *et al.* 2013). Thus it is noteworthy that one isolate of this species co-occurred in *Albugo laibachii* lesions on *Arabidopsis thaliana*, indicating only no or only limited antagonism against this specialised white blister rust species (Thines *et al.* 2009). It is commonly believed that most *Pseudozyma* species have lost pathogenicity, which is seemingly supported by recent genomic analyses (Lefebvre *et al.* 2013). However, it should be noted that if a different start codon is taken for translation than the one predicted, all *Pseudozyma* yeasts included by Lefebvre *et al.* (2013) have a functional copy of PEP1, a conserved effector among smut fungi of the *Ustilaginales* (Sharma *et al.* 2014, Hemetsberger *et al.* 2015), suggesting the possibility of a misannotation of the start codon. In-depth bioinformatic analyses and functional testing will be needed to clarify this situation.

Deducing the conspecificity of *Moesziomyces bullatus* with *Pseudozyma aphidis* and *P. rugulosa* was not possible for Wang *et al.* (2015), as they did not include sequences from the type host of *M. bullatus*, *Echinochloa crus-galli*, but only from *M. verrucosus* on *Paspalum distichum*, which they erroneously assumed to be conspecific with *M. bullatus*. However, the smut sexual morphs from the type host, *E. crus-galli* from Germany, are placed in two of the four subclusters

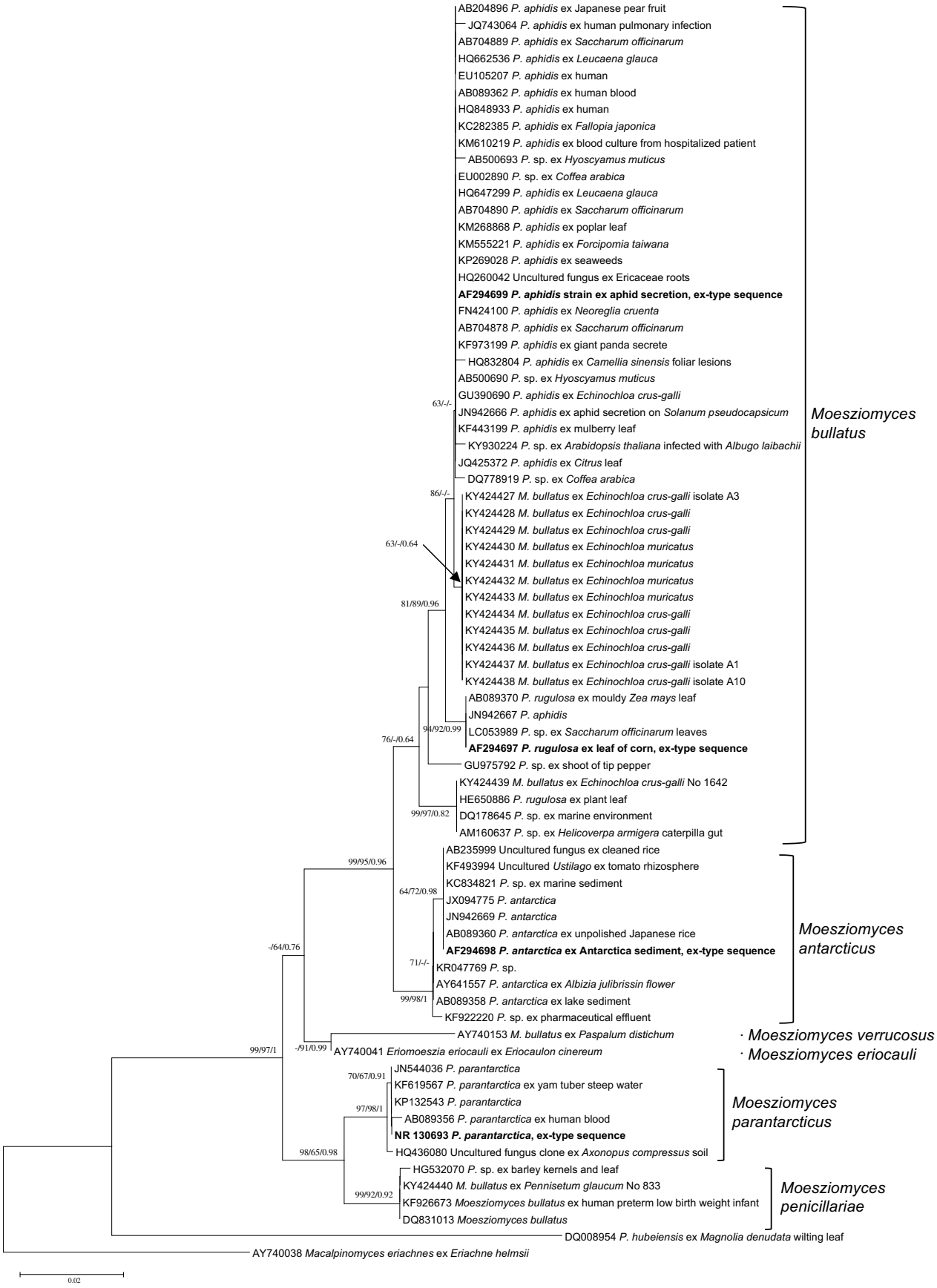

Fig. 1. Phylogenetic tree based on Minimum Evolution analyses of nrITS sequences of *Moesziomyces* spp., rooted with *Macalpinomyces eriachnes*. Numbers on branches denote bootstrap support in Minimum Evolution, Maximum Likelihood and *a posteriori* probabilities from Bayesian Analyses. Values below 55 % are not shown. The bar indicates expected substitutions per site. GenBank numbers precede taxon names, and are followed by the name of the host or isolation source of the fungus.

Fig. 2. Sori and spores of *Moesziomyces bullatus* on *Echinochloa crus-galli*. **A, D.** Sori. **B, E.** Teliospore balls. **C, F.** Teliospores. A–C (GLM-F105812), D–F (GLM-F105814).

of a larger cluster, which is interpreted here as representing *M. bullatus*. It is noteworthy that three of the four subclusters of *M. bullatus* contain environmental samples from various sources. In conjunction with the ease of cultivation observed for *M. bullatus* from *E. crus-galli*, it is concluded that unlike the vast majority of genera of *Ustilaginales*, the asexual yeast morph plays a major role as a proliferating life-cycle stage in *Moesziomyces* and that the plant-parasitic dikaryophase is probably mainly important for maintaining the possibility of sexual recombination. As the two subclades previously referred to as *M. aphidis* and *M. rugulosus* are interspersed with the morphologically identically lineages of *M. bullatus* from *E. crus-galli*, they are probably better included in *M. bullatus* until more sequence data become available. It seems probable that, with the inclusion of additional smut samples from *Echinochloa*, additional smut-causing members of the subclades will be discovered. Sampling in Africa seems to be promising in this respect, as the species diversity of *Echinochloa* is highest on this continent. Also, the notion that yeasts of the subclade containing the ex-type culture of *Pseudozyma aphidis* can withstand high temperatures, such as the human body temperature, is suggestive of a subtropical to tropical origin of this lineage.

Further, our investigations show that the older name *M. eriocauli* for *Eriomoeszia eriocauli*, should be taken up again, as this species was found embedded within *Moesziomyces*.

With the synonymy of the generic name *Eriomoeszia* and the reappraisal of the hardly used *Moesziomyces* names of the smut fungi of *Paspalum* and *Pennisetum*, *Moesziomyces* now includes six species. It is, however, likely that additional species will have to be added because smut samples from some *Poaceae* genera listed as host plants for the *M. bullatus* complex in Vánky (2012) could not be included in the current study, such as smuts from *Leersia, Panicum, Polytrias*, and *Uranthoecium*. Given the apparently high host specificity of *Moesziomyces* species, it seems likely that these pathogens represent species independent from *M. bullatus*.

TAXONOMY

Based on the phylogenetic data presented here, the following nomenclature and taxonomic changes are made.

Moesziomyces antarcticus (Goto *et al.*) Q.M. Wang *et al.*, *Stud. Mycol.* **81**: 81 (2015).

Basionym: *Sporobolomyces antarcticus* Goto *et al.*, *Mycologia* **61**: 759 (1969).

Synonyms: *Candida antarctica* (Goto *et al.*) Kurtzman *et al.*, *Yeasts*: 86 (1983).

Vanrija antarctica (Goto *et al.*) R.T. Moore, *Bibltheca Mycol.* **108**: 167 (1987).

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6 *Ustilago* species causing leaf-stripe smut revisited. IMA Fungus 9: 49–73.

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ARTICLE

Ustilago species causing leaf-stripe smut revisited

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Abstract: Leaf-stripe smuts on grasses are a highly polyphyletic group within *Ustilaginomycotina*, occurring in three genera, *Tilletia*, *Urocystis*, and *Ustilago*. Currently more than 12 *Ustilago* species inciting stripe smuts are recognised. The majority belong to the *Ustilago striiformis*-complex, with about 30 different taxa described from 165 different plant species. This study aims to assess whether host distinct-lineages can be observed amongst the *Ustilago* leaf-stripe smuts using nine different loci on a representative set. Phylogenetic reconstructions supported the monophyly of the *Ustilago striiformis*-complex that causes leaf-stripe and the polyphyly of other leaf-stripe smuts within *Ustilago*. Furthermore, smut specimens from the same host genus generally clustered together in well-supported clades that often had available species names for these lineages. In addition to already-named lineages, three new lineages were observed, and described as new species on the basis of host specificity and molecular differences: namely *Ustilago jagei* sp. nov. on *Agrostis stolonifera*, *U. kummeri* sp. nov. on *Bromus inermis,* and *U. neocopinata* sp. nov. on *Dactylis glomerata*.

Key words:
DNA-based taxonomy
host specificity
molecular species discrimination
multigene phylogeny
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INTRODUCTION

The term “stripe smut” is commonly used to refer to *Ustilaginomycotina* species that cause dark brown to black linear sori of varying length in the leaves of grasses (*Poaceae*). Black spore masses are released after the spores have matured beneath the epidermis in the mesophyll of the host leaves (Fischer 1953, Vánky 2012). The spore release process of sori is unknown, but may be facilitated either by the withering of dead epidermal cells or by enzymatic action, as in the white blister rusts (Heller & 2009). Of the smut genera that infect grasses, *Ustilago* is the most prevalent (Stoll *et al.* 2003, 2005, Vánky 2012).

The term stripe smut does not reflect phylogenetic relatedness, as at least two other genera, *Tilletia* and *Urocystis*, contain species that manifest similar symptoms. The vast majority of leaf-stripe smuts belong to *Ustilago*, including *U. agropyri, U. bahuichivoensis, U. bethelii, U. calamagrostidis, U. calcarea, U. davisii, U. deyeuxiicola, U. echinata, U. filiformis, U. phlei, U. scrobiculata, U. serpens s. lat., U. sporoboli-indici, U. striiformis s. lat., U. trebouxii, U. trichoneurana,* and *U. ulei* (Vánky 2012). Of these species, *U. striiformis s. lat.*, with the type species described on *Holcus lanatus,* is a complex occurring on 164 species of *Poaceae* representing 44 different genera (*Achnatherum*, *Agropyron*, *Agrostis*, *Alopecurus*, *Ammophila, Anthoxanthum, Arctagrostis, Arrhenatherum, Avena, Beckmannia, Brachypodium*, *Briza*, *Bromus*, *Calamagrostis*, *Cleistogenes*, *Cynosurus*, *Dactylis*, *Danthonia*, *Deschampsia*, *Deyeuxia*, *Elymus*, *Festuca*, *Helictotrichon*, *Hierochloë*, *Holcus*, *Hordeum*, *Hystrix*, *Koeleria*, *Leymus*, *Lolium*, *Melica*, *Milium*, *Pennisetum*, *Phalaris*, *Phleum*, *Piptatherum*, *Poa*, *Polypogon*, *Puccinellia*, *Sesleria*, *Setaria*, *Sitanion*, *Trisetaria*, and *Trisetum*). Based on host specificity and minor differences in spore size and surface ornamentation, approximately 30 different taxa have been described in the *U. striiformis* species complex on various host plants (Vánky 2012, Savchenko *et al.* 2014a). *Ustilago serpens* probably represents an overlooked species complex, occuring on five host genera: *Agropyron*, *Brachypodium*, *Bromus*, *Elymus,* and *Leymus*. Whether other species with large warts on their spores also belong to this complex, such as *U. echinata* and *U. scrobiculata,* is currently unclear.

Ustilago striiformis s. lat. on *Alopecurus pratensis* has often been the sole representative of this group in phylogenetic analyses (Stoll *et al.* 2005, Begerow *et al.* 2006, McTaggart *et al.* 2012a). Stoll *et al.* (2005) supported the recognition of *U. calamagrostidis,* a parasite of several species of *Calamagrostis*, as separate from *U. striiformis*.

The morphological difference was mainly in spore size and ornamentation. Savchenko *et al.* (2014a) provided a more detailed analysis of the *U. striiformis* species complex using several host-fungus combinations and phylogenetic reconstructions based on the nrITS and partial LSU regions. However, while two additional species were proposed as distinct in the *U. striiformis*-complex, the phylogenetic resolution was too low to draw further conclusions regarding host specificity and potential species boundaries. To resolve undescribed lineages within this species complex, Savchenko *et al.* (2014a) suggested that several additional gene loci and host-fungus combinations should be included. However, in line with Vánky (2012), Savchenko *et al*. (2014a) suggested that it would be difficult to distinguish between these lineages based on morphological characters. DNA-based characteristics, such as diagnostic SNPs, along with host specificity might be a solution towards characterizing and describing previously-named and new species (Denchev *et al.* 2009, Piątek *et al*. 2013). The aim of this study was to use a multigene phylogeny to infer the phylogenetic differentiation in the leaf stripe smuts in the genus *Ustilago*, particularly those in the *U. striiformis* species complex.

MATERIAL AND METHODS

Plant and fungal material

Specimens used in the study are listed in Table 1. The names of the hosts and fungi was derived from the latest version of The International Plant Names Index (www.ipni.org), Index Fungorum (www.indexfungorum.org/) and Vánky (2012), and partly following a broad generic concept for *Ustilago* (Thines 2016). A majority of the samples were collected in Germany (about 76) and most collections were not older than 20 years. Samples are deposited in Herbarium Senckenbergianum Görlitz (GLM). All host identifications were confirmed by ITS sequences.

DNA extraction and PCR

About 2–20 mg of infected plant tissue was taken from fungarium samples, placed in 2 mL plastic reaction tubes and homogenized in a mixer mill (MM2, Retsch) using a combination of three to five 1 mm and two 3 mm metal beads at 25 Hz for 5–10 min. Genomic DNA was extracted using the BioSprint 96 DNA Plant Kit (Qiagen, Hilden) loaded to a KingFisher Flex robot (Thermo Scientific, Dreieich).

The complete nrITS of all DNA extracts were amplified using PCR following the procedure of White *et al.* (1990). The primer pairs M-ITS1 (Stoll *et al.* 2003) / ITS4 (White *et al.* 1990) or M-ITS1 / smITS-R1 (Kruse *et al.* 2017a) were used as the reverse and forward primers, respectively. For DNA samples from historic specimens, including type specimens, the *Ustilaginaceae*-optimised reverse primer ITS-US3R (5'TATCAAAACCCGGCAGGGAAG3'), located at the ITS2 region, was used.

The NL1 and NL4 primer pair (O'Donnell 1993) were used to amplify the Large Subunit (LSU) of the nrDNA with an annealing temperature of 53 °C. For other loci, the following regions were amplified with their respective primer pairs and annealing temperatures in brackets: *myosin* R0.5/F3 (55 °C), *map* R6/F2 (56 °C), *rpl*3 R1/F1 (53 °C), *tif*2 R3/F3 (53 °C), *ssc*1 R1/F2 (53 °C), *sdh*1 R3/F2 (53 °C), *rpl*4A R1/F4 (53 °C), and *atp*2 R4/F6 (53 °C) (Kruse *et al.* 2017b).

The plant ITS was amplified using the primer pair ITS1P and ITS4 (Ridgway *et al.* 2003) at 53 °C annealing temperature. The cycling reaction was performed in a thermocycler (Eppendorf Mastercycler 96 vapo protect; Eppendorf, Hamburg) with an initial denaturation at 95 °C for 4 min, 36 PCR cycles of denaturation at 95 °C for 40 s, annealing between 53–56 °C (depending on the specific primer pair) for 40 s and elongation at 72 °C for 60 s, followed by a final elongation at 72 °C for 4 min. For DNA samples older than 50 years, PCR cycles were increased to 46 cycles and a larger amount of DNA (1.5 µL of extracted DNA in a reaction volume of 11 µL) was used. The resulting amplicons were sequenced at the Biodiversity and Climate Research Centre (BiK-F) laboratory using the abovementioned PCR primers. However, amplicons from M-ITS1/smITS-R1 were sequenced using the ITS4 reverse primer. The resulting sequences were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/, Table 1).

Alignments and phylogenetic tree reconstruction

We used 93 samples (including 62 of the *Ustilago striiformis* -complex) for the phylogenetic analysis; 93 had sequences from nrITS, *atp*2 (ATP synthase subunit 2) and *ssc*1 (member of the heat shock proteins), and 70 had additional sequences from *myosin* (myosin group I), *map* (methionine aminopeptidase), *rpl*3 (ribosomal protein L3), *tif*2 (initial translation factor of eIF4A), *sdh*1 (succinate dehydrogenase ubiquinone flavoprotein), and *rpl*4A (ribosomal protein L4-A) (Table 1). About two thirds of the samples (62) belonged to the *U. striiformis* species complex. *Sporisorium inopinatum* (syn. *Langdonia inopinata*) was chosen as outgroup, according to the findings of McTaggart *et al.* (2012a).

Alignments were made on individual loci using mafft v. 7 (Katoh & Standley 2013) using the G-INS-i algorithm. Both leading and trailing gaps of the alignments were removed manually. Two different sets of concatenated alignments for the phylogenetic constructions were generated. The first multigene-alignment includes three loci (ITS, *atp*2, and *ssc*1) from 93 smut samples. The resulting total alignment was 1502 bp (ITS: 643 bp, *atp*2: 595 bp, *ssc*1: 264 bp). The second multigene-alignment included nine genes with a final alignment of 3156 bp (ITS: 643 bp, *atp*2: 595 bp, *ssc*1 264 bp, *map*: 251 bp, *myosin*: 257 bp, *rpl*4A: 415 bp, *rpl*3: 218 bp, *sdh*1: 269 bp, *tif*2: 244 bp).

The diagnostic bases for the *U. striiformis* species complex for all gene markers were determined using the above mentioned alignments. One further ITS alignment was created (443 bp), with the sequence of the type specimen of *U. bromina* (Table 1), the *U. bromina* sequences from GenBank (KF381006-8) and sequences from the same host-fungus-combination from this study, to check if all specimens were sequence-identical with the type collection of *U. bromina* on *Bromus inermis* (data not shown).

For phylogenetic tree constructions, Minimum Evolution (ME) analysis was done using Mega 6.06 (Tamura *et al.* 2013) with the Tamura-Nei substitution model and assuming

Table 1. Smut specimens used for phylogenetic analysis.

			Collection details				gene loci								
DNA-no.	**Species**	**Host**	**Location**	**Date**	**Collector**	**Fungarium no.**	**ITS**	**atp2**	**ssc1**	**map**	**myosin**	**rpl4A**	**rpl3**	**sdh1**	**tif2**
2354	*Sporisorium* aff. *inopiatum* (*Langdonia*)	*Aristida adscensionis*	Zambia	12 Apr. 2001	C., T. & K. Vánky	M-0215944	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	KY929824	KY929964	KY930127
474	*Ustilago agrostidis-palustris*	*Agrostis* cf. *gigantea*	Germany, Bavaria	22 Jun. 2012	J. Kruse	GLM-F105832	KY929551	KY930157	KY929994	KY929709	KY929639	KY929849	KY929779	KY929919	KY930082
1374		*Agrostis gigantea*	Switzerland	9 Jul. 2004	V. Kummer	GLM-F107425	KY929582	KY930188	KY930025	KY929729	KY929659	KY929869	KY929799	KY929939	KY930102
2395		*Agrostis* sp.	Germany, Lower Saxony	12 Jul. 2014	J. Kruse & H. Jage	GLM-F107439	KY929596	KY930202	KY930039	KY929739	KY929669	KY929879	KY929809	KY929949	KY930112
2287	*Ustilago airae-caespitosae*	*Deschampsia caespitosa*	Polen	13 Jul. 1994	H. Scholz	B 70 0014901	KY929526	KY930132	KY929969	KY929688	KY929618	KY929828	KY929758	KY929898	KY930061
2401		*Deschampsia caespitosa*	Austria, Upper Austria	15 Aug. 2014	J. Kruse	GLM-F107444	KY929601	KY930207	KY930044	KY929744	KY929674	KY929884	KY929814	KY929954	KY930117
2402		*Deschampsia caespitosa*	Austria, Upper Austria	15 Aug. 2014	J. Kruse	GLM-F107445	KY929602	KY930208	KY930045	KY929745	KY929675	KY929885	KY929815	KY929955	KY930118
477	*Ustilago alopecurivora*	*Alopecurus pratensis*	Germany, Hesse	22 May 2010	J. Kruse	GLM-F105834	KY929553	KY930159	KY929996	KY929711	KY929641	KY929851	KY929781	KY929921	KY930084
1376		*Alopecurus pratensis*	Germany, Saxony-Anhalt	20 May 2013	H. Jage	GLM-F107426	KY929583	KY930189	KY930026	–	–	–	–	–	–
1822	*Ustilago* aff. *andropogonis* (*Sporisorium*)	*Bothriochloa ischaemum*	Germany, Saxony-Anhalt	25 Jul. 2004	H. Jage & H. John	GLM-F062665	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	KY929764	KY929904	KY930067
432	*Ustilago perennans*	*Arrhenatherum elatius*	Germany, Schleswig-Holstein	21 Jun. 2007	J. Kruse	GLM-F105817	KY929536	KY930142	KY929979	KY929697	KY929627	KY929837	KY929767	KY929907	KY930070
2398	*Ustilago brizae*	*Briza media*	Austria, Tirol	21 Jul. 2014	J. Kruse	GLM-F107442	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	KY929812	KY929952	KY930115
2399		*Briza media*	Germany, Bavaria	19 Jul. 2014	J. Kruse	GLM-F107443	KY929600	KY930206	KY930043	KY929743	KY929673	KY929883	KY929813	KY929953	KY930116
498	*Ustilago bromina*	*Bromus inermis*	Germany, Saxony-Anhalt	04 Jun. 2011	J. Kruse	GLM-F105843	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	KY929785	KY929925	KY930088
500		*Bromus inermis*	Germany, Thuringia	15 Jun. 2013	J. Kruse	GLM-F105844	KY929563	KY930169	KY930006	KY929716	KY929646	KY929856	KY929786	KY929926	KY930089
1180		*Bromus inermis*	Germany, Berlin	May 1983	H. Scholz	HUV No 498 (TUB)	KY929613	KY930219	KY930056	–	–	–	–	–	–
2070		***Bromus inermis***	**Germany, Berlin**	**Aug. 1892**	**P. Sydow**	**B 70 0014775**	**KY929525**	–	–	–	–	–	–	–	–
2275		*Bromus inermis*	Germany, Brandenburg	17 Jul. 2005	H. & I. Scholz	B 70 0014755	KY929524	KY930131	KY929968	–	–	–	–	–	–

Table 1. (Continued).

			Collection details				gene loci								
DNA-no.	Species	Host	Location	Date	Collector	Fungarium no.	ITS	atp2	ssc1	map	myosin	rpl4A	rpl3	sdh1	tif2
2276		*Bromus inermis*	Germany, Thuringia	10 Sep. 1999	I. Scholz	B 70 0021843	KY929527	KY930133	KY929970	–	–	–	–	–	–
1591	*Ustilago* aff. *bromivora*	*Bromus rigidus*	Greece	23 Apr. 2013	C. & F. Klenke	GLM-F107429	KY929586	KY930192	KY930029	KY929731	KY929661	KY929871	KY929801	KY929941	KY930104
3370		*Bromus sterilis*	Spain, Andalusia	2 May 2015	J. Kruse	GLM-F107449	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	KY929818	KY929958	KY930121
442	*Ustilago calamagrostidis*	*Calamagrostis epigejos*	Germany, Lower Saxony	03 Aug. 2011	J. Kruse	GLM-F105818	KY929537	KY930143	KY929980	–	–	–	–	–	–
445		*Calamagrostis epigejos*	Germany, Baden-Württemberg	20 Jul. 2013	J. Kruse	GLM-F105819	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	KY929768	KY929908	KY930071
1383		*Calamagrostis epigejos*	Germany, Saxony-Anhalt	29 Jun. 2013	H. Zimmermann, U. Richter	GLM-F107427	KY929584	KY930190	KY930027	KY929730	KY929660	KY929870	KY929800	KY929940	KY930103
1912		*Calamagrostis epigejos*	Germany, Saxony-Anhalt	09 Aug. 1996	H. Jage	GLM-F048100	KY929530	KY930136	KY929973	KY929691	KY929621	KY929831	KY929761	KY929901	KY930064
1182	*Ustilago corcontica*	*Calamagrostis villosa*	Germany, Saxony	22 Aug. 1987	W. Dietrich	HUV No 794 (TUB)	KY929615	KY930221	KY930058	–	–	–	–	–	–
1611		*Calamagrostis villosa*	Germany, Saxony-Anhalt	26 Jul. 2003	H. & U. Richter	GLM-F107434	KY929591	KY930197	KY930034	–	–	–	–	–	–
1825	*Ustilago cruenta* (*Sporisorium*)	*Sorghum bicolor*	Greece	11 May 2006	H-W, Otto	GLM-F078871	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	KY929766	KY929906	KY930069
3375	*Ustilago cynodontis*	*Cynodon dactylon*	Spain, Andalusia	3 May 2015	J. Kruse	GLM-F107450	KY929607	KY930213	KY930050	KY929749	KY929679	KY929889	KY929819	KY929959	KY930122
3376		*Cynodon dactylon*	Spain, Andalusia	3 May 2015	J. Kruse	GLM-F107451	KY929608	KY930214	KY930051	KY929750	KY929680	KY929890	KY929820	KY929960	KY930123
1596	*Ustilago* aff. *dactyloctaenii (Sporisorium)*	*Dactyloctenium australe*	South-Africa	22 Feb. 2000	V. Kummer	GLM-F107430	KY929587	KY930193	KY930030	KY929732	KY929662	KY929872	KY929802	KY929942	KY930105
478	*Ustilago denotarisii*	*Arrhenatherum elatius*	Germany, Schleswig-Holstein	13 May 2007	J. Kruse	GLM-F105835	KY929554	KY930160	KY929997	–	–	–	–	–	–
481		*Arrhenatherum elatius*	Germany, Rhineland-Palatinate	23 May 2010	J. Kruse	GLM-F105836	KY929555	KY930161	KY929998	–	–	–	–	–	–
483		*Arrhenatherum elatius*	Germany, Lower Saxony	31 Jul. 2011	J. Kruse	GLM-F105837	KY929556	KY930162	KY929999	–	–	–	–	–	–
486		*Arrhenatherum elatius*	Germany, Thuringia	04 Jun. 2012	J. Kruse	GLM-F105838	KY929557	KY930163	KY930000	–	–	–	–	–	–
488		*Arrhenatherum elatius*	Germany, Bavaria	16 May 2013	J. Kruse	GLM-F105839	KY929558	KY930164	KY930001	–	–	–	–	–	–

Table 1. (Continued).

			Collection details				gene loci								
DNA-no.	**Species**	**Host**	**Location**	**Date**	**Collector**	**Fungarium no.**	**ITS**	**atp2**	**ssc1**	**map**	**myosin**	**rpl4A**	**rpl3**	**sdh1**	**tif2**
447	*Ustilago echinata*	*Phalaris arundinacea*	Germany, Lower Saxony	01 Jul. 2010	J. Kruse	GLM-F105820	KY929539	KY930145	KY929982	KY929699	KY929629	KY929839	KY929769	KY929909	KY930072
449		*Phalaris arundinacea*	Germany, Lower Saxony	29 Aug. 2011	J. Kruse	GLM-F105821	KY929540	KY930146	KY929983	KY929700	KY929630	KY929840	KY929770	KY929910	KY930073
1914		*Phalaris arundinacea*	Switzerland, St. Gallen	26 Jul. 2000	H. Jage	GLM-F048338	KY929531	KY930137	KY929974	KY929692	KY929622	KY929832	KY929762	KY929902	KY930065
451	*Ustilago* aff. *filiformis*	*Glyceria fluitans*	Germany, Lower Saxony	17 May 2007	J. Kruse	GLM-F105822	KY929541	KY930147	KY929984	KY929701	KY929631	KY929841	KY929771	KY929911	KY930074
454		*Glyceria fluitans*	Germany, Bavaria	24 Jun. 2012	J. Kruse	GLM-F105823	KY929542	KY930148	KY929985	KY929702	KY929632	KY929842	KY929772	KY929912	KY930075
455		*Glyceria fluitans*	Germany, Bavaria	10 May 2013	J. Kruse	GLM-F105824	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	KY929773	KY929913	KY930076
456	*Ustilago filiformis*	*Glyceria maxima*	Germany, Lower Saxony	01 Jul. 2010	J. Kruse	GLM-F105825	KY929544	KY930150	KY929987	KY929704	KY929634	KY929844	KY929774	KY929914	KY930077
472	*Ustilago jagei* sp. nov.	*Agrostis rupestris*	Switzerland, Grisons	02 Aug. 2009	J. Kruse	GLM-F105830	KY929549	KY930155	KY929992	–	–	–	–	–	–
473		*Agrostis stolonifera*	Germany, Bavaria	20 May 2012	J. Kruse	GLM-F105831	KY929550	KY930156	KY929993	–	–	–	–	–	–
476		*Agrostis stolonifera*	Germany, Hesse	22 May 2010	J. Kruse	GLM-F105833	KY929552	KY930158	KY929995	KY929710	KY929640	KY929850	KY929780	KY929920	KY930083
551		*Agrostis* sp.	Germany, Lower Saxony	11 Jun. 2010	J. Kruse	GLM-F107423	KY929580	KY930186	KY930023	KY929727	KY929657	KY929867	KY929797	KY929937	KY930100
2396		*Agrostis stolonifera*	Germany, Bavaria	20 Jul. 2014	J. Kruse	GLM-F107440	KY929597	KY930203	KY930040	KY929740	KY929670	KY929880	KY929810	KY929950	KY930113
2397		*Agrostis stolonifera*	Germany, Hesse	27 Jun. 2014	J. Kruse	GLM-F107441	KY929598	KY930204	KY930041	KY929741	KY929671	KY929881	KY929811	KY929951	KY930114
494		*Agrostis* sp.	Germany, Bavaria	04 Jul. 2013	J. Kruse	GLM-F105841	KY929560	KY930166	KY930003	KY929713	KY929643	KY929853	KY929783	KY929923	KY930086
1375		***Agrostis stolonifera***	**Germany, Saxony-Anhalt**	**16 Sep. 2001**	**H. Jage**	**GLM-F047379**	**KY929528**	**KY930134**	**KY929971**	**KY929689**	**KY929619**	**KY929829**	**KY929759**	**KY929899**	**KY930062**
1612	***Ustilago kummeri* sp. nov.**	***Bromus inermis***	**Germany, Brandenburg**	**19 Jun. 2010**	**V. Kummer**	**GLM-F107435**	**KY929592**	**KY930198**	**KY930035**	**KY929736**	**KY929666**	**KY929876**	**KY929806**	**KY929946**	**KY930109**
1948		*Bromus inermis*	Germany, Saxony-Anhalt	17 Jul. 2001	H. Jage, W. Lehman	GLM-F047380	KY929529	KY930135	KY929972	KY929690	KY929620	KY929830	KY929760	KY929900	KY930063
501	*Ustilago loliicola*	*Lolium perenne*	Germany, Bavaria	14 May 2013	J. Kruse	GLM-F105845	KY929564	KY930170	KY930007	–	–	–	–	–	–
2288A		*Festuca pratensis*	Germany, Hesse	25 May 2014	J. Kruse	GLM-F107437	KY929594	KY930200	KY930037	–	–	–	–	–	–

Table 1. (Continued).

			Collection details				gene loci								
DNA-no.	Species	Host	Location	Date	Collector	Fungarium no.	ITS	atp2	ssc1	map	myosin	rpl4A	rpl3	sdh1	tif2
3386		*Festuca arundinacea*	Germany, Hesse	02 Nov. 2014	J. Kruse	GLM-F107454	KY929611	KY930217	KY930054	KY929753	KY929683	KY929893	KY929823	KY929963	KY930126
2815A	*Ustilago maydis*	*Zea mays*	Germany, Saxony-Anhalt	10 Jul. 2007	H. Jage	GLM-F107446	KY929603	KY930209	KY930046	KY929746	KY929676	KY929886	KY929816	KY929956	KY930119
1404	*Ustilago milii*	*Milium effusum*	Germany, Saxony-Anhalt	02 Jun. 2002	H. Jage	GLM-F107428	KY929585	KY930191	KY930028	–	–	–	–	–	–
2303		*Milium effusum*	Germany, Saxony	03 Jun. 2012	W. Dietrich	GLM-F107438	KY929595	KY930201	KY930038	KY929738	KY929668	KY929878	KY929808	KY929948	KY930111
3385		*Milium effusum*	Germany, Hesse	11 Jun. 2015	J. Kruse	GLM-F107453	KY929610	KY930216	KY930053	KY929752	KY929682	KY929892	KY929822	KY929962	KY930125
503	*Ustilago neocopinata* sp. nov.	*Dactylis glomerata*	Germany, Lower Saxony	01 Jul. 2010	J. Kruse	GLM-F105846	KY929565	KY930171	KY930008	–	–	–	–	–	–
505		*Dactylis glomerata*	Germany, Bavaria	20 Jun. 2010	J. Kruse	GLM-F105847	KY929566	KY930172	KY930009	–	–	–	–	–	–
506		*Dactylis glomerata*	Germany, Lower Saxony	19 May 2011	J. Kruse	GLM-F105848	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	KY929787	KY929927	KY930090
508		*Dactylis glomerata*	Germany, Bavaria	19 Jul. 2011	J. Kruse	GLM-F105849	KY929568	KY930174	KY930011	KY929718	KY929648	KY929858	KY929788	KY929928	KY930091
510		*Dactylis glomerata*	Germany, Bavaria	24 May 2012	J. Kruse	GLM-F105850	KY929569	KY930175	KY930012	KY929719	KY929649	KY929859	KY929789	KY929929	KY930092
512		***Dactylis glomerata***	**Germany, Bavaria**	**15 Jun. 2012**	**J. Kruse**	**GLM-F107413**	**KY929570**	**KY930176**	**KY930013**	–	–	–	–	–	–
521		*Dactylis glomerata*	Germany, Thuringia	15 Jun. 2013	J. Kruse	GLM-F107414	KY929571	KY930177	KY930014	–	–	–	–	–	–
463	*Ustilago nuda*	*Hordeum vulgare*	Germany, Bavaria	12 May 2012	J. Kruse	GLM-F105826	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	KY929775	KY929915	KY930078
884	*Sporisorium* aff. *occidentale*	*Andropogon gerardii*	USA	30 Jul. 1989	not known	HUV No 758 (TUB)	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	KY929825	KY929965	KY930128
471	*Ustilago salweyi*	*Holcus mollis*	Germany, Bavaria	11 Jun. 2012	J. Kruse	GLM-F105829	KY929548	KY930154	KY929991	KY929708	KY929638	KY929848	KY929778	KY929918	KY930081
489		*Holcus mollis*	Germany, Bavaria	16 May 2013	J. Kruse	GLM-F105840	KY929559	KY930165	KY930002	KY929712	KY929642	KY929852	KY929782	KY929922	KY930085
523		*Holcus lanatus*	Germany, Lower Saxony	24 May 2009	J. Kruse	GLM-F107415	KY929572	KY930178	KY930015	KY929720	KY929650	KY929860	KY929790	KY929930	KY930093
524		*Holcus lanatus*	Germany, Lower Saxony	22 May 2010	J. Kruse	GLM-F107416	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	KY929791	KY929931	KY930094
525		*Holcus lanatus*	Germany, Lower Saxony	27 May 2010	J. Kruse	GLM-F107417	KY929574	KY930180	KY930017	KY929722	KY929652	KY929862	KY929792	KY929932	KY930095
531		*Holcus lanatus*	Germany, Bavaria	17 May 2012	J. Kruse	GLM-F107418	KY929575	KY930181	KY930018	–	–	–	–	–	–

Table 1. (Continued).

			Collection details				gene loci								
DNA-no.	**Species**	**Host**	**Location**	**Date**	**Collector**	**Fungarium no.**	**ITS**	**atp2**	**ssc1**	**map**	**myosin**	**rpl4A**	**rpl3**	**sdh1**	**tif2**
541		*Holcus mollis*	Germany, Saxony	03 Jun. 2011	J. Kruse	GLM-F107419	KY929576	KY930182	KY930019	KY929723	KY929653	KY929863	KY929793	KY929933	KY930096
543		*Holcus mollis*	Germany, Saxony-Anhalt	05 Jun. 2011	J. Kruse	GLM-F107420	KY929577	KY930183	KY930020	KY929724	KY929654	KY929864	KY929794	KY929934	KY930097
544		*Holcus mollis*	Germany, Saxony-Anhalt	05 Jun. 2011	J. Kruse	GLM-F107421	KY929578	KY930184	KY930021	KY929725	KY929655	KY929865	KY929795	KY929935	KY930098
545		*Holcus mollis*	Germany, Lower Saxony	17 Aug. 2011	J. Kruse	GLM-F107422	KY929579	KY930185	KY930022	KY929726	KY929656	KY929866	KY929796	KY929936	KY930099
497	*Ustilago scaura*	*Helictotrichon pubescens*	Germany, Rhineland-Palatinate	23 May 2010	J. Kruse	GLM-F105842	KY929561	KY930167	KY930004	KY929714	KY929644	KY929854	KY929784	KY929924	KY930087
3384		*Helictotrichon pubescens*	Germany, Hesse	10 Jun. 2015	J. Kruse	GLM-F107452	KY929609	KY930215	KY930052	KY929751	KY929681	KY929891	KY929821	KY929961	KY930124
1359	*Ustilago* aff. *schroeteriana* (*Sporisorium*)	*Paspalum virgatum*	Costa Rica	15 Mar. 1991	T. & K. Vánky	HUV No 888 (TUB)	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	KY929826	KY929966	KY930129
1608	*Ustilago scrobiculata*	*Calamagrostis epigejos*	Germany, Brandenburg	17 Aug. 2011	V. Kummer & C. Buhr	GLM-F107431	KY929588	KY930194	KY930031	KY929733	KY929663	KY929873	KY929803	KY929943	KY930106
1609		*Calamagrostis epigejos*	Germany, Thuringia	27 May 2010	V. Kummer	GLM-F107432	KY929589	KY930195	KY930032	KY929734	KY929664	KY929874	KY929804	KY929944	KY930107
1610		*Calamagrostis epigejos*	Germany, Brandenburg	24 Jun. 2007	V. Kummer	GLM-F107433	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	KY929805	KY929945	KY930108
467	*Ustilago serpens*	*Elymus repens*	Germany, Schleswig-Holstein	31 Jul. 2012	J. Kruse	GLM-F105827	KY929546	KY930152	KY929989	KY929706	KY929636	KY929846	KY929776	KY929916	KY930079
469		*Elymus repens*	Germany, Thuringia	15 Jun. 2013	J. Kruse	GLM-F105828	KY929547	KY930153	KY929990	KY929707	KY929637	KY929847	KY929777	KY929917	KY930080
3110		*Elymus repens*	Germany, Brandenburg	29 Jun. 2014	V. Kummer	GLM-F107447	KY929604	KY930210	KY930047	–	–	–	–	–	–
1305	*Ustilago* aff. *sorghi* (*Sporisorium*)	*Sorghum plumosum*	Australia	20 Feb. 1996	A. A. Mitchell, C. & K. Vánky	HUV No 970 (TUB)	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	KY929827	KY929967	KY930130
1951	*Ustilago* aff. *syntherismae*	*Digitaria sanguinalis*	Germany, Saxony-Anhalt	01 Oct . 2004	H. Jage	GLM-F064759	KY929534	KY930140	KY929977	KY929695	KY929625	KY929835	KY929765	KY929905	KY930068
1617		*Digitaria sanguinalis*	Germany, Brandenburg	11 Aug. 2001	V. Kummer	GLM-F107436	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	KY929807	KY929947	KY930110
553	*Ustilago trichophora*	*Echinochloa crus-galli*	Germany, North Rhine-Westphalia	04 Oct . 2010	J. Kruse	GLM-F107424	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	Kruse *et al.* 2017b	KY929798	KY929938	KY930101

Table 1. (Continued).

DNA-no.	Species	Host	Collection details			Fungarium no.	gene loci							
			Location	Date	Collector		ITS	atp2	ssc1	map	myosin	rpl4A	rpl3	sdh1	tif2
1957		*Echinochloa crus-galli*	Germany, Saxony-Anhalt	01 Oct . 2003	H. Jage	GLM-F062638	KY929532	KY930138	KY929975	KY929693	KY929623	KY929833	KY929763	KY929903	KY930066
3347	*Ustilago* aff. *vanderystii* (*Sporisorium*)	*Hyparrhenia hirta*	Spain, Andalusia	22 Apr. 2015	J. Kruse	GLM-F107448	KY929605	KY930211	KY930048	KY929747	KY929677	KY929887	KY929817	KY929957	KY930120

Type specimens are printed in bold face.

complete deletion at 80 % cut-off with 1000 bootstrap replicates. All other parameters were set to default values. Maximum Likelihood (ML) analysis was done using RAxML (Stamatakis 2014) with parameters set to default values and Bayesian analysis was done using MrBayes 3.2 (Ronquist & Huelsenbeck 2003) running five times with model 6 (GTR) using four incrementally heated chains for 10 million generations, sampling every 1000th tree discarding the first 30 % of the obtained trees, all other parameters were set to default on the TrEase webserver (http://www.thines-lab.senckenberg.de/trease).

To account for potentially deviating evolutionary properties, the analysis in ME was done also on a partitioned concatenated dataset. As no supported differences within the topology of the trees were observed in comparison with the un-partitioned dataset, the other analyses were carried out without partitioning.

Morphological examination

For light microscopy, fungarium specimens (GLM-F107417, GLM-F105836, GLM-F107435, GLM-F107413, GLM-F047379, GLM-F105827) were transferred to 60 % lactic acid on a slide. Morphological examination was carried out using a Zeiss Imager M2 AX10 microscope (Carl Zeiss, Göttingen). Measurements of the spores were performed at x400. The measurements are reported as maxima and minima in parentheses, and the mean plus and minus the standard deviation of a number of measurements is given in parenthesis. The means are placed in italics.

RESULTS

Phylogenetic inference

The LSU sequence data were excluded from further analysis since sequences were identical for all members of the *Ustilago striiformis* species complex (data not shown). All other loci showed SNPs within the *U. striiformis* cluster. The diagnostic bases (SNPs) with their specific positions are given in Fig. 6.

There were no supported conflicts in the topology of the trees of the single loci and the concatenated trees. Thus, the datasets were combined and used as concatenated for further analysis. The multigene tree based on nine different loci (Fig. 1) showed strong to maximum support for a monophyly of the *U. striiformis* species complex. If multiple specimens from one host species were included, these grouped together with strong to maximum support, except for the clades corresponding to *U. scaura s. lat.* (ME 64, ML 63, BA 0.99), *U. brizae* (ME 63, ML 68, BA 0.99), and *U. agrostidis-palustris* (ME 71, ML 68, BA 0.99), which received weak to strong support (Fig. 1).

A phylogenetic reconstruction (Fig. 2) with an additional 21 specimens but based on only half of the characters per specimen (ITS, *atp*2, and *ssc*1) revealed the same groups as the double-sized alignment, but expectedly with weaker statistical support. For example, the three weak to strongly supported lineages shown in Fig. 1 still grouped together, but with no or weak support (*U. brizae* – ME 64, ML -, BA 0.79; *U. scaura s. lat.* – ME -, ML -, BA 0.79; *U. agrostidis-palustris* – no support), highlighting the importance of gene selection.

In the phylogenetic reconstruction based on fewer genes (Fig. 2) additional specimens were included, which further supported the high degree of genetic differentiation in conjunction with the host species infected. Specimens from *Festuca* and *Lolium* grouped together with strong support, while the monophyly of the clade containing samples from *Alopecurus* species was unsupported to weakly supported (ME 64, ML -, BA -). Two monophyletic groups were absent from the tree with more loci (Fig. 1): one on *Calamagrostis villosa* and another on *Arrhenatherum elatius*. Both of these groups were highly supported (*Calamagrostis*: ME 76, ML 94, BA 0.99; *Arrhenatherum*: ME 92, ML 99, BA 1) in the tree based on fewer loci (Fig. 2).

In both phylogenetic trees (Figs 1–2), *U. cynodontis* was inferred as the sister species to the whole *U. striiformis* species complex. To illustrate the relationships within this species complex further, two additional phylogenetic

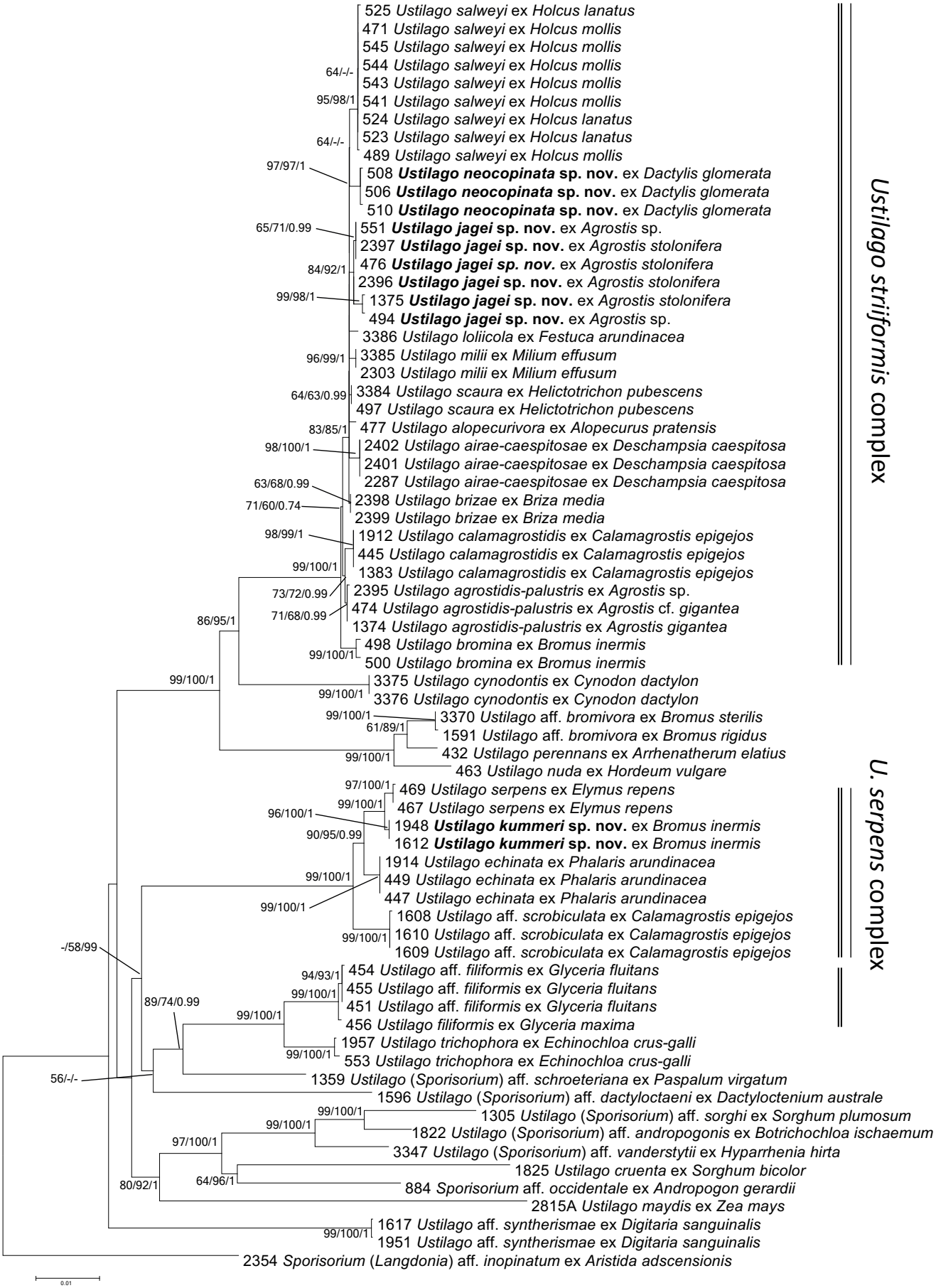

Fig. 1. Phylogenetic tree based on Minimum Evolution analysis of nine loci (ITS, *myosin*, *map*, *rpl*3, *tif*2, *ssc*1, *sdh*1, *rpl*4*A*, *atp*2). Numbers on branches denote support in Minimum Evolution, Maximum Likelihood and Bayesian Analyses, in the respective order. Values below 55 % are denoted by '-'. The bar indicates the number of substitutions per site.

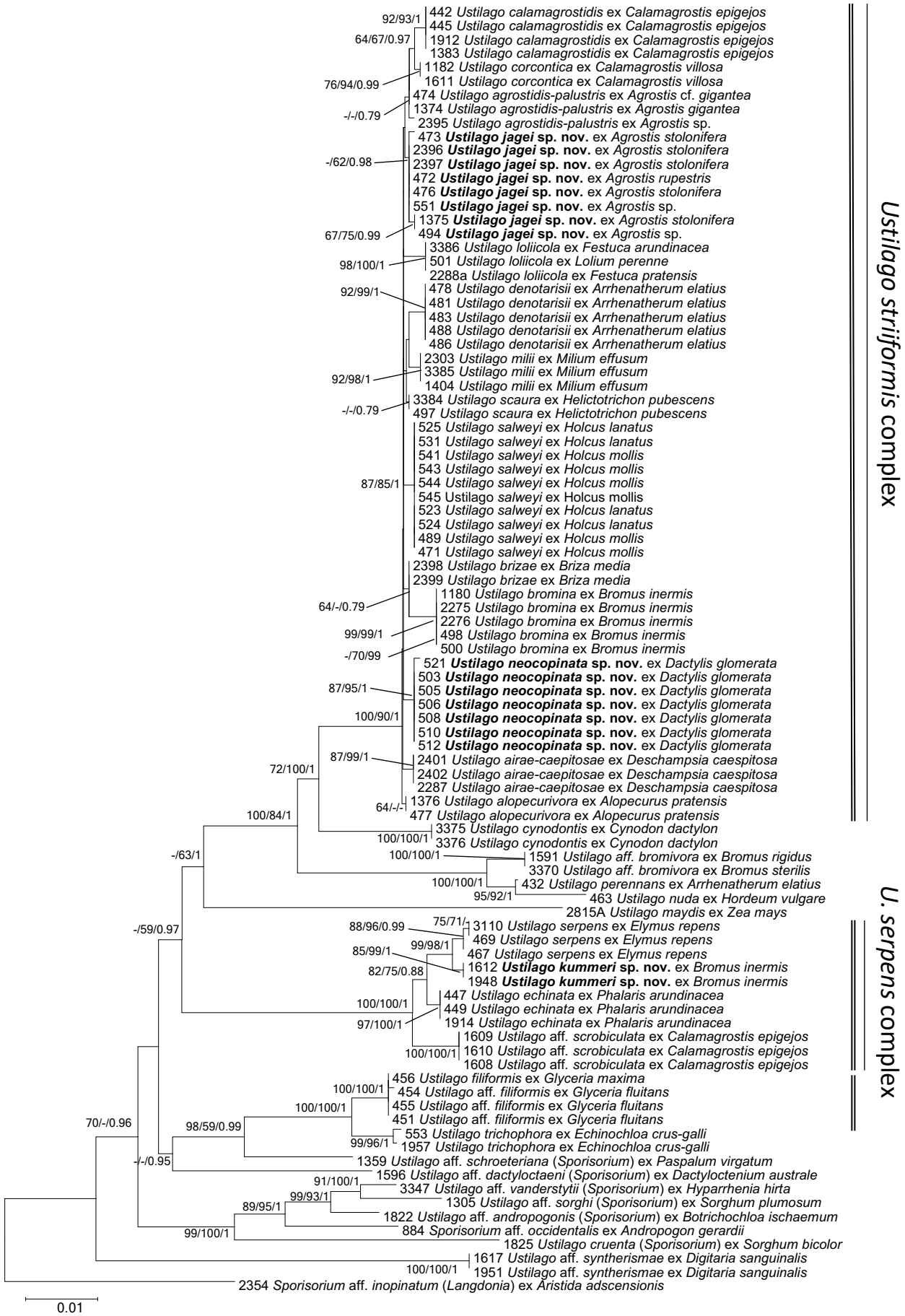

Fig. 2. Phylogenetic tree based on Minimum Evolution analysis of three loci (ITS, *ssc*1, *atp*2). Numbers on branches denote support in Minimum Evolution, Maximum Likelihood and Bayesian Analyses, in the respective order. Values below 55 % are denoted by '-'.. The bar indicates the number of substitutions per site.

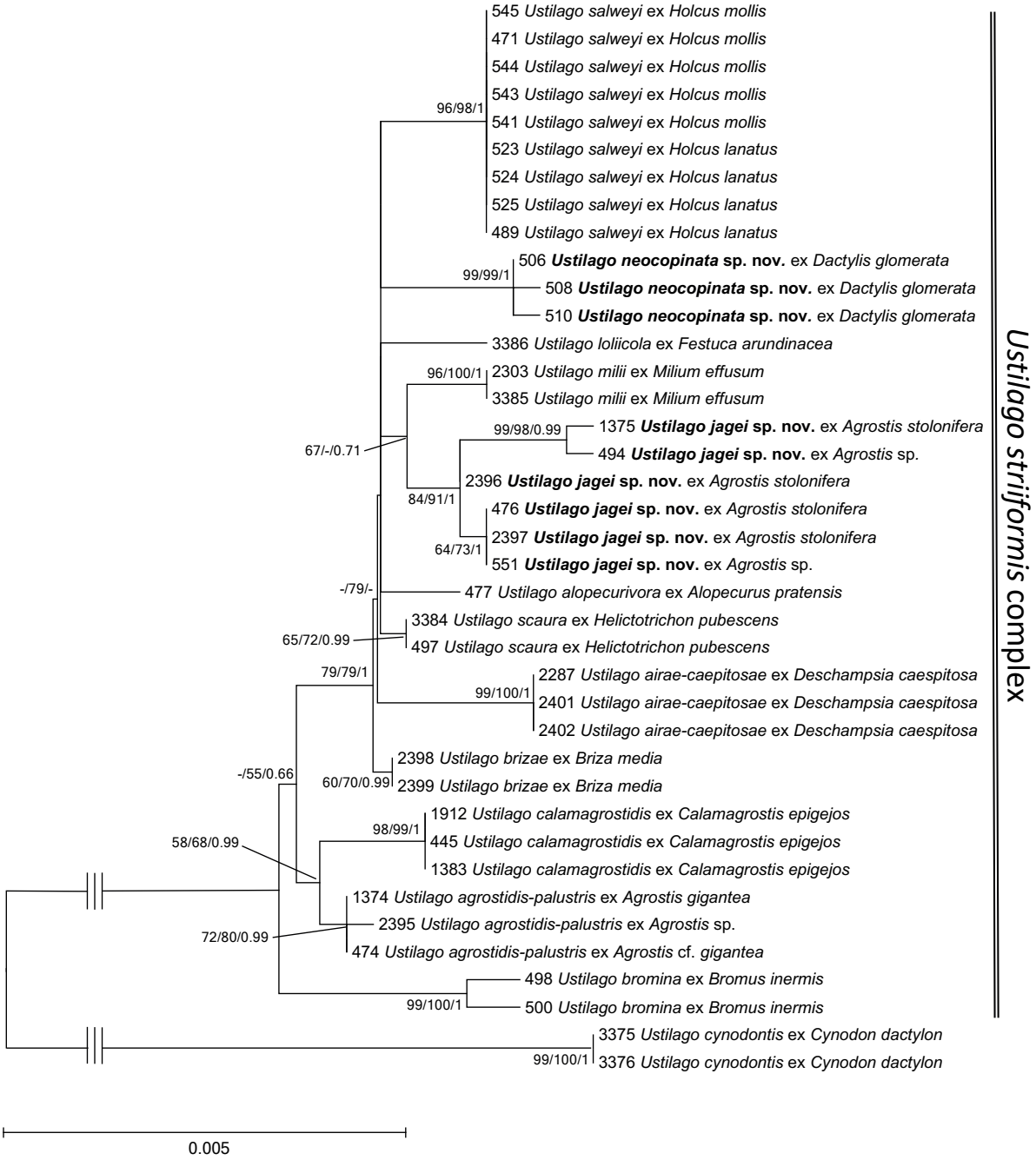

Fig. 3. Phylogenetic tree based on Minimum Evolution analysis of nine loci (ITS, *myosin*, *map*, *rpl*3, *tif*2, *ssc*1, *sdh*1, *rpl*4*A*, *atp*2) detailed showing the *Ustilago striiformis*-complex with the outgroup *U. cynodontis*. Numbers on branches denote support in Minimum Evolution, Maximum Likelihood and Bayesian Analyses, in the respective order. Values below 55 % are denoted by '-'. The bar indicates the number of substitutions per site.

trees with a reduced sampling and *U. cynodontis* as outgroup are shown in Figs 3 (9 loci) and 4 (3 loci). The support values and the topology were comparable to the phylogenetic reconstructions in Figs 1–2. In both phylogenetic trees, *U. serpens* on *Elymus repens* and on *Bromus inermis* grouped together with high to maximum support. This group clustered with two further lineages with larger echinulate spores compared to the *U. striiformis* species complex, which is considered a synapomorphy of this lineage.

The resolution on the backbone was rather low, as highlighted also by the ambiguous placement of *U. maydis*, which was resolved as a sister group to the pathogens on the majority of panicoid hosts in the tree based on 9 loci (Fig. 1) with moderate to maximum support, while being inferred as a sister to the clade containing the *U.* species complex as well as the *U. nuda* species group with lacking to maximum support in the tree based on three loci (Fig. 2).

Morphology

The degree of overlap in morphological characteristics was too high in both species complexes to provide easily accessible characteristics for species delimitation (Fig. 5). The individual measurements are included in the species descriptions below and summarized in Table 3.

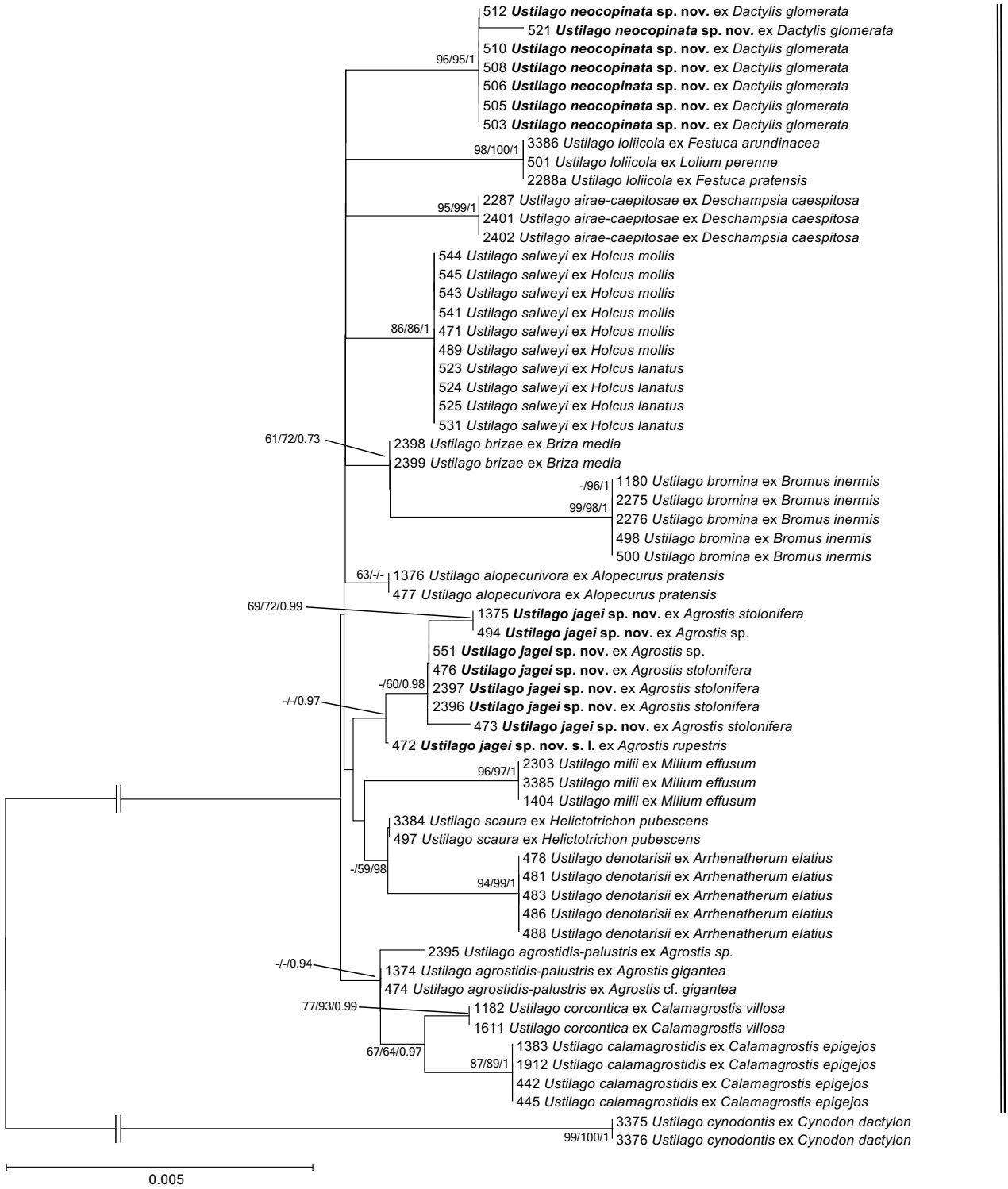

Fig. 4. Phylogenetic tree based on Minimum Evolution analysis of three loci (ITS, *ssc*1, *atp*2) detailed showing the *Ustilago striiformis*-complex with the outgroup *U. cynodontis*. Numbers on branches denote support in Minimum Evolution, Maximum Likelihood and Bayesian Analyses, in the respective order. Values below 55 % are denoted by '-'.. The bar indicates the number of substitutions per site.

TAXONOMY

Based on our phylogenetic analyses, the following nomenclature and taxonomic changes are proposed for leaf stripe smuts caused by species of *Ustilago*. The positions given for the diagnostic bases refer to specific positions in the alignments as highlighted in the alignment consensus sequences in Fig. 4. Only selected synonyms are given here. For a complete synonymy reference should be made to Vánky (2012) and references therein.

Ustilago agrostidis-palustris W. H. Davis ex Ciferri, *Ann. Mycol.* **29**: 54 (1931).

Type: **USA:** *Wisconsin*: Madison, on cultivated 'redtop' (i.e. *Agrostis* "*palustris* Huds.", now *Agrostis gigantea*),

8 July 1921, *W. H. & J. J. Davis* (BPI 166994 – ***lectotype designated here*,** MBT 380628).

Confirmed host: *Agrostis gigantea.*

Confirmed distribution: Germany and USA.

Notes: *Ustilago agrostidis-palustris* can be distinguished from other leaf stripe smuts of the *U. striiformis* species complex based on its host specific occurrence on *Agrostis gigantea s. lat*. Furthermore, it differs in one diagnostic base from all other species of the *U. striiformis*-complex included in this study – in the *sdh*1 gene there is a C instead of a T at position 138 (Table 2, Fig. 6).

Ustilago airae-caespitosae (Lindr.) Liro, *Ann. Acad. Sci. Fenn.*, ser. A **17** (1): 71 (1924).

Basionym: *Tilletia airae-caespitosae* Lindr., *Acta Soc. Fauna Flora Fenn.* **26**:15 (1904).

Type: **Finland**: *Nyland*: Helsingfors, Hagasund, on *Aira caespitosa* (i.e. *Deschampsia caespitosa*), 10 Aug. 1902, *J. I. Lindroth* [Vestergren, Micr. Rar. Sel. no. 806; Sydow, Ustil. no. 316] (M-0236198 – ***lectotype designated here,*** MBT 380628; from one of the several duplicate collections treated as "lectotype" by Lindeberg, *Symb. Bot. Upsal.* **16** (2): 135, 1959).

Confirmed host: *Deschampsia caespitosa*.

Confirmed distribution: Austria and Finland.

Notes: Within the *Ustilago striiformis* species complex, *U. airae-caespitosae* can be distinguished from other species based on the host-specific occurrence on *Deschampsia caespitosa*. Furthermore, it differs in six diagnostic bases from all other species within the *U. striiformis* species complex included in this study – in the *atp*2 gene it has an A instead of a G at position 22 and 94, in the *map* gene there is a T instead of a C at position 227, in the *myosin* gene there is an A instead of a G at position 133, in the *rpl*3 gene a T instead of a C at position 199, and an A instead of a G at position 576 in the ITS region (Table 2, Fig. 6).

Ustilago alopecurivora (Ule) Liro, *Ann. Acad. Sci. fenn.*, ser. A **17** (1): 72 (1924).

Basionym: *Tilletia alopecurivora* Ule, *Hedwigia* **25**: 113 (1886).

Synonyms: *Uredo longissima* var. *megalospora* Riess, *in* Rabenhorst, *Herb. Viv. Myc.* no. 1897 (1854).

Ustilago megalospora (Riess) Cif., *Nuovo Giorn. Bot. Ital.* **40**: 261 (1933).

Type: **Germany**: *Bavaria:* Coburg, Hofgarten, on *Alopecurus pratensis*, June 1879, *E. Ule* (B – holotype lost); *Berlin*: Charlottenburg-Nord, Kolonie Königsdamm, slope of ditch, 9 Aug. 1988, *H. Scholz* (B 70 0014985 – ***neotype designated here***, MBT 380629).

Confirmed host: *Alopecurus pratensis*.

Confirmed distribution: Germany.

Notes: Within the *U. striiformis* species complex, *U. alopecurivora* can be distinguished from other species based on the host-specific occurrence on *Alopecurus pratensis*. Furthermore, *U. alopecurivora* differs in three diagnostic bases from all other species within the *U. striiformis* species complex included in this study – in the *atp*2 gene, there is an A instead of a G at position 358, in the *map* gene there is a G instead of a T at position 192, and in the *myosin* gene there is a T instead of a C at position 83 (Table 2, Fig. 6).

Ustilago brizae (Ule) Liro, *Ann. Acad. Sci. Fenn.*, Ser. A **17** (1): 74 (1924).

Basionym: *Tilletia brizae* Ule, *Verh. Bot. Ver. Prov. Brandenb.* **25**: 214 (1884).

Type: **Germany**: *Bavaria*: Coburg, Rögener Berg, on *Briza media*, July 1879, *E. Ule* [Rabenhorst, Fungi Eur. no. 3604] (M-0147750 – ***lectotype designated here***, MBT 380630; from one of the several duplicate collections treated as "lectotype" by Lindeberg, *Symb. Bot. Upsal.* **16**(2): 135, 1959).

Confirmed hosts: *Briza media*.

Confirmed distribution: Austria and Germany.

Notes: Within the *U. striiformis* species complex, *U. brizae* can be distinguished from other species based on the host-specific occurrence on *Briza media*. Furthermore, *U. brizae* differs in one diagnostic base from all other species within the *U. striiformis* species complex included in this study, except *U. bromina* on *Bromus inermis,* in having a C instead of a T at position 621 in the ITS region, and differs from *U. bromina* by having an A instead of a G at position 223 in the ITS region (Table 2, Fig. 6).

Ustilago corcontica (Bubák) Liro, *Ann. Acad. Sci. Fenn.*, Ser. A **17** (1): 383 (1924).

Basionym: *Tilletia corcontica* Bubák, *Houby Ceské*, *Hemibasidii* **2**: 47 (1912).

Type: **Czech Republic**: on the crest of Riesengebirge Mts, on *Calamagrostis halleriana* (i.e. *C. villosa*), 20 July 1872, *J. Gerhardt* (BPI 172761 – ***lectotype designated here***, MBT 380631; one of the "isolectotypes" of Lindeberg, *Symb. Bot. Upsal.* **16**(2): 114, 1959).

Confirmed host: *Calamagrostis villosa*.

Confirmed distribution: Czech Republic and Germany.

Notes: Within the *U. striiformis* species complex, *U. corcontica* can be distinguished from other species based on the host-specific occurrence on *Calamagrostis villosa*. Furthermore, *U. corcontica* differs in one diagnostic bases from all other species within the *U. striiformis* species complex included in this study – in the *atp*2 gene there is an T instead of a C at position 535 (Table 2, Fig. 6).


A
C
E
B
10 µm
D
10 µm
F
10 µm
G
I
K
H
10 µm
J
10 µm
L
10 µm

Ustilago denotarisii A. A. Fischer v. Waldheim, *Aperçu Syst. Ustil.*: 22 (1877); as "de Notarisii".

Type: **Italy**: on *Arrhenatherum* spp. (not located but could also not be confirmed as lost; a neotype may need to be designated for this species in the future).

Confirmed hosts: *Arrhenatherum* species.

Confirmed distribution: Germany and Italy.

Notes: Spores globose to ovoid, standard range (9.0–)10.5–(av. *11.2)*–12.0 (–13.5) × (8.0–) 9.0–(av. *9.7*)–10.5(–12.0) µm, length/breadth ratio of 1.10- (av. *1.20)* -1.38, olive-brown, and finely echinulate. Within the *U. striiformis* species complex, *U. denotarisii* can be distinguished from other species based on the host-specific occurrence on *Arrhenatherum* species. Furthermore, *U. denotarisii* differs in two diagnostic bases from all other species within the *U. striiformis* species complex included in this study – in the *atp*2 gene there is an A instead of a G at position 346, and in the gene *ssc*1 there is an A instead of a C at position 182 (Table 2, Fig. 6).

Ustilago echinata J. Schröt., *Abh. Schles. Ges. Vaterl. Kult., Abth. Naturwiss.*: 48: 4 (1870 ["1869"].

Type: **Poland**: *Silesia:* 'Schwarzwasserbruch', near Legnica, on *Phalaris arundinacea*, June 1869, *W. G. Schneider* [Rabenhorst, Fungi Eur. no. 1497] (FR – ***lectotype designated here*****,** MBT 380632; one of the several duplicate collections previously treated as "lectotype" in Rabenhorst, Fungi Eur. No. 1497).

Reported hosts: *Glyceria grandis, Phalaris arundinacea*, and *Scolochloa festucacea*.

Confirmed host: *Phalaris arundinacea.*

Known distribution: Asia, North America, and Europe.

Notes: This species shares one sequence motif (AACCCAAC) at positions 20-27 in the ITS region with other coarsely ornamented stripe smuts (*U. serpens* clade in Fig. 1), and many SNPs which distinguish *U. echinata* from species of the *U. striiformis*-complex. Within the *U. serpens*-complex, *U. echinata* can be distinguished from other species based on its host-specific occurrence on *Phalaris arundinacea* (type host). Whether the other hosts of a similar ecotype are infected by the same species could not be clarified in the current study, but the high degree of host specificity observed in *Ustilago* renders it possible that specimens from other host genera will have to be described as new species. Furthermore, *U. echinata* differs in eight diagnostic bases from all other species within the *U. serpens* species complex included in this study – in the *atp*2 gene there is a G instead of an A at position 85, in the *map* gene there is an A instead of a G at position 208, in the *myosin* gene there is a C instead of an A at position 141 and a T instead of a C at position 156, in the *rpl*3 gene there is a T instead of a C at position 91 and an A instead of a G at position 146, in the *sdh*1 gene there is an A instead of a G and at positions 58 and 256, and in the ITS locus there is a C instead of an A at position 19, a C instead of a T at position 38, an A instead of a gap at position 186 and 596 and a G instead of an A at positions 188 and 604 (Tab. 2, Fig. 6).

Due to the generally narrow host specificity of smut fungi, it is conceivable that *U. echinata* will be revealed to be a species group.

Ustilago jagei J. Kruse & Thines, **sp. nov.**
MycoBank MB 819627
(Fig. 5A–B)

Etymology: Named after mycologist Horst Jage from Kemberg (Germany), who has made significant contributions to the knowledge of phytopathogenic fungi and has enabled well-sampled phylogenetic investigations in various plant pathogens by his outstanding collections.

Diagnosis: Within the *U. striiformis* species complex, *U. jagei* can be distinguished from other species based on its host-specific occurrence on *Agrostis stolonifera s. lat*. Furthermore, *U. jagei* differs in two diagnostic bases from all other species within the *U. striiformis* species complex included in this study – in the *atp*2 gene there is an A instead of a G at position 466 and in the gene *rpl*3 there is an A instead of a G at position 92 (Table 2, Fig. 6).

Type: **Germany**: *Saxony-Anhalt*: Dessau, Kühnauer Sea, southern shore east-southeast of Großkuhnau, wayside, on *Agrostis stolonifera*, 16 Sept. 2001, *H. Jage* (GLM-F047379 – holotype).

Description: *Sori* as long narrow streaks parallel to vascular bundles, mostly in the leaves, rarely ascending into the inflorescence, initially covered by the epidermis of the plants, which soon frays. *Spore mass* dark brown to almost black, powdery. Infection systemic, infected plants usually sterile. *Spores* globose to ovoid, (9.5–) 10.0–(av. *10.9)* –11.5(–13.5) x (7.5–) 8.5–(av. *9.3*)–10.0(–11.5) µm, length/breadth ratio 1.04-(av. *1.24)*-1.5, olive-brown, finely echinulate (Table 3, Figs 3–4).

Confirmed hosts: *Agrostis rupestris* and *A. stolonifera*.

Fig. 5. Sori and spores of *Ustilago jagei* (A–B), *U. denotarisii* (C–D), *U. neocopinata* (E–F), *U. salweyi* (G–H), *U. kummeri* (I–J), and *U. serpens s. str.* (K–L). **A.** Sori of *U. jagei* on *Agrostis stolonifera* (GLM-F047379); **B.** Teliospores seen by LM; **C.** Sori of *U. denotarisii* on *Arrhenatherum elatius* (GLM-F105836); **D.** Teliospores seen by LM; **E.** Sori of *U. neocopinata* on *Dactylis glomerata* (GLM-F107413); **F.** Teliospores seen by LM; **G.** Sori of *U. salweyi* on *Holcus lanatus* (GLM-F107417); **H.** Teliospores seen by LM; **I.** Sori of *U. kummeri* on *Bromus inermis* (GLM- F107435); **J.** Teliospores seen by LM; **K.** Sori of *U. serpens s. str.* on *Elymus repens* (GLM-F105827); and **L.** Teliospores seen by LM.

Table 2. Diagnostic bases within the *Ustilago striiformis* and the *Ustilago serpens* complexes.

	Gen Loci																	
	atp2		***map***		***ssc1***		***myosin***		***rpl4A***		***rpl3***		***sdh1***		***tif2***		**ITS**	
***U. striiformis*-complex**	**Pos.**	**Base**	**Pos.**	**Base**	**Pos.**	**Base**	**Pos.**	**Base**	**Pos.**	**Base**	**Pos.**	**Base**	**Pos.**	**Base**	**Pos.**	**Base**	**Pos.**	**Base**
on *Agrostis gigantea*	x	x	x	x	x	x	x	x	x	x	x	x	138	C / T	x	x	x	x
on *Agrostis stolonifera* and *A. rupestris*	466	A / G	x	x	x	x	x	x	x	x	92	A / G	x	x	x	x	x	x
on *Alopecurus pratensis*	358	A / G	192	G / T	x	x	83	T / C	x	x	x	x	x	x	x	x	x	x
on *Arrhenatherum elatius*	346	A / G	x	x	182	A / C	x	x	x	x	x	x	x	x	x	x	x	x
on *Bromus inermis*	191, 244	G / A	x	x	232	C / T	x	x	228, 311 292	A / G C / T	x	x	x	x	23	A / G	621	C / T
on *Calamagrostis epigejos*	91	A / G	x	x	x	x	x	x	x	x	x	x	x	x	65	T / C	102	T / C
on *Calamagrostis villosa*	535	T / C	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
on *Dactylis glomerata*	x	x	x	x	69, 198	A / G	x	x	120	T / C	40	A / G	x	x	x	x	617	A / G
on *Deschampsia caespitosa*	22, 94	A / G	227	T / C	x	x	133	A / G	x	x	199	T / C	x	x	x	x	576	A / G
on *Festuca* spp. and *Lolium* spp.	x	x	x	x	210, 214, 231 243	A / G T / C	x	x	x	x	x	x	x	x	x	x	x	x
on *Holcus* spp.	x	x	x	x	x	x	x	x	85	T / C	133	T / C	x	x	x	x	103	A / G
on *Milium effusum*	301	A / G	x	x	x	x	x	x	x	x	x	x	x	x	x	x	206	T / C
***U. serpens*-complex**	**Pos.**	**Base**	**Pos.**	**Base**	**Pos.**	**Base**	**Pos.**	**Base**	**Pos.**	**Base**	**Pos.**	**Base**	**Pos.**	**Base**	**Pos.**	**Base**	**Pos.**	**Base**
on *Bromus inermis*	x	x	X	x	x	x	x	x	x	x	x	x	x	x	x	x	260 629	C / T G / A
on *Calamagrostis epigejos*	175 181, 429, 496 352	G / A T / C A / G	92 203	A / C C / T	88, 99 93 255	C / G G / A G / T	70 225	A / G T / C	7 91 187	C / G T / C G / T	28	G / T	13 25 100	G / A G / T A / G	x	x	215 522 597 606	C / T T / - A / T A / G
on *Elymus repens*	x	x	x	x	x	x	93	A / G	232	T / C	x	x	x	x	x	x	157 170	A / G C / T
on *Phalaris arundinacea*	85	G / A	208	A / G	x	x	141 156	C / A T / C	x	x	91 146	T / C A / G	58, 256	A / G	x	x	19 38 186, 596 188, 604	C / A C / T A / - G / A

Slash (/) = instead of, x = no diagnostic bases.

atp2 – 595bp

CTTVGAGTCVAGRGGGTCBA C**R**GCRGGGTARATACCRAGC TCGGCRATACCACGVGAHAR BACRGTGGTGGCGTCCAAGT GKGC**R**AAGGT**R**GT**R**GCGGGR GCAGGRTCMGTCAAGTCATC GGCGGGCACRTARACGGCCT GBACSGARGTRATGGARCCC
TTCTTGGTGGTRGT**R**ATKCG **Y**TCCTGCATG**R**SACCCATGT CRGTRGCDAGGGTRGGCTGR TAWCCKACRGCBGARGGRAT ACG**R**CCRAGMARRGCSGAGG TCTCGGAACCRGCYTGGGTG AARCGGAAAATRTTGTCRAT **R**AARAGAAGCACRTCCTGDC
CCTCYTCGTCACGGAAGTAC TCRGC**R**ATGGT**R**AKACC**V**GT VAGRGCRACRCGGGCRCGGG CWCCRGGRGGCTCGTTCATC TGACCGAAVACRAGVGCKAC CTTGGAKT**Y**RCCBTYVAGGT TGATRACACCRGTCTCRATC ATYTC**R**TGGTARAGATCRTT
RCCCTCACGRGTDCG**Y**TCAC CGACACCRGTGAARACVGAG TAACCACCGTRRGC**Y**TTRGC RACGTTGTTRATSAGYTCYT GRATRAGYACVGTCTTDCCG ACMCCRGCRCCACCR

ITS 643bp

HCGTYGCGTCSRGCTYHN**N**V MYYNNNYBHYHNHBNYC**Y**CC GAAGYYYYGAYANNKTATCA AAACCCGGCAGGNWVGMNNN NNNNVRGVRNNVRARANNNN W**BR**AGCTTTCGTCCGTCNTY KCNNCYDTHVAANNNNNNNR CTMATSYWTTTCRAGR**R**AGC
CAYGRYDMAYGGCNN**N**N**N**NN NNNNNNNVVMYCCTCAVTAC CRAKC**Y**RYCVDCYY**Y**YHNNN NNNNNNNNNNNNNNNNNNN NRARRRRNNNNBBKNCGY**Y** CGAAACDATTCGCGGCCCTC AAACMGGCATGCTCCCCAGA TTARATCTGCMGGGAGCGCA
AGGTGCGTTCAAAGATTCGA TGATTCRCTTCTGCAATTCA CATTACTTATCGCAATTCGC TGCGTTCTTCATCGAKGGGA RAACCAAGAGATCCGTTGCC AAAAGTTVNTTTKNNNNNWW TTAGACGACCGYATTACCAG YCGNNNNNNNNNNNNNNNAW
RAYNNNNNNNNNNNNTYHHA ATCCTANNTSWTCNAAAARK K**N**KTNNNNDDDDDNNNNNNN BRYMNRBYNNBBYBBHHNNN NNNNNNNNNNNNNNN**NN**NNN NNNNNNNNNNNNNNN**NN**NNN NNN**N**N**N**NNNNNNNNNN**N**CDB **B**DNYBNNY**R**NRNCYRMCCSR
TGC

ssc1 264bp

CCRTCVGCRGCRGTVGARAA SACYTGCGACTTCTTGGTDG GGATVGTGGTRTTRCGGTTR ATVAGRCG**V**GTGAAVACACC RCCVAG**N**GTCTG**R**ATACC**S**A GVGARAGVGGVGTRACRTCG AGSARVARAAYRTCGGTRAC CTGRCCGGAVAGVACACCAC
CCTGRATSGARGCRCCRATR G**M**VACRGCCTCRTCKGG**R**TT RACRCCCTT**R**CTN**R**GGTCRC GCTTGAARAT**RY**YCTTGACV GT**Y**TCRAGVACCTT**K**GGCAT DCGG

map 251bp

TYCGHKCCGARATCAARGCN CACMTCAARAATGTBCAGCA RGAYACBGGCAARCTYGCCA ACTCGGTYGMMAAGGAGCGY GARATGTCBAC**M**AARWYGAT YACCGARCTYGCYCGHTCCA TCACYYTSCTCAARAAYACB CCYATGAGYGTCACBGCBCG
HGARGAYCCYTACGCYGCCA AYCARBMSGTC**K**WYCGYCAR CT**Y**CAGC**R**CCAGGTCAAYGA GGARAA**Y**GCYCTBCARAART CSATYATCATC

myosin 257bp

CTCTTRTTCTGRTTDGGCTT GATKGTDCKRATRTAVGAHG GCTGNGCNYKCATVARVTTY TCNACHARV**R**CRTTKGCRCT NG**H**YTTKATACG**R**TCRCCVG CRGTRGGVGGVCKCTTYTTR YTRTYRGGRTYN**R**GDCGDTC **N**GGRAARAGYTTYTG**V**ARRT
ASRAGTTDKBRCTYGARTCR ACVARRTCVARRATRTCYTT RAGVAGYGMRTCYTTRTTBT TRTY**B**GTCATDCCYTSRACR TTGTACATGACRTCRCC

rpl4A 415bp

SCTNKM**N**GARRTHCCBYTSG TYRTBKCBGAYSMBGCYGAG RRYYTSACSAARACCAAGGA GGCYRTYRCBCTBCTCAAGK CBVT**Y**AAYGC**Y**TWCRMSGAY GTYRYYAAGGTSTCSAACT**Y** GCGCAAGRTYCGYGCNGGYG TKGGYAAGMTSCGCAACCGY
CGCCAYACSCAGCGHCGYGG TCCYCT**K**GTBATYTACAACM AGGAYGCCGGYATYGTYAAG GCBTTCC**R**YAA**H**GTBCCYGG YGTKGAGCTSTGCTCNGYYG ASCGYCTSAACMTSCTCCAG CTYGCYCCYGG**H**GGACAYMT BGGYCGHTTY**R**TCATYTTCA
CBSAGTCYGCYTTYGGCCGC CTCGACRAGGTSTTYGCYGC CAAGWMBRGYTTCAYBCTNC CYAAGGCYAAGATYGCYAAC ACBGAYGTBACSCGC

rpl3 218bp

CANYGGYGCYTSSATCTSBC GHGAGCT**B**GAGCGCATCAA**R** AAGTACTGCACYGTBGTBCG TSTBCTYGCYCACACYCAGR TBCGCAAGAC**BR**GYCTSAAG CAGAAGAAGKCRCAYCTBAT GGAGRTBCARAT**Y**AACGGWG GYTCB**R**TYGYYGAVAARGTC
GACTTYGCSAAGGAGCACTT YGAGAAGACBTTYGMBGT**Y**A AGTCVRTBTTYGAGSASA

sdh1 269bp

RTCYTGVAGRGG**R**AGVCCRG CWCG**B**GARACCATRGCCATR CCRTCACCVGTGCAVGTRTG VGCVGARGTGGCVGAGAAGT ARGCACGDCCRTADCCRCC**D** GTGGCVAGVACRGTCTT**R**TG RGCRCGGAADCGGTGRAYKG TRCCRTCCTCCATRTTRAGD
GCRGYVACACCGACRCAYTC GCCRTCYTCCATGATVARGT CRAGVGCRAARTACTCGATR AARAAGTTKGTGTTRTGKCG RAGCGACTGDCCRTAVAGSG TGTGMAGCA

tif2 244bp

TGTCGTARACACGRCCRGGV GT**R**CCRACRACGACCTGRGC RCCBTCRTTGAGCTTRGCCA TGTC**Y**TCRCGRACGTTRGTR CCRCCRATGCARGCGTGGCA GTYRAYCTTCATGTAGTCRC CRAGNGCRATRACGACCTTY TGRATCTGCTGRGCVAGCTC
RCGRGTRGGVGCRAGGATVA GVGCYTGRACVGCCTKGATS YYRGGGTCRATDCGCTGVAR RATGGCRATSGAGAAGGTRG CBGT

Fig. 6. Alignment consensus sequences for the alignments used in this study with positions of diagnostic bases highlighted in bold face.

Confirmed distribution: Germany and Switzerland.

Notes: It seems possible that *U. jagei* on *Agrostis stolonifera s. lat*. represents a species complex, and further investigations with more specimens and additional gene loci are needed to clarify this situation.

Ustilago kummeri J. Kruse & Thines, **sp. nov.**
MycoBank MB 819628
(Fig. 5I–J)

Etymology: Named after the mycologist Volker Kummer from Potsdam (Germany), who has made significant contributions to the knowledge of phytopathogenic fungi and has enabled well-sampled phylogenetic investigations in various plant pathogens by his outstanding ability to recognise easily overlooked plant pathogens.

Diagnosis: Differs from species of the *U. striiformis* species complex in the larger spores and taller warts. Furthermore, *U. kummeri* shares one sequence motif at positions 20-27 (AACCCAAC) with other coarsely ornamented stripe smuts, and many SNPs distinguishing it from species of the *U. striiformis* species complex. Within the *U. serpens*-complex, *U. kummeri* can be distinguished from other species based on the host-specific occurrence on *Bromus inermis*. Furthermore, *U. kummeri* differs in two diagnostic bases from *U. serpens* on *Elymus repens* – in the ITS region there is an C instead of a G at position 260 and G instead of an A at position 629 (Table 2, Fig. 6).

Type: **Germany**: *Brandenburg*: Middlemark, Uetz: Hinterer Werder, southwest corner between Sacrow-Paretzer-Channel und Havel-Channel, on *Bromus inermis*, 19 June 2010, *V. Kummer* (GLM-F107435 – holotype; VK 2577/17 – isotype).

Description: *Sori* as long, narrow streaks parallel to vascular bundles, mostly in the leaves, rarely ascending to the inflorescence, initially covered by the epidermis of the plants, which soon frays. *Spore mass* dark brown, powdery. Infection systemic, infected plants mostly sterile. *Spores* ovoid to globose, (11.0-) 12.0- (av. *13.0)* -14.0 (-15.5) × (9.0-) 10.5- (av. *11.5)* -12.0 (-13.5), length/breadth ratio 1.04- (av. *1.15*) -1.41, olive-brown, coarsely verrucose to echinulate (Table 3, Figs 5–6).

Confirmed host: *Bromus inermis*.

Confirmed distribution: Germany.

Notes: It seems likely that additional species will be discovered in the *U. serpens* clade once more stripe-smuts with coarse spore ornamentation will be scrutinised.

Ustilago loliicola Ciferri, *Fl. Ital. Crypt.*, *Par. I. Fungi, Fasc.* **17**: 345 (1938).

Type: **Germany**: *Berlin*: Berlin-Weissensee, on *Lolium perenne,* Sept. 1877, *E. Ule* [Rabenhorst, Fungi Eur. no. 2491] (FR – ***lectotype designated here***, MBT 380633; from one of the several duplicate collections treated as “lectotype” by Lindeberg, *Symb. Bot. Upsal.* **16** (2): 136, 1959).

Confirmed hosts: *Festuca arundinacea s. lat*. and *Lolium perenne*.

Confirmed distribution: Germany.

Notes: Within the *U. striiformis* species complex, *U. loliicola* can be distinguished from other species based on the specific occurrence on the closely related hosts *Festuca arundinacea s. lat.* and *Lolium perenne*. Furthermore, *U. loliicola* differs in four diagnostic bases from all other species within the *U. striiformis* species complex included in this study – in the *ssc*1 locus there is an A instead of a G at positions 210, 214 and 231, and a T instead of a C at position 243 (Table 2, Fig. 6).

Ustilago milii (Fuckel) Liro, *Ann. Acad. Sci. Fenn.*, ser. A **17** (1): 78 (1924).
Basionym: *Tilletia milii* Fuckel, *Jb. nassau. Ver. Naturk.* **23-24**: 40 (1870).

Type: **Germany**: *Hesse*: Rabenkopf Mt., near Oestrich, on *Milium effusum*, *L. Fuckel* [Fungi Rhenani no. 2410] (FR – ***lectotype designated here,*** MBT 380634, from one of the several duplicate collections treated as “lectotype” in Fuckel, Fungi Rhenani no. 2410).

Confirmed host: *Milium effusum*.

Confirmed distribution: Germany.

Notes: Within the *U. striiformis* species complex, *U. milii* can be distinguished from other species based on the host-specific occurrence on *Milium effusum*. Furthermore, *U. milii* differs in two diagnostic bases from all other species within the *U. striiformis* species complex included in this study – in the *atp*2 gene there is an A instead of a G at position 301, and in the ITS there is a T instead of a C at position 206 (Table 2, Fig. 6).

Ustilago neocopinata J. Kruse & Thines, **sp. nov.**
MycoBank MB819630
(Fig. 5E–F)

Etymology: Highlights the unexpected finding that there are several distinct and host-specific species within the *U. striiformis* species complex.

Diagnosis: Within the *U. striiformis* species complex, *U. neocopinata* can be distinguished from other species based on the host-specific occurrence on *Dactylis glomerata*. Furthermore, *U. neocopinata* differs in five diagnostic bases from all other species within the *U. striiformis* species complex included in this study – in the *ssc*1 gene there is an A instead of a G at positions 69 and 198, in the *rpl*4A gene there is a T instead of a C at position 120, in the *rpl*3 gene there is an A instead of a G at position 40, and in the ITS region there is an A instead of a G at position 617 (Table 2, Figs 5–6).

Table 3. Measurements from 100 teliospores for four different species of the *Ustilago striiformis*-complex on *Agrostis stolonifera*, *Dactylis glomerata*, *Arrhenatherum elatius*, and *Holcus lanatus*, as well as two species of the *Ustilago serpens*-complex on *Elymus repens* and *Bromus inermis*.

	***Ustilago striiformis*-complex**												***Ustilago serpens*-complex**					
	***U. jagei* sp. nov.**			***U. denotarisii***			***U. neocopinata* sp. nov.**			***U. salveii***			***U. serpens***			***U. kummeri* sp. nov.**		
	on *Agrostis stolonifera*			**on *Arrhenatherum elatius***			**on *Dactylis glomerata***			**on *Holcus lanatus***			**on *Elymus repens***			**on *Bromus inermis***		
	spores			**spores**			**spores**			**spores**			**spores**			**spores**		
No.	length	width	l/b	length	width	l/b	length	width	l/b	length	width	l/b	length	width	l/b	length	width	l/b
1	10.5	9.5	1.11	10	9	1.11	11	10.5	1.05	11.5	10.5	1.1	11	10.5	1.05	14	13	1.08
2	10	7.5	1.33	11.5	9	1.28	9.5	9.5	1	11.5	10	1.15	12	10	1.2	13.5	11	1.23
3	10.5	8.5	1.24	9	8	1.13	11	10	1.1	10	9.5	1.05	12.5	10	1.25	14.5	12	1.21
4	13.5	9.5	1.42	10	8	1.25	10.5	9.5	1.11	11	9.5	1.16	13	12	1.08	14	12.5	1.12
5	11	9	1.22	10.5	8.5	1.24	10.5	10	1.05	12	9.5	1.26	12.5	10.5	1.19	14	12	1.17
6	11	10	1.1	11.5	9	1.28	11	9.5	1.16	12	9	1.33	13	12.5	1.04	11.5	11.5	1
7	9.5	8	1.19	10.5	9.5	1.11	10	8.5	1.18	11	9	1.22	12.5	11.5	1.09	14	12	1.17
8	11	8	1.38	10.5	9.5	1.11	10.5	10	1.05	10.5	9	1.17	12.5	9.5	1.32	14	13.5	1.04
9	10.5	10	1.05	11.5	10	1.15	10.5	10	1.05	10	10	1	13.5	11	1.23	13	12.5	1.04
10	11.5	9	1.28	11.5	8.5	1.35	10.5	10.5	1	10.5	9.5	1.11	13	11	1.18	13.5	13.5	1
11	11.5	10	1.15	11	8	1.38	11	10	1.1	10.5	9.5	1.11	14.5	13.5	1.07	13.5	11.5	1.17
12	11.5	8	1.44	11	10	1.1	11	11	1	10.5	10	1.05	14.5	12	1.21	12.5	11	1.14
13	12	8	1.5	10.5	9	1.17	12	10.5	1.14	12.5	9	1.39	15.5	11	1.41	13.5	12.5	1.08
14	12	10.5	1.14	12	9	1.33	10	10	1	10	8	1.25	13	12.5	1.04	12	12	1
15	10	8.5	1.18	10.5	9	1.17	10.5	10.5	1	11	10	1.1	12.5	12	1.04	13.5	12.5	1.08
16	12	11.5	1.04	10.5	9.5	1.11	10.5	9.5	1.11	10.5	9.5	1.11	13	12.5	1.04	12	11.5	1.04
17	11	8	1.38	12	9	1.33	10	9	1.11	10	9	1.11	12	11.5	1.04	13.5	13	1.04
18	11	9.5	1.16	12.5	10.5	1.19	10	9.5	1.05	11.5	9.5	1.21	13	10.5	1.24	14.5	13	1.12
19	11	9	1.22	10	9	1.11	10.5	9.5	1.11	10	9	1.11	13	11.5	1.13	13	11.5	1.13
20	12	9.5	1.26	12.5	11	1.14	11	10.5	1.05	10.5	9.5	1.11	13	12	1.08	13.5	13	1.04
21	11	9.5	1.16	12.5	11.5	1.09	10.5	10	1.05	10.5	9	1.17	12.5	11	1.14	14.5	12	1.21
22	13	9.5	1.37	13.5	12	1.13	11.5	10.5	1.1	10	8.5	1.18	12	11.5	1.04	13.5	12.5	1.08
23	12.5	10	1.25	13.5	10	1.35	11	11	1	11.5	9	1.28	13	11.5	1.13	13	12.5	1.04
24	11.5	10	1.15	11.5	10.5	1.1	10	9.5	1.05	10	9.5	1.05	13.5	12	1.13	12.5	12	1.04
25	10.5	8.5	1.24	11.5	9.5	1.21	11	10.5	1.05	11	9.5	1.16	13	10.5	1.24	15	13.5	1.11
26	10.5	10	1.05	12.5	11	1.14	11	10.5	1.05	9.5	9	1.06	12	10.5	1.14	13	11.5	1.13
27	11	9	1.22	12.5	11.5	1.09	11	10	1.1	10.5	9	1.17	12.5	12	1.04	13.5	13.5	1
28	10.5	10.5	1	11	10.5	1.05	10.5	9	1.17	10	9.5	1.05	13	12	1.08	13.5	11.5	1.17
29	11	9.5	1.16	11	11	1	11	11	1	10	9	1.11	14	12.5	1.12	13.5	11.5	1.17
30	10.5	7.5	1.4	11	9.5	1.16	10	10	1	10	9	1.11	12	11	1.09	13	12.5	1.04
31	10.5	9	1.17	11	9	1.22	10	9.5	1.05	10	9	1.11	12.5	11.5	1.09	13	11	1.18
32	10	8.5	1.18	11.5	10.5	1.1	10	9.5	1.05	11	10	1.1	14.5	12.5	1.16	14	13	1.08
33	10.5	9.5	1.11	11	8.5	1.29	10.5	10.5	1	10	9	1.11	13	11.5	1.13	14	13	1.08
34	10.5	9.5	1.11	11.5	9	1.28	10.5	10.5	1	11	8.5	1.29	14	12.5	1.12	14	13	1.08
35	11.5	10	1.15	12.5	9.5	1.32	11	10	1.1	11	10	1.1	12	11.5	1.04	12.5	12	1.04
36	12	9	1.33	10.5	8.5	1.24	10.5	9.5	1.11	10	9	1.11	14.5	11.5	1.26	13	11	1.18
37	11	9.5	1.16	12.5	10.5	1.19	11	10.5	1.05	10	8.5	1.18	12.5	11.5	1.09	15	13.5	1.11
38	10.5	9	1.17	11	9.5	1.16	10.5	10	1.05	10.5	9.5	1.11	14	12	1.17	14	13	1.08
39	9.5	9	1.06	10.5	10	1.05	11	10	1.1	10.5	10.5	1	13	10	1.3	14	13.5	1.04
40	10	8.5	1.18	12.5	10.5	1.19	10.5	9.5	1.11	10	9	1.11	11.5	11	1.05	13	13	1
41	10.5	9.5	1.11	11	9.5	1.16	10	9.5	1.05	10	9	1.11	13.5	10.5	1.29	14.5	12.5	1.16
42	11.5	10.5	1.1	11.5	11	1.05	10	10	1	10.5	10	1.05	12.5	9.5	1.32	13	12	1.08
43	11	10.5	1.05	10	10	1	10.5	9.5	1.11	10.5	9.5	1.11	13.5	11	1.23	13.5	11.5	1.17
44	10	9	1.11	11	9.5	1.16	10.5	10	1.05	10	10	1	14	12	1.17	14	11.5	1.22
45	10.5	8.5	1.24	11.5	10	1.15	10.5	9.5	1.11	12	10	1.2	13.5	10.5	1.29	13	11.5	1.13
46	10.5	8	1.31	11.5	11.5	1	10.5	9.5	1.11	10.5	10	1.05	14	12	1.17	13.5	12	1.13
47	12.5	10.5	1.19	11	10.5	1.05	9.5	8.5	1.12	9.5	9.5	1	12	11.5	1.04	12.5	11.5	1.09
48	11	9.5	1.16	10.5	10	1.05	10	10	1	11.5	10	1.15	13.5	12	1.13	13.5	11.5	1.17
49	11	9.5	1.16	11.5	9.5	1.21	10.5	9.5	1.11	10.5	10	1.05	13	11.5	1.13	13	12	1.08
50	10	9.5	1.05	11.5	9.5	1.21	11	10.5	1.05	10.5	8	1.31	13.5	12	1.13	13	12.5	1.04
51	10	9.5	1.05	10	8.5	1.18	10	9	1.11	11	9	1.22	14	11.5	1.22	12.5	10.5	1.19
52	10.5	8.5	1.24	10.5	8	1.31	10	11	0.91	10.5	10.5	1	13.5	10.5	1.29	14.5	12	1.21

Table 3. (Continued).

	***Ustilago striiformis*-complex**												***Ustilago serpens*-complex**					
	***U. jagei* sp. nov.**			***U. denotarisii***			***U. neocopinata* sp. nov.**			***U. salweyi***			***U. serpens***			***U. kummeri* sp. nov.**		
	on *Agrostis stolonifera*			**on *Arrhenatherum elatius***			**on *Dactylis glomerata***			**on *Holcus lanatus***			**on *Elymus repens***			**on *Bromus inermis***		
	spores			**spores**			**spores**			**spores**			**spores**			**spores**		
No.	length	width	l/b	length	width	l/b	length	width	l/b	length	width	l/b	length	width	l/b	length	width	l/b
53	12	9.5	1.26	10	8.5	1.18	9	9	1	11.5	10	1.15	14	11.5	1.22	14	12	1.17
54	10.5	10.5	1	11	8.5	1.29	10	9.5	1.05	10.5	9	1.17	13	12	1.08	13.5	12	1.13
55	10.5	10.5	1	12	9.5	1.26	10	9.5	1.05	10	10	1	12	11	1.09	14	12	1.17
56	11	10	1.1	9.5	9	1.06	9.5	9.5	1	10.5	10.5	1	12	12	1	13	13	1
57	10.5	9	1.17	10	8.5	1.18	11.5	10.5	1.1	11	9.5	1.16	13	10.5	1.24	14	13	1.08
58	10	10	1	11.5	9.5	1.21	10	9	1.11	10.5	10	1.05	14.5	10.5	1.38	13	12.5	1.04
59	11	10	1.1	11	10	1.1	10	7.5	1.33	10.5	9.5	1.11	13	11.5	1.13	13.5	12	1.13
60	10.5	10.5	1	12	9.5	1.26	10	10	1	10	9.5	1.05	13	12	1.08	14.5	12	1.21
61	10.5	8.5	1.24	11	10	1.1	10.5	10	1.05	10.5	9.5	1.11	13.5	10	1.35	14.5	13	1.12
62	11.5	9	1.28	10.5	10	1.05	11	9	1.22	10.5	10	1.05	13	11.5	1.13	14.5	13	1.12
63	10.5	8.5	1.24	10.5	9.5	1.11	10.5	9.5	1.11	9.5	9.5	1	12.5	12	1.04	13.5	12	1.13
64	10.5	9.5	1.11	10.5	10	1.05	11	10.5	1.05	10	9.5	1.05	12.5	12	1.04	14	12.5	1.12
65	10	10	1	10	8.5	1.18	10.5	10.5	1	11	9	1.22	14.5	10.5	1.38	13	12.5	1.04
66	10.5	8.5	1.24	11.5	11	1.05	11	11	1	10.5	8.5	1.24	11.5	10.5	1.1	13	12.5	1.04
67	11	10.5	1.05	11	9.5	1.16	11.5	10	1.15	11	9.5	1.16	15	12	1.25	13.5	12.5	1.08
68	10.5	8.5	1.24	11	9.5	1.16	11	11	1	10.5	9.5	1.11	12.5	11	1.14	14	13	1.08
69	10	10	1	11.5	10.5	1.1	11.5	11	1.05	10	9	1.11	14	11	1.27	14.5	14	1.04
70	10	10	1	11.5	11	1.05	10	9.5	1.05	11	10	1.1	12	11	1.09	13.5	12.5	1.08
71	11	9	1.22	11	10	1.1	9	9	1	10.5	9	1.17	13	10.5	1.24	13	12.5	1.04
72	10	10	1	10.5	10	1.05	10	9.5	1.05	11	10.5	1.05	13	12	1.08	13.5	12.5	1.08
73	10	10	1	13	10	1.3	11	10	1.1	9.5	9	1.06	11.5	11.5	1	13.5	13	1.04
74	10.5	8	1.31	11	9	1.22	10.5	9.5	1.11	11	9.5	1.16	14	11	1.27	13.5	13	1.04
75	10	9.5	1.05	11.5	10	1.15	10.5	9.5	1.11	10.5	10.5	1	12	10.5	1.14	15.5	13.5	1.15
76	11.5	9	1.28	12	10.5	1.14	11	9.5	1.16	10.5	10	1.05	11.5	9	1.28	13	12.5	1.04
77	11	10	1.1	10.5	10	1.05	9	9	1	11.5	9.5	1.21	12.5	11	1.14	14	12.5	1.12
78	11.5	9.5	1.21	10.5	10	1.05	9.5	9.5	1	9.5	8.5	1.12	11	10.5	1.05	14.5	12	1.21
79	11	9	1.22	11.5	8.5	1.35	11	10	1.1	10	9	1.11	13	11	1.18	13.5	12	1.13
80	11.5	9.5	1.21	10.5	9.5	1.11	10	8.5	1.18	11	8.5	1.29	11.5	11	1.05	13.5	12.5	1.08
81	9.5	9.5	1	11	9.5	1.16	10.5	10	1.05	11.5	9	1.28	11.5	11	1.05	13.5	10.5	1.29
82	11	9.5	1.16	10.5	10	1.05	11	9.5	1.16	10.5	10	1.05	12.5	11	1.14	13	13	1
83	10.5	10.5	1	11.5	9	1.28	11.5	9.5	1.21	11	9.5	1.16	12.5	12	1.04	14.5	13	1.12
84	11.5	10	1.15	11	9	1.22	10	9.5	1.05	10	9.5	1.05	12	9.5	1.26	13.5	12.5	1.08
85	11	10	1.1	10	10	1	11	9.5	1.16	10	8	1.25	13.5	11	1.23	14	13	1.08
86	11	9	1.22	10.5	9.5	1.11	11.5	11	1.05	9.5	7.5	1.27	14	9.5	1.47	14	12	1.17
87	11	10.5	1.05	10.5	9	1.17	11.5	10.5	1.1	11	8.5	1.29	12.5	12	1.04	15	13.5	1.11
88	11	9	1.22	11	9.5	1.16	10	9	1.11	10	9	1.11	12	12	1	12.5	12.5	1
89	10	7.5	1.33	11.5	8.5	1.35	9.5	9	1.06	10	9.5	1.05	15	12.5	1.2	13.5	11.5	1.17
90	11	9.5	1.16	10	9.5	1.05	11	9.5	1.16	11	10	1.1	14.5	12	1.21	12	12	1
91	10.5	9	1.17	13.5	11	1.23	11	9.5	1.16	11	10.5	1.05	12	11.5	1.04	13.5	13	1.04
92	10.5	9	1.17	13.5	10.5	1.29	9.5	9.5	1	11.5	10	1.15	14	11	1.27	13	12	1.08
93	10	8.5	1.18	13	10.5	1.24	9.5	7.5	1.27	11.5	9.5	1.21	13.5	11	1.23	14	12.5	1.12
94	11	9.5	1.16	11.5	10.5	1.1	11	11	1	10.5	9.5	1.11	13	11	1.18	13.5	11.5	1.17
95	10.5	9	1.17	11	11	1	11	10	1.1	10.5	10	1.05	14.5	12	1.21	14.5	12	1.21
96	10.5	7.5	1.4	12	10.5	1.14	10.5	8.5	1.24	10.5	9.5	1.11	12.5	10.5	1.19	14.5	13	1.12
97	11.5	9.5	1.21	11	11	1	10.5	10	1.05	10.5	9.5	1.11	12.5	11.5	1.09	13	11	1.18
98	11.5	9.5	1.21	11	9	1.22	13	11	1.18	11	10	1.1	11.5	11	1.05	13.5	11.5	1.17
99	13.5	11.5	1.17	10.5	10	1.05	10	9	1.11	10.5	9.5	1.11	12.5	11.5	1.09	14.5	13.5	1.07
100	10.5	9.5	1.11	11	10	1.1	10	10	1	11	9.5	1.16	13.5	12.5	1.08	13.5	11.5	1.17

Type: **Germany**: *Bavaria*: Upper Franconia, Kronach county, Wallenfels, in the direction of the sewage treatment plant downstream of Stumpfenschneidmühle, on *Dactylis glomerata*, 15 July 2012, *J. Kruse* (GLM-F107413 – holotype).

Description: *Sori* as long small streaks parallel to vascular bundles, mostly in the leaves, very rarely ascending to the inflorescence, initially covered by the epidermis of the plants, which soon frays. *Spore mass* dark brown to almost black, powdery. Infection systemic, infected plants mostly sterile. *Spores* mostly globose, rarely ovoid, (9.0–) 10.0– (av. *10.5*)–11.0 (–13.0) × (7.5–) 9.0– (av. *9.8*) –10.5 (–11) µm, length/breadth ratio 1.00- (av. *1.07*) -1.18, olive-brown, finely echinulate (Table 3, Figs 5–6).

Notes: As the host is widespread throughout the Holarctic region, it is conceivable that the species will prove to have a much wider distribution range than currently known.

Ustilago salweyi Berk. & Broome, *Ann. Mag. Nat. Hist.* **5**: 463 (1850).
(Fig. 5G–H)

Type: **UK**: *Channel Islands:* Guernsey, St Martin's, on *Holcus lanatus* [originally misidentified as *Dactylis glomerata fide* Hubbard, *in* Stevenson, *Plant Dis. Rep.* **30**: 57, 1946], 1847, *T. Salwey* (K-M – holotype; K-M00022071 – isotype).

Synonyms: *Uredo striiformis* Westend., *Bull. Acad. R. Sci. Belg., cl. sci.* **18**: 406 (1852); as "*striaeformis"*.
Uredo salveii (Berk. & Broome) Oudem.*, Prodromus Florae Bataviae*, 2nd edn,**4**: 180 (1866).
Tilletia debaryana A.A. Fisch. Waldh., *in* Rabenhorst, *Fungi eur.* no. 1097 (1867).
Tilletia striiformis (Westend.) Magnus, *Malpighia* **1**: 8 (1875).
Ustilago striiformis (Westend.) Niessl, *Hedwigia* **15**: 1 (1876).
Tilletia salveii (Berk. & Broome) P. Karst., *Bidrag. Kännedom. Finlands Naurt. Folk.* **6**: 102 (1884).

Confirmed hosts: *Holcus lanatus* and *H. mollis*.

Confirmed distribution: Belgium, Germany, and UK.

Notes: Spores globose to ovoid, standard range (9.5–)10.0–(av. *10.6*) –11.0 (–12.5) × (7.5–) 9.0–(av. *9.4*)–10.0(–10.5) µm, finely echinulate, length/breadth ratio 1.00–(av. *1.15*)–1.39. Within the *U. striiformis* species complex, *U. salweyi* can be distinguished from other species based on the host-specific occurrence on *Holcus lanatus* and *H. mollis*. Furthermore, *U. salweyi* differs in three diagnostic bases from all other species within the *striiformis* species complex included in this study – in the *rpl*4A gene there is a T instead of a C at position 85, in *rpl*3 there is a T instead of a C at position 133, and in the ITS region there is an A instead of a G at positions 103 (Table 2, Fig. 6).

The original host was misidentified as *Dactylis glomerata*, but this was found to be incorrect and actually *Holcus lanatus* by the leading grass specialist C.E. Hubbard (in Stevenson 1946). David Hawksworth also studied the type materials in K-M and concurs. Hosts in their vegetative stage can be misidentified, as some characteristics, such as leaf shape, ligula, and general habit can be modified as a consequence of infection.

Ustilago scaura Liro **s. lat.** , *Ann. Acad. Sci. Fenn., ser.* A, **17**(1): 73 (1924).

Replaced name: Tilletia avenae Ule, *Verh. Bot. Vereins Prov. Brandenburg* **25**: 214 (1884).

Type: **Germany**: *Bavaria*: Coburg, Fortress, on *Avena pratensis* (i.e. *Helictotrichon pratense*), June 1879, *E. Ule* (s. n. – lost); *Hesse*: county Tann/Rhön, at Galgenmount, on *Avena pubescens* [now, *Helictotrichon pubescens*], 16 Sept. 1990, *H. Scholz* (B 70 0014830 ***– neotype designated here***, MBT 380637).

Non *Ustilago avenae* (Pers.) Rostrup, *Overs. K. danske Vidensk. Selsk. Forh. Medlemmers Arbeider*: 13 (1890).

Confirmed host: *Helictotrichon pubescens, H. pratense?*

Confirmed distribution: Germany.

Notes: Within the *U. striiformis* species complex, *U. scaura s. lat.* can be distinguished from other species based on the host-specific occurrence on *Helictotrichon pratense and H. pubescens*. Furthermore, *U. scaura s. lat*. differs in one diagnostic base from all other species within the *U. salweyi* species complex included in this study, except *U. denotarisii* on *Arrhenatherum* spp.*,* in having a T instead of a C at position 628 in the ITS region, and from *U. denotarisii* on *Arrhenatherum elatius* in having a 13 nucleotide deletion at positions 222-241 in the ITS alignment (Table2, Fig. 6).

Since the type has been lost, we designate a neotype for *Ustilago scaura* with material on the closely related *H. pubescens*.

Ustilago scrobiculata Liro, *Ann. Acad. Sci. Fenn.*, *ser*. A **17**(1): 68 (1924).

Type: **Finland**: *Nyland*: Pornainen, Kirveskoski, on *Calamagrostis arundinacea,* 9 Aug. 1916, *T. Putkonen & J. I. Liro* (H – lectotype, designated by Lindeberg, *Symb. Bot. Upsal.* **16** (2): 130 (1959).

Synonym: ? *Ustilago deyeuxiae* L. Guo, *Mycosystema* **6**: 51 (1993).

Reported hosts: *Calamagrostis* spp. (see Vánky 2012: 1265).

Reported distribution: Asia and Europe.

Notes: This species shares one sequence motif with other coarsely ornamented stripe smuts (AACCCAAC at positions 20-27), which distinguishes it from species of the *Ustilago striiformis* species complex, and many additional single SNPs. Within the *U. serpens* species complex, *U. scrobiculata* differs in 21 diagnostic bases from other species (Table 2, Fig. 6). It seems possible that *U. deyeuxiae* has not been sampled

on *Calamagrostis arundinacea*, as the host of *U. deyeuxiae* is given as "*Deyeuxia arundinacea*" by Guo (1993), which is often seen as a synonym of *D. pyramidalis* in Asian literature (e.g. Shenglian *et al.* 2006). Thus, it seems possible that the species needs to be reconsidered as independent from *U. scrobiculata* once sequence data from the type specimen become available.

Ustilago serpens (P. Karst.) B. Lindeb., *Symb. Bot. Upsal.* **16**(2): 133 (1959).

Basionym: *Tilletia serpens* P. Karst., *Fungi Fenn. Exs.*, *fasc.* **6** : no. 599 (1866).

Type: **Finland**: Merimasku, on "*Dactylis glomerata*" [re-determined as *Elymus repens* by Lindeberg, *Symb. Bot. Upsal.* **16**(2): 133, 1959], July 1862, *P. Karsten* [Fungi Fenn. Exs no. 599] (HUV 10432 ***– lectotype designated here;*** MBT 380638 from one of the several duplicate collections treated as "lectotype" by Lindeberg, *Symb. Bot. Upsal.* **16**(2): 133, 1959).

Confirmed host: *Elymus repens.*

Confirmed distribution: Finland and Germany.

Notes: The spores are small to medium sized, (11.5–) 13.0–(av. *13.5*) –14.5 (–15.5) × (10.5–) 11.5 (av. *12.5*) –13.0 (–14.0) µm, with a length/breadth ratio of 1.00–(av. 1.09)–1.23 and with coarsely verrucose ornamentation. This species shares one sequence motif with other coarsely ornamented stripe smuts (AACCCAAC at position 20-27), which distinguishes it from species of the *U. striiformis* species complex and many additional SNPs. Within the *U. serpens*-complex, *U. serpens* can be distinguished from other species based on four diagnostic bases: in the *myosin* gene there is an A instead of G at position 93, in *rpl4*A gene there is a T instead of a C at position 232 and in the ITS locus there is a C instead of a T at position 260, and a G instead of an A at position 629 (Table 2, Fig. 6).

Vánky (2012) lists several additional hosts for *U. serpens*. Due to the narrow specialization of stripe-smut revealed in this study, however, it seems likely that these harbour several distinct species. Until sequence data become available for these host-pathogen combinations, *Ustilago* on these other hosts is probably best referred to as *U. serpens s. lat.*

DISCUSSION

In this study, the closely related species of the *Ustilago striiformis*-complex and some other leaf stripe *Ustilago* smuts were investigated using multigene phylogenetic reconstructions to clarify their relationships. In total, 62 specimens of the *U. striiformis* species complex (incl. *U. calamagrostidis*) and four other leaf stripe smuts (*U. echinata, U. filiformis, U. scrobiculata,* and *U. serpens s. lat.*) were studied.

Phylogenetic analyses provided strong support for the polyphyly of the leaf-stripe smuts within *Ustilago*. However, the multilocus-based phylogenetic trees support the monophyly of the *U. striiformis* species complex, in contrast to the analysis by Savchenko *et al.* (2014a), where it was concluded that the *U. striiformis* group was polyphyletic and the segregation of two species was necessary to render it monophyletic. That interpretation was mainly based on a combined LSU-ITS tree of *U. striiformis* species, where *U. bromina* and *U. nunavutica* were located outside the *U. striiformis s. lat*. clade. Because of this conflicting result, the ITS region of the type specimen of *U. bromina* was sequenced (Table 1) and compared with the deposited GenBank sequences of Savchenko *et al.* (2014a). The type specimen of *U. bromina* on *Bromus inermis* had an ITS sequence nearly identical (except for a base exchange in a poly A/T region) with the other specimens identified as this species in the current study. It differed in nine bases compared to the three sequences labelled as *U. bromina* in Savchenko *et al.* (2014a). It is conceivable that these specimens belong to another undescribed smut species (the three sequences were obtained from material from Israel and USA, while the type collection was from Germany), or the quality of the sequences was not optimal; almost all differences in the sequences from Savchenko *et al.* (2014a) in comparison to the sequences from this study were located behind a poly A/T site, which necessitated re-sequencing for several of the specimens used in this study. Furthermore, misidentification of the host plant seems also possible, as no records were found for the occurrence of *Bromus inermis* in the floras of Israel (http://flora.org.il/en/plants/) or Palestine (Feinbrun-Dothan 1986).

Ustilago nunavutica was the second species that led Savchenko *et al.* (2014a) to assume that the *U. striiformis* species complex was polyphyletic. Comparing the ITS and LSU sequences of *U. nunavutica* with sequences from the current study, the LSU sequence used by Savchenko *et al.* (2014a) showed several SNPs (data not shown), while all other *U. striiformis* samples investigated in this study were identical in the LSU region. In contrast, the ITS sequence of *U. nunavutica* has only few SNPs in comparison to other members of the *U. striiformis* species complex and is identical with *U. neocopinata*. It seems possible that the LSU sequence of *U. nunavutica* either was of bad quality or shows the amplification of a contaminant smut fungus. However, as the genera *Puccinellia* and *Dactylis* are not closely related (Schneider *et al.* 2009) and very high host specificity has been revealed for the closely related species of the *U. striiformis* species complex in this study, it is unlikely that *U. neocopinata* and *U. nunavutica* are conspecific.

In agreement with Stoll *et al.* (2005) and Spooner & Legon (2006), we found that *U. calamagrostidis* and *U. corcontica* belonged to the *U. striiformis* species complex. However, further resolution within the *U. striiformis* species complex was only achieved when the protein-coding loci introduced by Kruse *et al.* (2017b) were employed. The trees revealed a host genus or host species specific occurrence for almost all lineages within the *U. striiformis* species complex, thus they should be treated as distinct species, supported by the observations of Liro (1924). All specimens from a single host species formed a clade according to the host species (or the host genus, in case of *Holcus*), with the exception of the rather closely related species *Lolium perenne* and *Festuca arundinacea* (Malik & Thomas 1966, Catalán *et al.* 2004, Hand *et al.* 2010). As most of these clades received high to

maximum support, they should be considered to represent distinct species, which can be distinguished based on the host and diagnostic SNPs (Fig. 6). For most of the 14 lineages of the *U. striiformis* species complex validly published names are available, necessitating the description of only two new species in this complex, *U. neocopinata* on *Dactylis glomerata* and *U. jagei* on *Agrostis stolonifera s. lat*. Vánky (2012) and Savchenko *et al.* (2014a) mentioned that different species on different hosts within this complex vary remarkably in spore shape, size, and ornamentation. However, morphological variation was observed to be high even within the same host species in the current study and also by Vánky (2012). Thus it is difficult to distinguish these closely related species based on morphology, necessitating the consideration of hosts and SNPs for diagnosis. The host range of at least two species of *Ustilago* parasitic to *Agrostis* could not be inferred with certainty, as both ITS and chloroplast loci did not resolve closely related species in the *A. stolonifera* and *A. gigantea* clusters (Amundsen & Warnke 2012).

While investigating synonymies of the *U. striiformis* species complex, it was found that the name *U. salweyi* is the correct name for the stripe smut on *Holcus lanatus*. Stevenson (1946) flagged *U. salweyi* as a "*nomen ambiguum"*, although no action was taken to formally reject the name. Following the ICN (McNeill *et al.* 2012), the name *U. salweyi* has priority over *Uredo striiformis* as it was published two years earlier (Berkeley & Broome 1850: 463). Although the group generally referred to as the *U. striiformis*-group does not contain a species with that as the correct name, as it is still included as a synonym we feel that it is best to continue to use "*U. striiformis*-group" or "species complex" for these fungi as it is so well established and recalls the symptoms all species of the complex exhibit, although this feature is shared by some leaf-stripe smuts not belonging to this complex.

The species within the *U. striiformis* species complex have sometimes been recognised as special forms based on infection trials (Liro 1924, Davis 1930, 1935, Fischer 1940). However, it has been shown for various biotrophic pathogens that the special form concept, in which there is a population continuum with somewhat specialised forms, cannot be upheld (Göker *et al.* 2004, Lutz *et al.* 2005, Kemler *et al.* 2009, Thines *et al.* 2009, Ploch *et al.* 2011, Savchenko *et al.* 2014b, Choi & Thines 2015).

Similar to the situation in the *U. striiformis* species complex, *Ustilago serpens s. lat.* on different hosts clustered in phylogenetically distinct subgroups. As the type host for *U. serpens* is *Elymus repens*, the collections from *Bromus inermis* warrants recognition as a new species. *Ustilago serpens* is another example illustrating the narrow host specialization among smut fungi. As for both the coarsely ornamented stripe-smuts (*U. serpens* clade) and the finely ornamented stripe smuts (*U. striiformis* clade) only a subset of the known hosts could be included in the current study. It is therefore conceivable that some older names published for specific host-pathogen combinations in these groups warrant recognition and several new species await discovery.

With respect to the global phylogeny of *Ustilago* it is noteworthy that even based on nine loci the backbone of the phylogenetic tree was only poorly resolved. Conflicting supported topologies were inferred with respect to the phylogenetic position of *U. maydis* in the reconstructions based on three (sister to a clade comprising, among others, the *U. nuda* and the *U. salweyi* clade) and nine loci (sister to a clade comprising the majority of smuts on panicoid grasses).

This highlights the high degree of uncertainty that there still is with respect to the global phylogeny of *Ustilago s. lat*. (Thines 2016). Considering the diversity of anatomical characteristics and disease syndromes caused, many of which have arisen several times independently (such as the stripe-smut habit; McTaggart *et al.* 2012a, b, c), any splitting of *Ustilago s. lat*. into smaller genera as suggested by McTaggart *et al.* (2012a, 2016) is probably premature and might become obsolete due to the high degree of parallel evolution and associated homoplasy.

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7 (2507) Proposal to reject the name *Ramularia gibba* (Ustilaginomycotina: Entylomatales). Taxon 66: 515–516.

Julia Kruse & Marco Thines

Statement of Joint Authorship

On the publication: (2507) Proposal to reject the name *Ramularia gibba* (Ustilaginomycotina: Entylomatales)

Status: accepted & printed

Name of the journal: Taxon

Involved authors: Julia Kruse (JK), Marco Thines (MT)

What did the PhD student or the Co-Authors contributed to this work?

(1) Development and planning

PhD student JK: 80 %

Co-Author MT: 20 %

(2) Performance of the individual investigations and experiments

PhD student JK: 80 %, morphological investigations, comparison of type collections

Co-Author MT: 20 %

(3) Preparation of the data collection and figures

PhD student JK: 95%, comparison of collections

Co-Author MT: 5%, helped with collection search

(4) Analyse and interpretation of data

PhD student JK: 80%, literature search, analysed double infection of smut type specimen

Co-Author MT: 20%, important feedback on several points and new ideas for interpretation

(5) Writing the manuscript

PhD student JK: 70%

Co-Author MT: 30%

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Verification of the statements above

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(2507) Proposal to reject the name *Ramularia gibba* (*Ustilaginomycotina*: *Entylomatales*)

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(2507) ***Ramularia gibba*** Fuckel in Hedwigia 5: 50. 1866, nom. rej. prop.

Lectotypus (vide Braun, Monogr. Cercosporella, Ramularia 2: 298. 1998): Germany, "ca Hostrichum" [Oestrich], on *Ranunculus repens* L. Fuckel, Fungi Rhenani Exs. No. 1636 (HAL; isolectotypi: FH, FR, G, TUB)

Ramularia gibba Fuckel (l.c.) was described as growing on *Ranunculus repens* L., based on *Fungi Rhenani Exsiccati* No. 1636 with the following description (our emphasis, see below):

"1636. *Ramularia* ***gibba*** Fckl. Caespitibus minutissimis, punctiformibus, gregariis, niveis, **in macula flavescente, demum hemisphaerico-turgida**; hyphis simplicibus, erectis; sporidiis fusiformibus, rectis, triguttulatis, hyalinis, hypharum longitudine."

As de Bary (in Bot. Zeitung (Berlin) 32: 83–84. 1874) already noted, two different fungi are present on the specimens of No. 1636 from Fuckel's *Fungi Rhenani Exsiccati* and he expressed the opinion that the portion of the description highlighted in bold applied to the teleomorph *Entyloma microsporum* (Unger) J. Schröt. (basionym *Protomyces microsporus* Unger, Exanth. Pfl.: 343. 1833, non *Protomyces microsporus* Pass. in Atti Reale Accad. Lincei, Rendiconti Cl. Sci. Fis., ser. 4, 7(2): 43. 1891) and the other part of the description to a *Ramularia* species (anamorphic *Ascomycota*) and that Fuckel probably considered both sets of characters to apply to the same fungi in different developmental stages.

Braun (l.c.) referred only to the anamorph when he combined *Ramularia gibba* into *Entylomella gibba* (Fuckel) U. Braun. Recently, as a consequence of ending dual naming for fungal teleomorphs and anamorphs, thus also ending priority for sexual morphs (McNeill & al. in Regnum Veg. 154. 2012), Rossman & al. (in IMA Fungus 7: 6. 2016) combined *Ramularia gibba* Fuckel into *Entyloma* as *Entyloma gibbum* (Fuckel) Rossman & Castl., based on the synonymy in Vánky (Smut Fungi World: 201–202. 2012), who gave *Entylomella gibba* as the asexual morph of *Entyloma ranunculi-repentis* Sternon (L'heterogenite Gen. Ramularia: 34. 1925), following Braun's (l.c) synonymy.

If a type specimen contains parts belonging to more than one taxon, Art. 9.14 of the *ICN* permits the selection of parts of the original type that correspond most nearly to the description as type material. However, in the case of *Ramularia gibba*, such selection cannot readily be done and is not desirable, as the species description and the species name itself are chimeric, in the sense of referring to two different organisms. Both of which are present as pathogens in components of the exsiccate within the same leaves of the lectotype and the isolectotype specimens. Thus also actions according to Art. 9.11 or 9.14 could not readily resolve the problem, and consequently *Ramularia gibba* is proposed for rejection. The name *Ramularia gibba* has hardly been used since its publication, probably also because de Bary (l.c.) highlighted the mixed nature of the species description and the type specimen. Instead, the names *Entyloma microsporum*, based on *Protomyces microsporus*, and *Entyloma ranunculi-repentis* were used in most taxonomic treatments, e.g., Vánky (l.c.). *Protomyces microsporus* forms protuberances on the leaves of the host by causing hypertrophy (and no *Ramularia*-like anamorph) and thus there is a widely used name for the "*gibba*-part" of the species that predates *Ramularia gibba*. However that the latter name antedates the widely used name *Entyloma ranunculi-repentis* and also the less frequently used older name *Fusidium eburneum* that corresponds to the "*Ramularia*-part", i.e., to the *Ramularia*-like anamorph not formed by *E. microspurum*. As mentioned, Braun (l.c.) combined *Ramularia gibba* into *Entylomella* Höhn.

(in Ann. Mycol. 22: 191. 1924) as *Entylomella gibbum*, stating that this species was the anamorph of *E. ranunculi-repentis*, and Rossman & al. (l.c.), acting in accordance with the ending of dual nomenclature for different fungal morphs (McNeill & al., l.c.), but not recognising the chimeric nature of the species and its description, proposed its transfer to *Entyloma* as *Entyloma gibbum*.

Due to its chimeric nature, which cannot be sensibly resolved by doing a secondary leptotypification, *Ramularia gibba* is proposed for rejection. The rejection of *Ramularia gibba* would not have adverse impact on the taxonomy of *Entyloma*, as the name has barely been used since its publication.

8 Broad host range species in specialised pathogen groups should be treated with suspicion – a case study on *Entyloma* infecting *Ranunculus*. Persoonia 41: 175-201.

Julia Kruse, Marcin Piątek, Matthias Lutz & Marco Thines

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What did the PhD student or the Co-Authors contributed to this work?

(1) Development and planning

PhD student JK: 70 %

Co-Author MP & ML: each 5 %

Co-Author MT: 20 %

(2) Performance of the individual investigations and experiments

PhD student JK: 45 %, collections trips, DNA extraction, PCR, sequencing, primer designing and testing (molecular work)

Co-Author MP: 40 %, measurements/comparison and pictures of species, providing material

Co-Author ML: 5 %, DNA extraction, providing material

Co-Author MT: 10 %, primer designing

(3) Preparation of the data collection and figures

PhD student JK: 47 %, phylogenetic trees, species list (excel), diagnostic bases (power point)

Co-Author MP: 47 %, Plates of species

Co-Author MT: 6 %, helped with phylogenetic trees

(4) Analyse and interpretation of data

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(5) Writing the manuscript

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Broad host range species in specialised pathogen groups should be treated with suspicion – a case study on *Entyloma* infecting *Ranunculus*

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Key words

Entyloma microsporum complex
Entyloma ranunculi-repentis complex
host specificity
multigene analyses
new primers
six new taxa
smut fungi

Abstract Plant pathogenic smut fungi in the broader sense can be divided into the *Ustilaginomycetes*, which cause classical smut symptoms with masses of blackish spores being produced in a variety of angiosperms, and the *Exobasidiomycetes*, which are often less conspicuous, as many do not shed large amounts of blackish spores. The leaf-spot causing members of the genus *Entyloma* (*Entylomatales*, *Exobasidiomycetes*) belong to the latter group. Currently, 172 species that all infect eudicots are included in the genus. Vánky (2012) recognised five *Entyloma* species on species of *Ranunculus* s.lat. Two have been reported only from *Ficaria verna* s.lat., while three, *E. microsporum*, *E. ranunculi-repentis*, *E. verruculosum*, have been reported to have a broad host range, encompassing 30, 26, and 5 species of *Ranunculus*, respectively. This broad host range is in contrast to the generally high host specificity assumed for species of *Entyloma*, indicating that they may represent complexes of specialised species. The aim of this study was to investigate *Entyloma* on *Ranunculus* s.lat. using multigene phylogenies and morphological comparisons. Phylogenetic analyses on the basis of up to four loci (ITS, *atp*2, *ssc*1, and *map*) showed a clustering of *Entyloma* specimens according to host species. For some of these *Entyloma* lineages, names not currently in use were available and reinstated. In addition, *Entyloma microsporum* s.str. is neotypified. Six novel species are described in this study, namely, *Entyloma jolantae* on *Ranunculus oreophilus*, *E. klenkei* on *R. marginatus*, *E. kochmanii* on *R. lanuginosus*, *E. piepenbringiae* on *R. polyanthemos* subsp. *nemorosus* (type host) and *R. repens*, *E. savchenkoi* on *R. paludosus*, and *E. thielii* on *R. montanus*. For all species diagnostic bases and morphological characteristics are provided. The results in this study once more highlight the importance of detailed re-investigation of broad host-range pathogens of otherwise specialised plant pathogen groups.

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INTRODUCTION

The smut fungi in a broad sense (*Ustilaginomycotina*) contain more than 1 600 plant parasitic species in two major classes, the *Ustilaginomycetes*, the smut fungi in a strict sense and the *Exobasidiomycetes*, many of which do not cause typical smut symptoms with huge amounts of blackish spores being shed from sori in their host plants. Two more classes have been proposed recently (Wang et al. 2014), but as they might be embedded within the *Exobasidiomycetes* (Wang et al. 2015) or the sister group to the *Ustilaginomycetes* (Mishra et al. 2018), we do not treat them as separate classes here. *Entyloma* (*Entylomatales*, *Exobasidiomycetes*) is a species-rich genus with species that cause mostly inconspicuous, white to brown leaf spots. *Entyloma* currently comprises 172 species, restricted to dicotyledonous host plants belonging to 26 families (Vánky 2012, Denchev et al. 2013, Savchenko et al. 2014a, Rooney-Latham et al. 2017, Savchenko & Carris 2017). Because of their simple spore morphology, species delimitation in *Entyloma* is difficult (Savile 1947). A combination of spore morphology and host plant species is currently the most useful way to delineate species of *Entyloma* (Vánky 1994, 2012). Molecular phylogenetics has resolved species boundaries for many smut fungi (Vánky & Lutz 2007, Piątek et al. 2011, 2013, 2015a, b, 2016, Savchenko et al. 2013, 2014a, b, Vasighzadeh et al. 2014, Li et al. 2017, Kruse et al. 2018), including *Entyloma* (Begerow et al. 2002, Vánky & Lutz 2010, Savchenko et al. 2014a, Lutz & Piątek 2016). However, sequences of many *Entyloma* species are poorly represented in publicly available databases and many currently recognised species lack sequence data.

With about 600 species, *Ranunculus* is the largest genus of the family *Ranunculaceae* (Tamura 1995). *Ranunculus* species have a cosmopolitan distribution and mostly occur in temperate to arctic zones, where they grow in forests, meadows, peat bogs, on wet soils, as well as in lakes and rivers. Most species are herbaceous, some are annual, but the vast majority of species are perennial (Rastipishe et al. 2011). In the world monograph of smut fungi, Vánky (2012) recognised five different *Entyloma* species on *Ranunculus* s.lat., namely, *E. ficariae*, *E. majewskii*, *E. microsporum*, *E. ranunculi-repentis*, and *E. verruculosum*. Two species, *Entyloma ficariae* and *E. majewskii*, infect hosts in the genus *Ficaria* that is closely related to *Ranunculus* (Hörandl et al. 2005, Emadzade et al. 2010). Only three *Entyloma* species, *E. microsporum*, *E. ranunculi-repentis*, and *E. verruculosum*, were reported to infect species of the genus *Ranunculus* s.str. (Vánky 2012).

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Table 1 Smut specimens used for phylogenetic analysis.

Species	Host	Location	Location details	Date	Collector	DNA-no.	Fungarium no.	GenBank no.			
								ITS	*atp2*	*ssc1*	*map*
Entyloma bullosum	*Ranunculus paludosus*	Greece, Rhodes	eastcoast, SE of Archangelos: c. 1.5 km S Stegna, Phrygana, northeast slope, N36°11'49" E28°08'06", elev. c. 70 m a.s.l.	09.03.2016	J. Kruse	3471	GLM-F107632	MF924658	MH022782	MF939230	MF939296
	R. paludosus	Spain, Andalusia	Cazorla, Parque Natural Sierras de Cazorla, 2.2 km E of Burunchel, A-319, slip rocks at wayside, N37°56'50" W02°56'28", elev. c. 1200 m a.s.l	23.04.2015	J. Kruse	3211	GLM-F107633	MF924651	MH022775	MF939223	MF939289
	R. paludosus	Greece, Rhodes	c. 2.8 km NW of Lindos, Phrygana, way up to the mountain, hiking path, N36°05'48" W28°03'13", elev. c. 145 m a.s.l.	10.03.2016	J. Kruse	3467	GLM-F107634	MF924654	MH022778	MF939226	MF939292
	R. paludosus	Greece, Rhodes	eastcoast, c. 3.5 km NE of Archangelos: Tsambika, way up Kloster, northern slope, Phrygana, N36°14'16" E28°09'16", elev. c. 160 m a.s.l.	11.03.2016	J. Kruse	3468	GLM-F107635	MF924655	MH022779	MF939227	MF939293
	R. paludosus	Greece, Rhodes	c. 1 km S of Salakos, way up Mt Profitis Ilias, Phrygana, N36°17'03" E27°56'38", elev. c. 275 m a.s.l.	13.03.2016	J. Kruse	3469	GLM-F107636	MF924656	MH022780	MF939228	MF939294
	R. paludosus	Greece, Rhodes	c. 1 km NW of Siana, way up to Akramitis, open Phrygana, plateau, N36°09'23" E27°45'59", elev. c. 650 m a.s.l.	15.03.2016	J. Kruse	3470	GLM-F107637	MF924657	MH022781	MF939229	MF939295
E. eburneum	*R. bulbosus*	Germany, Baden-Württemberg	Hegau, county Konstanz, NE of Neuhausen, near Schoren, dry grasland, MTB/Q: 8118/41, elev. c. 500 m a.s.l.	28.05.2013	J. Kruse	107	GLM-F107639	MF924630	MH022754	MF939209	MF939275
	R. bulbosus	Germany, Baden-Württemberg	county Konstanz, peninsula Reichenau in the Undersea, E Oberzell, littoral, MTB/Q: 8320/2, elev. c. 400 m a.s.l.	31.05.2013	J. Kruse	108	GLM-F107640	MF924631	MH022755	MF939210	MF939276
	R. bulbosus	Germany, Bavaria	Oberfranken, S of Bayreuth, Swedebridge in direction to Studentwood, wayside, MTB/Q: 6035/34, elev. c. 360 m a.s.l.	12.06.2013	J. Kruse	109	GLM-F107641	MF924632	MH022756	–	–
	R. bulbosus	Germany, Baden-Württemberg	Swabian Alps, county Sigmaringen, Beuron, Leibertingen-Wildenstein, castle Wildenstein, N48°03'21" E9°00'0", MTB/Q: 7919/13, elev. c. 760 m a.s.l.	07.06.2014	J. Kruse	3049	GLM-F107642	MF924649	MH022773	MF939221	MF939287
	R. bulbosus	Germany, Hesse	Taunus, Bad Nauheim, Bad Nauheimer street, wayside, N50°22'50" E08°44'45", MTB/Q: 5618/12, elev. c. 175 m a.s.l.	09.11.2015	J. Kruse	3496	GLM-F107643	MF924665	MH022789	MF939237	MF939303
	R. bulbosus	Germany, Hesse	Main-Taunus-county, Hattersheim at Main, grasland at Welschenstream, Kuckuckspfad, wayside, N50°03'54" E08°30'03", MTB/Q: 5917/13, elev. c. 90 m a.s.l.	30.04.2016	J. Kruse	3621	GLM-F107644	MF924666	MH022790	MF939238	MF939304
	R. bulbosus	Italy, Liguria	Lower Varavalley, c. 1.5 km SW of Tavarone, circular path, Monte Alpe from Agriturismo Giandriale, east slope, meadow, N44°18'28" E09°31'58", elev. c. 725 m a.s.l.	10.05.2016	J. Kruse	3622	GLM-F107645	MF924667	MH022791	MF939239	MF939305
	R. bulbosus	Germany, Saxony-Anhalt	Kyffhäuser-northern area, county Sangerhausen: SW of Kelbra, Großes Rabental, wayside, N51°25'33" E11°01'14", MTB/Q: 4532/33	13.05.2008	H. Jage	2317	GLM-F095089	MF924639	MH022763	–	–
	R. repens	Germany, Baden-Württemberg	Swabian Alps, county Sigmaringen, Leibertingen-Wildenstein, S of Beuron, ascent castle Wildenstein, mixed forest, wayside, N48°02'49" E08°58'17", MTB/Q: 7919/42, elev. c. 682 m a.s.l.	06.06.2014	J. Kruse	3045	GLM-F107638	MF924646	MH022770	MF939218	MF939284
	R. repens	Germany, Lower Saxony	county Northeim, at the bottom of the Katlencastle, wayside near river, MTB/Q: 4326/21, elev. c. 110 m a.s.l.	23.04.2010	J. Kruse	110	GLM-F107648	MF924633	MH022757	MF939211	MF939277
	R. repens	Germany, Schleswig-Holstein	county Rendsburg-Eckernförde, Barkelsby, Schusterredder, wayside, MTB/Q: 1425/33, elev. c. 22 m a.s.l.	25.04.2011	J. Kruse	113	GLM-F107649	MF924634	MH022758	–	–
	R. repens	Germany, Hesse	Franfurt at Main, Sachsenhausen, Landwehrstreet,South-Cementery, N50°05'20" E08°41'43", MTB/Q: 5918/11, elev. c. 150 m a.s.l.	22.03.2014	J. Kruse	3048	GLM-F107654	MF924648	MH022772	MF939220	MF939286
	R. repens	Germany, Saxony-Anhalt	county Wittenberg, Zahna-Elster, E of Bad Zahna, Oßnitzbach, wet grasland, N51°55'24" E12°46'10", MTB/Q: 4042/41, elev. c. 100 m a.s.l.	17.08.2014	J. Kruse	3046	GLM-F107653	MF924647	MH022771	MF939219	MF939285
	R. repens	Germany, Bavaria	Upper Bavaria, Chiemgauer Alps, county Rosenheim, Priener cabin, climb down towards Berg, Via Alpina, firsforest, wayside, N47°41'41" E12°18'24", MTB/Q: 8339/22, elev. c. 1290 m a.s.l.	22.07.2014	J. Kruse	3044	GLM-F107652	MF924645	MH022769	–	–
	R. repens	Germany, Hesse	Rüsselsheim, county Groß-Gerau, Varkaustreet, forest cementary, wayside, N49°59'22" E08°26'10", MTB/Q: 6016/21, elev. c. 100 m a.s.l.	08.03.2015	J. Kruse	3641	GLM-F107651	MF924680	MH022804	MF939245	MF939311
	R. repens	Germany, Hesse	Wiesbaden, Stationstraße, Reisingeranlage, wayside, N50°04'21" E08°14'38", MTB/Q: 5915/12, elev. c. 110 m a.s.l.	21.03.2015	J. Kruse	3640	GLM-F107650	MF924679	MH022803	MF939244	MF939310
	R. repens	**Poland**	**Małopolska Province: Kraków-Pleszów, at Suchy Jar street**	**20.11.2010**	**M. Piątek**	3652	**KRAM F-59037**	**MF924689**	**MH022813**	–	–
	R. repens	Poland	Małopolska Province: near Bukowica Reserve, close to Wygiełzów	10.09.2014	J. & M. Piątek	3653	KRAM F-59038	MF924690	MH022814	–	–

Table 1 (cont.)

Species	Host	Location	Location details	Date	Collector	DNA-no.	Fungarium no.	GenBank no.			
								ITS	*atp2*	*ssc1*	*map*
E. eburneum (cont.)	*R. polyanthemos* subsp. *nemorosus*	Germany, Bavaria	Upper Bavaria, county Garmisch-Partenkirchen, c. 4.9 km NE of Mittenwald, Karwendel mountains, hiking path 266 from Rehbergalm to Hochland cabin, mixed mountain-forest, N47°27'37" E11°18'36", MTB/Q: 8533/24, elev. c. 1575 m a.s.l.	11.07.2016	J. Kruse	3659	GLM-F107647	MF924696	MH022820	MF939255	MF939321
	R. polyanthemos subsp. *nemorosus*	Austria, Salzburg	county Salzburg, Lungau, Prebersee, MTB/Q: 8849/1	14.08.2013	C. & F. Klenke	3631	GLM-F107646	MF924674	MH022798	–	–
E. ficariae	*Ficaria verna*	Germany, Schleswig-Holstein	Barkelsby, Schusterredder, wayside, MTB/Q: 1425/33, elev. c. 20 m a.s.l.	27.04.2008	J. Kruse	70	GLM-F107655	MF924702	MH022826	–	–
	Ficaria verna	Germany, Lower Saxony	Hannover, Misburg-North, Ludwig-Jahn-Street, country lane nearby the marlpit, wayside, MTB/Q: 3625/11, elev. c. 60 m a.s.l.	18.04.2011	J. Kruse	73	GLM-F107656	MF924703	MH022827	–	–
	Ficaria verna	Germany, Schleswig-Holstein	county Rendsburg-Eckernförde, Ascheffel, Old Station, near exit to the Asch Mt, mixed forest, wayside, MTB/Q: 1524/31, elev. c. 6 m a.s.l.	24.04.2011	J. Kruse	74	GLM-F107658	MF924704	MH022828	–	–
	Ficaria verna subsp. *chrysocephala*	Italy, Liguria	Varavalley, c. 2.5 km S of Varese Ligure, E of Stora, street from Sant Pietro Vare to Teviggo, shady wayside, N44°22'01" E09°37'39", elev. c. 530 m a.s.l.	08.05.2016	J. Kruse	3638	GLM-F107657	MF924677	MH022801	MF939242	MF939308
E. jolantae	***R. oreophilus***	**Poland**	**Małopolska Province: Tatra Mts, Mała Dolinka valley – northern slopes of Giewont Mt, elev. c. 1230 m a.s.l.**	**25.08.2008**	**J. & M. Piątek**	3650	**KRAM F-59030**	**MF924688**	**MH022812**	**MF939250**	**MF939316**
	R. oreophilus	Poland	Małopolska Province: Tatra Mts, Mała Dolinka valley – northern slopes of Giewont Mt, elev. c. 1260 m a.s.l.	25.08.2008	J. & M. Piątek	ML1535	KRAM F-59031	MF924714	MH022838	–	–
E. klenkei	***R. marginatus***	**Greece, Rhodes**	**c. 0.7 km W of Archipoli, Eparchiaki Odos Pastidas-Mesanagrou, field beneath street, N36°15'58" E28°03'11", elev. c. 185 m a.s.l.**	**13.03.2016**	**J. Kruse & V. Kummer**	3476	**GLM-F107659**	**MF924663**	**MH022787**	**MF939235**	**MF939301**
E. kochmanii	***R. lanuginosus***	**Italy, Liguria**	**Varavalley, c. 2 km NE of Caranza, Strada Provinciale from Caranza to passo della Cappelletta, canyon alluvial forest, N44°23'33" E09°38'44", elev. c. 840 m a.s.l.**	**09.05.2016**	**J. Kruse**	3639	**GLM-F107660**	**MF924678**	**MH022802**	**MF939243**	**MF939309**
E. majewskii	***Ficaria verna***	**Iran**	**Tehran Prov., 60 km E Tehran, Mts Elburz, 'Emamzadeh-Haskei', N35°50' E52°02', elev. c. 2610 m a.s.l.**	**17.05.1990**	**D. Ershad, T. Ványa & K. Ványa**	Efc34	**BRIP: HUV14888**	**MF924713**	**MH022837**	**MF939265**	**MF939331**
E. microsporum	*R. repens*	Germany, Lower Saxony	county Hildesheim, Brüggen, Kirschweg, Sieben Bergen, Mt Hohe Tafel, wayside, MTB/Q: 3924/42, elev. c. 395 m a.s.l.	08.05.2011	J. Kruse	95	GLM-F107667	MF924708	MH022832	MF939262	MF939328
	R. repens	Germany, Bavaria	Oberfranken, Bayreuth, cemetery Saas, Bärenleite, wayside, MTB/Q: 6035/3, elev. c. 360 m a.s.l.	24.05.2012	J. Kruse	96	GLM-F107668	MF924709	MH022833	MF939263	MF939329
	R. repens	Germany, Bavaria	Oberpfalz, national park Bavarian Wood, county Regen, W of Zwieseler Waldhaus, Watzlikhain, mixed mountain-forest on granite, MTB/Q: 6945/1, elev. c. 650 m a.s.l.	21.08.2012	J. Kruse	97	GLM-F107669	MF924710	MH022834	MF939264	MF939330
	R. repens	Germany, Bavaria	Oberpfalz, national park Bavarian Wood, county Regen, Zwieseler Waldhaus, Mittelsteig cabin, mixed mountain-forest on granite, MTB/Q: 6945/2, elev. c. 700 m a.s.l.	24.08.2012	J. Kruse	98	GLM-F107670	MF924711	MH022835	–	–
	R. repens	Germany, Bavaria	Oberfranken, Bayreuth, Eremitage, W of river Red Main, mixed forest, MTB/Q: 6035/42, elev. c. 375 m a.s.l.	02.05.2013	J. Kruse	99	GLM-F107671	MF924712	MH022836	–	–
	R. acris	Germany, Bavaria	Oberfranken, between Horbach at the Steinach and Leutendorf, flood hollow, MTB/Q: 5733/3, elev. c. 290 m a.s.l.	10.05.2013	J. Kruse	92	GLM-F107662	MF924705	MH022829	MF939261	MF939327
	R. repens	Germany, Baden-Württemberg	county Konstanz, Hegau, W of Singen, way up Mt Hohentwiel, wayside, MTB/Q: 8218/2, elev. c. 600 m a.s.l.	29.05.2013	J. Kruse	101	GLM-F107672	MF924624	MH022748	MF939205	MF939271
	R. repens	**Germany, Hesse**	**county Groß-Gerau, Ginsheim-Gustavsburg, Radweg zum Mainspitzdreieck, wayside circular path, N49°59'37" E08°17'46", MTB/Q: 6015/22**	**17.11.2013**	**J. Kruse**	1631	**GLM-F107661, KRAM F-59043**	**MF924636**	**MH022760**	**MF939213**	**MF939279**
	R. repens	Austria, Tyrol	district Kufstein, county Walchsee, Kaiserwinkel, hiking track, Wandberg cabin towards Niederkaseralm, firsforest, slope, wayside, N47°41'16" E12°19'07", MTB/Q: 8339/22, elev. c. 1380 m a.s.l.	21.07.2014	J. Kruse	3040	GLM-F107675	MF924643	MH022767	MF939216	MF939282
	R. repens	Germany, Bavaria	Upper Bavaria, Chiemgauer Alps, county Rosenheim, way up Priener cabin, N47°42'00" E12°17'54", MTB/Q: 8239/44, elev. c. 1280 m a.s.l.	22.07.2014	J. Kruse	3038	GLM-F107674	MF924642	MH022766	–	–
	R. acris	Germany, Bavaria	county Rottal-Inn, Simbach, road St 2112, grasland at roundabout, N48°16'23" E13°00'53", elev. c. 370 m a.s.l.	14.08.2014	J. Kruse	3037	GLM-F107663	MF924641	MH022765	MF939215	MF939281

Table 1 (cont.)

Species	Host	Location	Location details	Date	Collector	DNA-no.	Fungarium no.	GenBank no.			
								ITS	*atp2*	*ssc1*	*map*
E. microsporum (cont.)	*R. repens*	Austria, Upper Austria	Braunau am Inn, Hagenau in Inncounty, Hagenauer street, grasland, sidearm of Inn, wayside, N48°16'23" E13°06'01", MTB/Q: 7744/2, elev. c. 340 m a.s.l.	18.08.2014	J. Kruse	3036	GLM-F107673	MF924640	MH022764	–	–
	R. repens	Germany, Baden-Württemberg	Eschberg, county Waldshut, Buckmattstraße, meadow and forest edge around Liederbach, N08°10'49" E08°10'49", MTB/Q: 8315/31, elev. c. 400 m a.s.l.	03.07.2015	J. Kruse	3643	GLM-F107665	MF924682	MH022806	MF939246	MF939312
	R. repens	Austria, Carinthia	Völkermarkt, SW of Bad Eisenkappel-Vellach, Koschuta, Trögener land road, Trögener Klamm, towards Trögern, wet slope at wayside, N46°27'53" E14°30'18", elev. c. 720 m a.s.l.	06.07.2015	J. Kruse	3644	GLM-F107666	MF924683	MH022807	MF939247	MF939313
	R. repens	Germany, Hesse	Gießen, Lahntal, county Gießen, c. 5.5 km SW of Gießen, Allendorf at river Lahn, above parking at TSV Allendorf, street „In der Lache“, Slopeforest at stream Kleebach, wayside, N50°33'18" E08°36'55", MTB/Q: 5417/23, elev. c. 165 m a.s.l.	21.05.2016	J. Kruse	3642	GLM-F107664	MF924681	MH022805	–	–
	R. repens	Poland	Małopolska Province: Tatra Mts, between Hala Kalatówki glade and Hala Kondratowa glade, elev. c. 1250 m a.s.l.	17.07.2005	J. & M. Piątek	3646	KRAM F-59039	MF924685	MH022809	–	–
	R. repens	Slovakia	on tourist track from Lucky to Choc Mt	28.06.2008	J. & M. Piątek	3647	KRAM F-59040	MF924686	MH022810	–	–
	R. repens	Poland	Małopolska Province, Tatra Mts, Hala Gąsienicowa glade (near Murowaniec mountain hut), elev. c. 1510 m a.s.l.	24.09.2005	J. & M. Piątek	3649	KRAM F-59041	MF924687	MH022811	MF939249	MF939315
E. piepenbringiae	*R. polyanthemos* agg.	Germany, Bavaria	Upper Bavaria, county Weilheim, N Pähl, E of Hartschimmelhof, ‘Goaslweide’, region F3, MTB/Q: 8033/31, elev. c. 730 m a.s.l.	14.05.2013	J. Kruse	93	GLM-F107688	MF924706	MH022830	–	–
	R. polyanthemos agg.	Germany, Bavaria	Upper Bavaria, county Weilheim, N Pähl, E Hartschimmelhof, ‘Goaslweide’, region F4, MTB/Q: 8033/31, elev. c. 730 m a.s.l.	13.05.2013	J. Kruse	94	GLM-F107689	MF924707	MH022831	–	–
	R. repens	Germany, Baden-Württemberg	county Konstanz, communal Moos, S of Weiler, nearby Grey Reed, wayside, MTB/Q: 8219/4, elev. c. 445 m a.s.l.	30.05.2013	J. Kruse	102	GLM-F107694	MF924625	MH022749	MF939206	MF939272
	R. sp.	Spain, Andalusia	Cazorla, Parque Natural Sierras de Cazorla, c. 2.2 km S of Cazorla, hicking track, ascent Gilillo, slip rock, N37°53'30" W02°59'49", elev. c. 1185 m a.s.l.	24.04.2015	J. Kruse	3210	GLM-F107695	MF924650	MH022774	MF939222	MF939288
	R. polyanthemos* subsp. *nemorosus	**Germany, Bavaria**	**Oberallgäu, Einödsbach, Allgäu Alps, hiking path from Black Cabin to Rappensee cabin, meadow W of Rappensee cabin, N47°17'24" E10°14'40", MTB/Q: 8727/12, elev. c. 1900 m a.s.l.**	**26.07.2015**	**J. Kruse**	3493	**GLM-F107687**	**MF924664**	**MH022788**	**MF939236**	**MF939302**
	R. polyanthemos subsp. *nemorosus*	Germany, Bavaria	Upper Bavaria, county Garmisch-Partenkirchen, c. 2.8 km SE of Mittenwald, Karwendel mountains, hiking path 290 towards Brunnstein cabin, serpentines, light mixed mountain-forest, N47°24'44" E11°16'23", MTB/Q: 8533/43, c. 1260 m a.s.l.	06.07.2016	J. Kruse	3664	GLM-F107693	MF924701	MH022825	MF939260	MF939326
	R. polyanthemos subsp. *nemorosus*	Germany, Bavaria	Upper Bavaria, county Garmisch-Partenkirchen, c. 2.8 km SE of Mittenwald, Karwendel mountains, hiking path 290 towards Brunnstein cabin, serpentines, light mixed mountain-forest, N47°24'48" E11°16'33", MTB/Q: 8533/43, elev. c. 1380 m a.s.l.	06.07.2016	J. Kruse	3663	GLM-F107692	MF924700	MH022824	MF939259	MF939325
	R. polyanthemos subsp. *nemorosus*	Germany, Bavaria	Upper Bavaria, county Garmisch-Partenkirchen, c. 3.2 km SE of Mittenwald, Karwendel mountains, hiking path 291 from Brunnstein cabin towards Mt Brunnsteinspitze, scree, N47°24'33", E11°16'59", MTB/Q: 8533/43, elev. c. 1760 m a.s.l.	07.07.2016	J. Kruse	3662	GLM-F107691	MF924699	MH022823	MF939258	MF939324
	R. polyanthemos subsp. *nemorosus*	Germany, Bavaria	Upper Bavaria, county Garmisch-Partenkirchen, c. 2.8 km SE of Mittenwald, Karwendel mountains, meadow around Brunnstein cabin, N47°24'49" E11°16'41", MTB/Q: 8533/43, elev. c. 1475 m a.s.l.	08.07.2016	J. Kruse	3661	GLM-F107690	MF924698	MH022822	MF939257	MF939323
	R. polyanthemos agg.	Austria, Tyrol	Lechtal, N of Elbigenalp, wayside in mixed forest	26.08.2002	U. Fischer & M. Lutz	ML523	TUB-012566	MF924716	MH022840	MF939267	MF939333
	R. sp.	Switzerland	Kanton Bern, north bottom slope of Sustenpass, c. 4 km to Hotel Steingletscher, meadow	12.06.2003	W. Maier & M. Lutz	ML614	TUB-012567	MF924717	MH022841	MF939268	MF939334
	R. polyanthemos subsp. *nemorosus*	Slovenia	Triglav National Park, Lopucnica, way to Siebenseen cabin, tall herbaceous vegetation	02.08.2005	M. Kemler	ML838	TUB-012568	MF924718	MH022842	MF939269	MF939335
	R. repens	Germany, Baden-Württemberg	Tübingen, Bebenhausen, Goldersbachtal, littoral of lake, N48°33'30" E09°02'48", elev. c. 370 m a.s.l.	14.06.2002	M. Lutz	ML471	TUB-012570 MF924626	MF924715 MH022750	MH022839 MF939207	MF939266 MF939273	MF939332
E. ranunculacearum	*R. acris*	Germany, Bavaria	Oberfranken, county Bamberg, SE of Sandhof, Mönchsweiher, mixed forest on Keuper-Sandstone, MTB/Q: 6030/2, elev. c. 290 m a.s.l.	05.05.2012	J. Kruse	103	GLM-F107676	MF924627	MH022751	–	–

Table 1 (cont.)

Species	Host	Location	Location details	Date	Collector	DNA-no.	Fungarium no.	GenBank no.			
								ITS	*atp2*	*ssc1*	*map*
E. ranunculacearum (cont.)	*R. acris*	Germany, Bavaria	Oberpfalz, national park Bavarian Wood, county Regen, Zwieseler Waldhaus, Mittelsteig cabin, mixed mountainous forest on granite, MTB/Q: 6945/2, elev. c. 700 m a.s.l.	24.08.2012	J. Kruse	104	GLM-F107677	MF924628	MH022752	MF939208	MF939274
	R. acris	Germany, Baden-Württemberg	county Konstanz, Lake Constance, Radolfzell, SE of Möggingen, Mindelsea, circular path around sea, littoral and wayside, MTB/Q: 8220/1, elev. c. 420 m a.s.l.	30.05.2013	J. Kruse	105	GLM-F107678	MF924637	MH022761	MF939214	MF939280
	R. acris	**Germany, Saxony-Anhalt**	**county Wittenberg, Kemberg, district Rotta-Gniest, Heidestreet, wayside, N51°45'4" E12°35'33", MTB/Q: 4241/23, elev. c. 105 m a.s.l.**	**13.11.2013**	**J. Kruse**	1632	**GLM-F107680**	**MF924635**	**MH022759**	**MF939212**	**MF939278**
	R. acris	Germany, Hesse	Rheingau-Taunus-county, Eltville at river Rhein, Rheinsteig direction forest-restaurant Rausch, N50°02'46", E08°05'44", MTB/Q: 5914/41, elev. c. 160 m a.s.l.	08.03.2014	J. Kruse	1373i	GLM-F107679	MF924644	MH022768	MF939217	MF939283
	R. acris	Germany, Bavaria	Kirchdorf at Inn, Lower Bavaria, county Rottal-Inn, Hitzenau, Eckener street, wayside, N48°15'56" E12°58'53", MTB/Q: 7743/24, elev. c. 400 m a.s.l.	17.08.2014	J. Kruse	3043	GLM-F107681	MF924668	MH022792	–	–
	R. acris	Germany, Saarland	Mettlach-Orscholz, county Merzig-Wadern, Cloef-Street, surroundings of Cloef-Atrium and Varadeser Park, N49°30'20" E06°32'06", MTB/Q: 6405/33, elev. c. 395 m a.s.l.	29.09.2014	J. Kruse	3623	GLM-F107684	MF924653	MH022777	MF939225	MF939291
	R. acris	Germany, Hesse	Hoher Meißner, Meißner eastern slope, Fulda-Werra-uplands, Werra-Meißner-county, Frau Holle lake, circular path, N51°13'09" E09°52'07", MTB/Q: 4725/33, elev. c. 640 m a.s.l.	09.06.2015	J. Kruse	3315	GLM-F107683	MF924652	MH022776	MF939224	MF939290
	R. acris	Germany, Hesse	Hoher Meißner, Meißner eastern slope, Fulda-Werra-uplands, Werra-Meißner-county, Frau Holle lake, circular path, alpine meadow, N51°13'06" E09°52'13", MTB/Q: 4725/33, elev. c. 620 m a.s.l.	09.06.2015	J. Kruse	3314	GLM-F107682	MF924676	MH022800	MF939241	MF939307
E. ranunculi-scelerati	*R. sceleratus*	Germany, Bavaria	Oberpfalz, county Grafenwöhr, E of Hütten, littoral of lake, N49°40'52" E11°58'42", MTB/Q: 6337/22, elev. c. 410 m a.s.l.	01.05.2016	G. Hübner	3637	GLM-F107685	MF924669	MH022793	–	–
	R. sceleratus	Germany, Saxony-Anhalt	SSE of Elsnigk, S of Würflauer Schachtlake, near road B 185, MTB/Q: 4238/12	03.11.2004	H. Jage	3624	GLM-F074573	MF924670	MH022794	–	–
	R. sceleratus	Germany, Saxony-Anhalt	SE of Allstedt, Ziegelrodaer forest (N-part), aiport Allstedt (NW edge), MTB/Q: 4634/21	23.10.2005	H. Jage	3625	GLM-F076138	MF924671	MH022795	–	–
	R. sceleratus	Germany, Saxony-Anhalt	SW Sülldorf, Sülzetal, wet ditch right next to the brook Sülze (nearby salty area), MTB/Q: 3935/34	04.11.2005	H. Jage	3626	GLM-F076159	MF924629	MH022753	–	–
	R. sceleratus	Germany, Saxony-Anhalt	Lodersleben, near castle, in the Querne, MTB/Q: 4635/12	06.05.2005	H. John & H. Jage	3627	GLM-F076186	MF924673	MH022797	–	–
	R. sceleratus	Germany, Saxony-Anhalt	Friedersdorf near Lohsa (South), WSW of Neuhof, near Ballackmill, Maxlake (part of Ballacklakes), surceased, MTB/Q: 4652/14	26.05.2006	H. Jage	3628	GLM-F086008	MF924691	MH022815	–	–
	R. sceleratus	**Poland**	**Mazowieckie Province: Warszawa-Wesoła**	**17.07.2015**	**P. Mędykowski**	3654	**KRAM F-59032**	**MF924672**	**MH022796**	–	–
E. ranunculorum	***R. auricomus***	**Germany, Bavaria**	**Oberfranken, county Kulmbach, Lindau, Mt chain Rough Mt, wayside, MTB/Q: 5934/2, elev. c. 410 m a.s.l.**	**12.05.2012**	**J. Kruse**	106	**GLM-F107686**	**MF924638**	**MH022762**	–	–
	R. auricomus	Germany, Saxony-Anhalt	E of Dölkau, Burgholz (E-part) Jagen 29, alluvial forest, MTB/Q: 4638/24, elev. c. 25 m a.s.l.	19.04.1998	H. Jage	1768	GLM-F048093	MF924659	MH022783	MF939231	MF939297
E. savchenkoi	*R. paludosus*	Greece, Rhodes	c. 1 km S of Salakos, way up to Mt Profitis Ilias, *Quercus coccifera* forest, N36°16'59" E27°56'42", elev. c. 320 m a.s.l.	13.03.2016	J. Kruse	3472	GLM-F107696	MF924660	MH022784	MF939232	MF939298
	R. paludosus	Greece, Rhodes	c. 1 km NW of Siana, way up Akramitis, open Phrygana, plateau, N36°09'23" E27°45'59", elev. c. 650 m a.s.l.	15.03.2016	J. Kruse	3473	GLM-F107697	MF924661	MH022785	MF939233	MF939299
	R. paludosus	Greece, Rhodes	c. 1.2 km SE of Theologos, olive grove, N36°22'00" E28°02'45", elev. c. 40 m a.s.l.	16.03.2016	J. Kruse	3474	GLM-F107698	MF924662	MH022786	MF939234	MF939300
	R. paludosus	**Greece, Rhodes**	**eastcoast, c. 2.5 km N of Kalathos, street towards Masari, wayside, olive grove, N36°08'47" E28°03'33", elev. c. 15 m a.s.l.**	**20.03.2016**	**J. Kruse**	3475	**GLM-F107699** MF924697	**MF924675** MH022821	**MH022799** MF939256	**MF939240** MF939322	**MF939306**
E. thielii	*R. montanus*	Germany, Bavaria	Oberallgäu, Einödsbach, Rappensee cabin, near Rappensea, wayside, N47°17'11" E10°15'19", MTB/Q: 8727/21, elev. c. 2080 m a.s.l.	29.07.2015	J. Kruse	3632	GLM-F107705	MF924695	MH022819	MF939254	MF939320
	R. montanus	Germany, Bavaria	Upper Bavaria, county Garmisch-Partenkirchen, c. 4.9 km NE Mittenwald, Karwendel mountains, hiking path 266 from Rehbergalm to Hochland cabin, mixed mountainous forest, N47°27'37" E11°18'36", MTB/Q: 8533/24, elev. c. 1575 m a.s.l.	11.07.2016	J. Kruse	3660	GLM-F107704	MF924694	MH022818	MF939253	MF939319

Table 1 (cont.)

Species	Host	Location	Location details	Date	Collector	DNA-no.	Fungarium no.	GenBank no.			
								ITS	*atp2*	*ssc1*	*map*
E. thielii (cont.)	*R. montanus*	Germany, Bavaria	Upper Bavaria, county Garmisch-Partenkirchen, c. 4.9 km NE of Mittenwald, Karwendel mountains, hiking path 266 from Rehbergalm to Hochland cabin, mixed mountain-forest, N47°27'37" E11°18'36", MTB/Q: 8533/24, elev. c. 1575 m a.s.l.	11.07.2016	J. Kruse	3658	GLM-F107703	MF924693	MH022817	MF939252	MF939318
	R. montanus	**Germany, Bavaria**	**Upper Bavaria, county Garmisch-Partenkirchen, c. 2.8 km SE of Mittenwald, Karwendel mountains, meadows around Brunnstein cabin, N47°24'49" E11°16'41", MTB/Q: 8533/43, elev. c. 1475 m a.s.l.**	**08.07.2016**	**J. Kruse**	3657	**GLM-F107702**	**MF924692**	**MH022816**	**MF939251**	**MF939317**
	R. montanus	Germany, Bavaria	Upper Bavaria, county Garmisch-Partenkirchen, c. 2.8 km SE of Mittenwald, Karwendel mountains, meadows around Brunnstein cabin, N47°24'49" E11°16'41", MTB/Q: 8533/43, elev. c. 1475 m a.s.l.	08.07.2016	J. Kruse	3656	GLM-F107701	MF924719	MH022843	MF939270	MF939336
	R. montanus	Germany, Bavaria	Upper Bavaria, county Garmisch-Partenkirchen, c. 2.8 km SE of Mittenwald, Karwendel mountains, hiking path 290 from Brunnstein cabin towards Mittenwald, serpentines, thin mixed mountainous forest, N47°24'48" E11°16'33", MTB/Q: 8533/43, elev. c. 1380 m a.s.l.	08.07.2016	J. Kruse	3655	GLM-F107700	MF924684	MH022808	MF939248	MF939314
	R. montanus	Germany, Bavaria	Oberjoch, Iseler, elev. c. 1500 m a.s.l.	29.09.1997	M. Piepenbring	rr502	TUB-012586	MF924658	MH022782	MF939230	MF939296
Entyloma verruculosum	*R. lanuginosus*	Italy, Apulia	Monte Sant Angelo, Provinz Foggia, c. 12 km N of Monte Sant Angelo, road SP52b, Foresta Umbra, beech forest, N41°47'52" E15°58'44", elev. c. 720 m a.s.l.	19.04.2016	J. Kruse	3645	GLM-F107706	MF924651	MH022775	MF939223	MF939289

Type specimens are printed in **bold** face.

Considering the narrow host specificity for the species occurring on *Ficaria*, it is remarkable that these three *Entyloma* species are reported from about 46 mostly yellow flowered *Ranunculus* species, worldwide (Savchenko et al. 2012, Vánky 2012). *Entyloma microsporum* and *E. ranunculi-repentis* have the widest reported host range with 30 and 26 different *Ranunculus* host species, respectively (Vánky 2012). However, it is still to be demonstrated, whether these *Entyloma* species are indeed generalist species, like some biotrophic pathogens (Choi et al. 2009, Runge et al. 2011, Scholler et al. 2011, Morin et al. 2012), or represent complexes of specialised species that justify earlier attempts to split them into several species with narrow host spectra, specifically *Caeoma bullosum* on *R. chaerophyllos* and *E. pygmaeum* on *R. pygmaeus* (Saccardo 1915, Ciferri 1928), *E. ranunculacearum* on *R. acris*, *E. ranunculi-sclerati* on *R. sceleratus*, *E. ranunculorum* on *R. auricomus*, and *E. wroblewskii* on *R. polyanthemos* (Kochman 1934, 1936, Liro 1938). Only a small number of *Entyloma* spp. on *Ranunculus* species have been included in phylogenetic analyses (e.g., Begerow et al. 2000, 2002, 2006, Savchenko et al. 2014a, Savchenko & Carris 2017).

The aim of this study was to resolve the species boundaries of *Entyloma* species on *Ranunculus*, based on the combination of morphological, biological, and molecular markers, including four loci (ITS, *atp*2, *ssc*1, and *map*). For this, a broad set of host-fungus combinations was studied, including *Entyloma* specimen from eleven different *Ranunculus* species, mostly from Germany but also from the Mediterranean (Greece, Italy, Slovenia, Spain), and Central Europe (Austria, Poland, Slovakia).

MATERIALS AND METHODS

Specimen sampling, documentation, and nomenclature

This study is based on morphological and/or phylogenetic analyses of 96 *Entyloma* specimens from eleven different *Ranunculus* species and one *Ficaria* species that were either collected in different regions of Europe or obtained from private herbaria (Table 1). They were deposited in the herbarium Senckenbergianum Görlitz (GLM) and in the herbarium of the W. Szafer Institute of Botany, Polish Academy of Sciences, Kraków (KRAM F). The nomenclature of the host plant species is according to Euro+Med PlantBase (Euro+Med 2006–onwards), the nomenclature of the fungi is according to Index Fungorum (http://www.indexfungorum.org/) and Vánky (2012). The *Entyloma microsporum* complex and the *E. ranunculi-repentis* complex are defined as species complexes having sori forming swollen pustules filled with spores with cracked surfaces and sori forming flat leaf spots with tissue-embedded smooth spores, respectively.

Morphological examination

The morphology of sori and spores was studied using dry herbarium specimens. For each of the host species of the two presumed complexes, up to five specimens were analysed in detail, using those specimens for which four loci (ITS, *atp*2, *ssc*1, and *map*) could be obtained, with four exceptions: for *Entyloma* sp. on *Ranunculus auricomus* specimens included in the two loci (ITS and *atp*2) dataset were used; for *Entyloma* sp. on *R. oreophilus*, one of two specimens had only two loci available; for *Entyloma* sp. on *R. sceleratus*, four of five specimens had only two loci available; and for *E. eburneum* one of six specimens had only two loci available. The specimens morphologically analysed are listed in the respective species descriptions.

Preparations for light microscopy (LM) were done as follows. Thin freehand sections of sori with spores and conidiophores and conidia (if present) were mounted in 80 % lactic acid, heated to the boiling point, and then immediately examined using a Nikon Eclipse 80i light microscope (Nikon) at ×1000. Thirty spores were measured using the Nikon NIS-Elements BR 3.0 imaging software (Nikon). Measurements were rounded to the nearest 0.5 µm. LM micrographs were taken with a Nikon DS-Fi1 camera (Nikon). The species descriptions include combined values from all analysed specimens of the respective species.

DNA extraction, primer design, PCR, and sequencing

Genomic DNA was isolated from 96 *Entyloma* herbarium specimens (Table 1). For methods regarding isolation, homogenisation of fungal material, and DNA extraction see Lutz et al. (2004) as well as Kruse et al. (2017a). PCR amplification of the complete ITS nrDNA (internal transcribed spacers) was performed with the conditions outlined in White et al. (1990), using M-ITS1 (Stoll et al. 2003) as forward and ITS4 (White et al. 1990) or smITS-R2 (Kruse et al. 2017a) as reverse primers. Plant ITS was amplified using primer pair ITS1P/ITS4 (Ridgway et al. 2003) with an annealing temperature of 53 °C. The amplification of the *atp*2 (ATP synthase subunit 2) locus was done according to Kruse et al. (2017b), using the F8/R4 primer combination with an annealing temperature of 54 °C. For the *ssc*1 (member of the heat shock protein family) and *map* (methionine aminopeptidase) locus used in Kruse et al. (2017b) two new primer sets specific for the *Exobasidiomycetes* were designed in this study on the basis of unpublished genome sequences of *Exobasidium vaccinii* and *Pseudomicrostroma juglandis*. The set of primers designed along the lines described in Kruse et al. (2017b) was tested on a variety of *Exobasidiomycetes* genera (*Entyloma*, *Exobasidium*, and *Tilletia)* and *Ustilaginomycetes* (*Urocystis*) with an annealing temperature of 53 °C. For the primer combinations providing best results gradient PCRs were conducted (50 °C to 60 °C and 60 °C to 72 °C) using *Entyloma* sp. samples and the optimal temperature was selected based on amplification strength and the absence of unspecific amplification. For the amplification of the *ssc*1 locus of *Entyloma* spp. this revealed the optimal primer pair to be *ssc*1_F3ex (5'GWGGWGAAGACTTYGACTTGT3') and *ssc*1_R5ex (5'ACACCACCYTGRATSGAAGC3') with an annealing temperature of 58 °C. For the amplification of the *map* locus of *Entyloma* spp. *map*_F3ex (5'AGYTGCTRATRTCGTTCCACCA3') and *map*_R3ex (5'CCAYGCCAAYTTGGCCAAGAC3') with an annealing temperature of 60 °C gave the best results.

PCR conditions were according to Kruse et al. (2017b), but with 46 PCR cycles. The resulting amplicons were sequenced at the sequencing laboratory of the Senckenberg Biodiversity and Climate Research Centre (BiK-F, Senckenberg, Germany) using the primers used in PCR, except for the *map*_F3ex/*map*_R3ex amplicons which were sequenced with a shortened reverse primer: *map*_R3exShort (5'CCAAYTTGGCCAAGAC3'). Sequences were deposited in GenBank (accession numbers are given in Table 1).

Molecular phylogenetic reconstruction

In total 91 ITS, 91 *atp*2, 64 *ssc*1, and 64 *map* sequences from *Entyloma* species affecting members of the genus *Ranunculus* were used for phylogenetic reconstructions in two different datasets. In addition to *Entyloma* on *Ranunculus* some *Entyloma* species on *Ficaria verna* were included because initial analyses suggested that *Entyloma* species on *Ficaria verna* might belong to the *E. ranunculi-repentis* complex. The first dataset comprised all four loci for 66 *Entyloma* specimens. The second comprised only ITS and *atp*2 sequences for 96 *Entyloma* specimens. Alignments were done for each locus independently using MAFFT (Katoh & Standley 2013) v. 7, employing the G-INS-i algorithm, and subsequently leading and trailing gaps were removed. After this and after checking for supported phylogenetic conflicts between the loci using Minimum Evolution analysis as outlined below, the aligned sequences of the individual loci were concatenated to obtain the datasets for phylogenetic analyses. For dataset 1 the resulting total alignment contained 1 871 characters (ITS: 523, *atp*2: 480, *ssc*1: 394, *map*: 474) for dataset 2 the resulting total alignment contained 1 003 characters (ITS: 523, *atp*2: 480). The methods for phylogenetic analyses were according to Kruse et al. (2018) for reconstructions using Minimum Evolution, Maximum Likelihood, and Bayesian Inference. To determine diagnostic bases for the different *Entyloma* species, alignments were checked manually for differences between the different host-fungus combinations. Host plant determination was verified comparing their ITS sequences to those deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) using BLASTN (Altschul et al. 1997).

RESULTS

Molecular phylogenetic reconstruction

There were no strongly supported conflicts between the topologies of the trees obtained from single loci. Minimum Evolution, Maximum Likelihood, and Bayesian Analyses yielded consistent topologies for both datasets. The results of the phylogenetic reconstructions based on four and two loci are given in Fig. 1 and Fig. 2, respectively.

All analyses revealed three strongly supported major lineages. The first lineage corresponded to the *E. microsporum* complex and included specimens from *Ranunculus acris*, *R. paludosus*, *R. polyanthemos* subsp. *nemorosus*, and *R. repens*, with gross morphology that matched *E. microsporum*. The second lineage corresponded to the *E. ranunculi-repentis* complex and included specimens from *Ficaria verna*, *Ranunculus acris*, *R. auricomus*, *R. bulbosus*, *R. lanuginosus*, *R. marginatus*, *R. montanus*, *R. oreophilus*, *R. paludosus*, *R. polyanthemos* subsp. *nemorosus*, *R. repens*, and *R. sceleratus*, with gross morphology that matched *E. ranunculi-repentis*. The third lineage was represented by *E. verruculosum* on *R. lanuginosus*. Within both the *Entyloma microsporum* complex and the *E. ranunculi-repentis* complex, specimens from the same host plant species grouped together, with few exceptions. Within the *E. microsporum* complex the majority of specimens from *Ranunculus repens* formed a clade together with two accessions on *R. acris*. Two specimens from *R. repens* (ML471, 102) clustered with specimens from *R. polyanthemos* subsp. *nemorosus*. Within the *E. ranunculi-repentis* complex, specimens from *Ranunculus repens* clustered together with specimens from *R. bulbosus* and *R. polyanthemos* subsp. *nemorosus.*

Comparing the results from both datasets, support values for the topology inferred from two loci (ITS and *atp*2) were mostly lower than from four loci (ITS, *atp*2, *ssc*1, and *map*), and the topology was generally more resolved in the latter. Within the *E. microsporum* complex, a group of specimens on both *Ranunculus polyanthemos* subsp. *nemorosus* and *R. repens*, were a sister lineage to specimens on *R. acris* and *R. repens*. The specimens on *R. paludosus* formed the sister group to all specimens mentioned so far. Within the *E. ranunculi-repentis* complex support values for the relationships of the well-supported host-specific clades were generally low.

Diagnostic bases enable the molecular identification of species given on the basis of a defined alignment (Bennett et al. 2017, Kruse et al. 2018). Diagnostic bases for the different *Entyloma* species are given as an overview in Fig. 3 and detailed in Table 2.

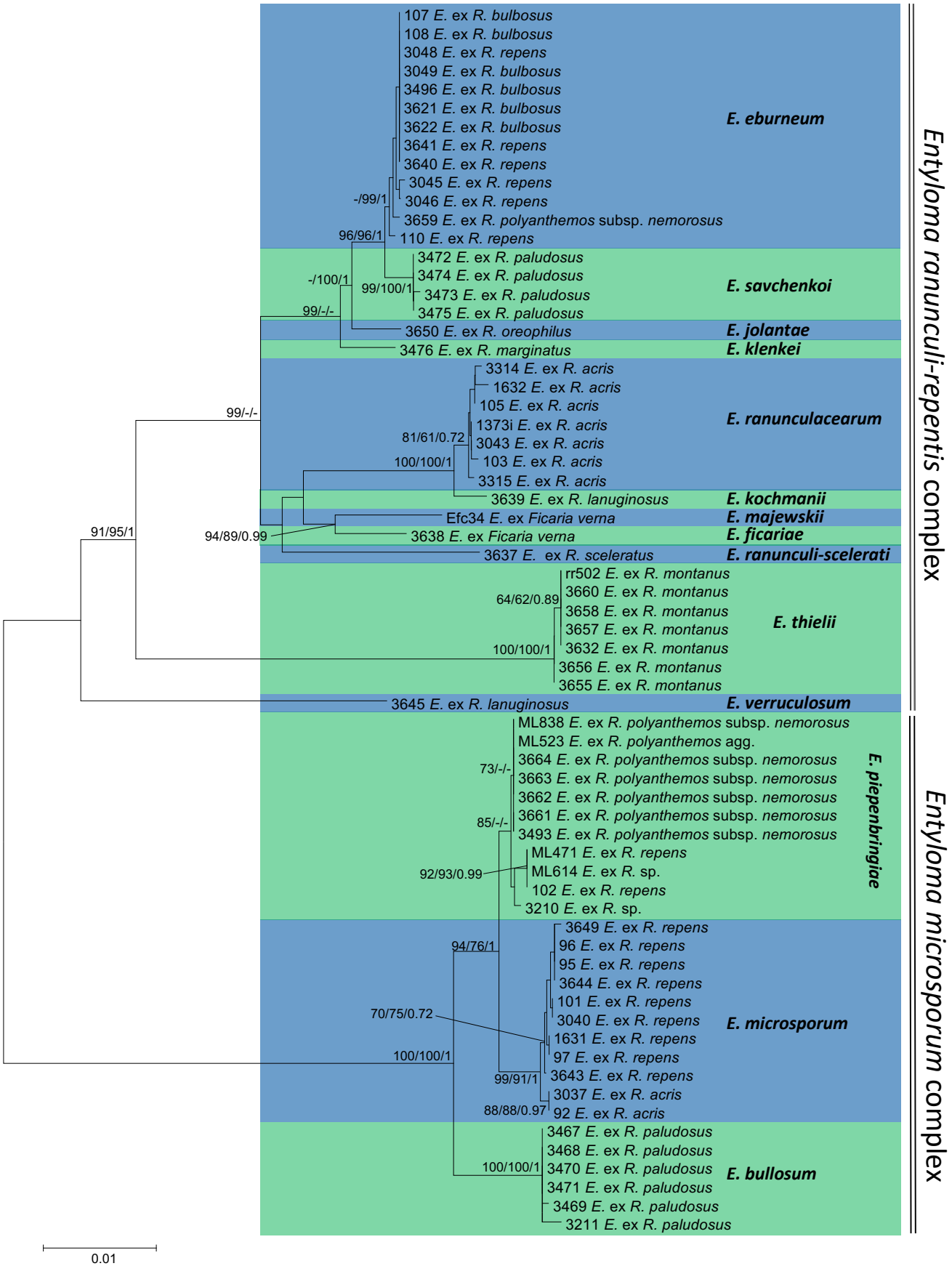

Fig. 1 Phylogenetic relationships of *Entyloma* species on *Ranunculus* spp., rooted with the specimens of the *Entyloma microsporum* complex, based on Minimum Evolution analyses of four loci (ITS, *atp*2, *ssc*1, and *map*). Numbers on branches denote bootstrap support in Minimum Evolution and Maximum Likelihood, as well as *a posteriori* probabilities from Bayesian Analyses, in the respective order. Values below 60 % are not shown. The scale bar indicates the number of substitutions per site.

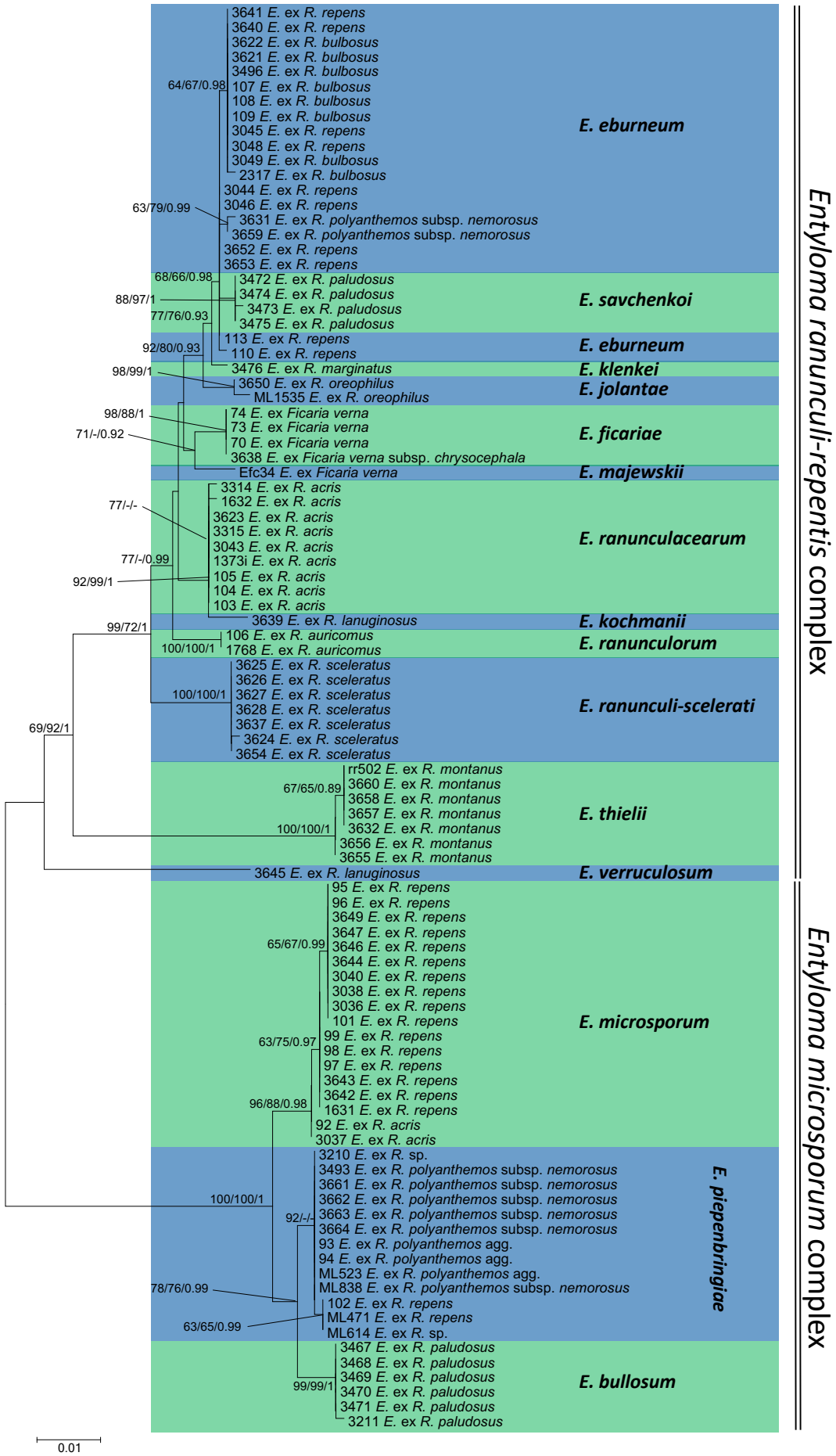

Fig. 2 Phylogenetic relationships of *Entyloma* species on *Ranunculus* spp., rooted with the specimens of the *Entyloma microsporum* complex, based on Minimum Evolution analyses of two loci (ITS and *atp*2). Numbers on branches denote bootstrap support in Minimum Evolution and Maximum Likelihood, as well as *a posteriori* probabilities from Bayesian Analyses. Values below 60 % are not shown. The scale bar indicates the number of substitutions per site.

atp2 – 480 bp

RGAGTCMARAGGGTCGAC**R**G C**R**GGGTA**R**ATRYC**B**AGSTCR GCAATACCACGRGCSAGCGA RGTGGTGGCGTCCAAGTGAG CGAAGGTSGTGGCKGGYGCA
GGRTCCGTCAAGTCATCRGC TGGYACGTATACGGCCTG**W**A C**S**GARGTRATGGAACCCTTC TTGGTCGT**R**GTAATGCGCTC TTGCATACCACCCATGTCGG
TGGASAGHGTRGGCTGGTA**R** CCTACGGCSGA**K**GGRATACG **R**CCGAGCAGGGCAGASGTCT CGGARCCRGCTTG**M**GTGAAR CGGAAAATGTTGTC**R**ATGAA
GAGCAGYACRTC**Y**TGTCCCC TRNNN**R**H**R**T**S**WR**N**KY**R**H**R**V**N** NNNWRWGT**S**AG**Y**B**BK**B**HD**DN N**B**H**N**RBBRWY**KH**R**DY**RN**Y**K**H** Y**R**N**N**ASM**RY**V**NNN**YTACTCA
CTCCTCGTCACGGAAGTACT C**R**GCAAT**V**GTCA**R**ACC**S**GT**R** AGGGCKACRCGGGCACG**V**GC **W**CCRGG**R**GGCTC**R**TTCATYT

ITS – 523 bp

TSAGACTGCAAAGAGTCTTT CGCCGRRTTAGAARCAGACR CW**NN**KG**Y**ACAAGAA**R**GTAAG GGCAGTTNNNNNRMCYGCCG CCTCAACGAATGACTTTATC
ACGTTGAGGACRCCTACRCA TN**YR**TRYAC**N**GGTCCAGCTA AT**R**MATTTGAGGTGAGC**B**GT **HN**N**N**NNN**NN**N**RV**AACRGSAA **S**CACCCAAGGRCCAA**Y**CCCA
R**R**CAAC**R**C**R**A**B**AAAGCATTG AGGGT**K**GAGAATTCATGACA CTCAAACAGGCATGCTCCTC GGAATACCAAGGWGCGCAAG GTGCGTTCAAAGATTCGATG
ATTCACTGAATTCTGCAATT CACATTACTTATCGCATTTC GCTGCGTTCTTCATCGATGG GAGAACCAAGAGATCCGTTG TCAAAAGTTGT**N**TT**K**TGTTT
CTACTTGYGTAG**K**TACAGTG ACATTCAK**Y**TAYTTNNNNNN TGT**R**TG**WR**TAAA**N**GGN**K**TGC **YM**G**Y**R**R**CRRACRARATACT**K** GSGYATCRGBCGTCACTARC
GGTGCACAGGTGTGTGGATA TGG

map – 474 bp

VGA**B**ARTTCMGGGTTGGG**R**T G**R**TT**S**AG**D**GC**R**TCBGA**R**CT**D** CCGTGCTCGTGCARGTA**D**CC GGCRGGCGTRAGRACGTAGA A**S**GACTCYTT**R**TAVK**K**YTTG
GTGAARCGCTTCTT**R**CGCTC GAGAATGCCCTGGTGRACGC ARATRACSGAVGGRTC**Y**TCC TTGCC**K**GG**R**TA**R**TC**R**ATCGT YTCGGGGTTGCGCARRGGYG
TGTCWGG**R**TC**N**ARMAGGTG**R** TCCGA**R**CGMGCAGC**R**AARGC RATCCAYTCKGC**R**TT**B**GGCT GMAG**B**GCRCGCAT**Y**TGYTGA CCYARRCC**R**AGCCACGTGTC
YTGWACCTGDGCCGACATGC G**H**CCGCTCCACTCRTCRAAC GT**Y**TGCCAGGC**R**CTCTGRAT GCT**K**CGAACGATGCCCTCCT CRAAGTGAGCRCTGTTCTGC
TGCAT**R**ATRATGATRGACTT TTGYAG**B**GCRTTYTCCTCGT TDACTTGCTTTTGCARCTGA CGGAARACRGCRTT

*ssc*1 – 394 bp

RWGGAAGCRCCAATGGCAAC AGCYTC**R**TCGGGGTT**R**ACAC CCTTRGAYGG**R**TCGCG**Y**TTG AAGATGC**Y**CTT**R**AC**D**GTCTC **R**AGDACCTTKGGCAT**R**CGCG
ACATWCC**R**CC**D**ACCATGATG ACGTCCTGHACRTCGYTVGC C**Y**T**R**ATGCCTGCRTCRGCRA TGGCCTTCTK**R**CA**Y**GGCTC**R** ACSGTRCG**Y**TC**R**A**Y**GAGCTT
GCCAAC**V**AG**M**CCCTCRAGYT GCGMKCGGCTCATCTT**K**GTR TTGATGTGCTTGGG**Y**CC**R**GA **R**GC**R**TC**D**GCGGTRATGTAGG G**S**AGYGARATGTCSGTTTGC
GC**M**GTCGA**V**GARAGYTCRAT CTTGGCCTTCTCRGC**R**GCCT CGCG**R**ATRCGMTGAATGGCC ATGCGRTCYTGMRARAGGTC RATGCCAGA**R**TCCY

Fig. 3 Consensus sequences for *atp2*, ITS, *map*, and *ssc1*, with diagnostic positions for *Entyloma* species on *Ranunculus* highlighted in **bold** type.

Morphology

The three major phylogenetic lineages could be distinguished by teliospore surface ornamentation. Spores from species in the *E. microsporum* complex had a cracked surface; those from the *E. ranunculi-repentis* complex were smooth; and those from *E. verruculosum* were verrucose. Species in the *E. microsporum* complex always formed sori in hard, swollen galls. Most species-specific lineages of the *E. ranunculi-repentis* complex produced an asexual morph, which was not observed in the *E. microsporum* and the *E. verruculosum* complexes. Morphological differences within the two species complexes were generally low. The morphological characterisation of the species is included in species descriptions, depicted in Fig. 4–16, and summarised in Table 3.

TAXONOMY

In this section an overview on accepted *Entyloma* species on *Ficaria* and *Ranunculus* is given, and six new species are introduced. We have refrained from designating formal epitypes in the current study. The progress in sequencing technologies has already enabled the sequencing of the whole genome of specimens from the mid-19th century Irish Potato Famine (Yoshida et al. 2013, 2014). Thus, it seems to be only a matter of time until cheap and reliable whole genome sequencing from historic specimens will become routine. However, if the historic specimens turn out to be demonstrably devoid of DNA that can be used for sequencing, the reference specimens given in this section could be designated as epitypes.

Entyloma microsporum complex

Entyloma bullosum (Sacc.) J. Kruse, M. Lutz, Piątek & Thines, *comb. nov.* — MycoBank MB823957; Fig. 4

Basionym. *Caeoma bullosum* Sacc., Nuovo Giorn. Bot. Ital., n.s. 22: 32. 1915.

Type. Malta, Uied il Kleigha, on *Ranunculus* '*chaerophyllos*' (= *R. paludosus*), Mar. 1914, *A. Caruana-Gatto* (type could not be located, probably lost); – Greece, Rhodes, eastern coast, SE of Archangelos, c. 1.5 km S Stegna, Phrygana, northeast slope, N36°11'49" E28°08'06", elevation c. 70 m a.s.l., on *Ranunculus paludosus*, 9 Mar. 2016, *J. Kruse* (GLM-F107632 neotype designated here; MycoBank MBT380639; ex-type sequences available in GenBank: MF924658 (ITS), MH022782 (*atp2*), MF939296 (*ssc*1), MF939230 (*map*)).

Sori in the leaves, rarely leaf petioles, forming distinct, rounded, hard, swollen pustules on leaves, 1–2 mm diam, markedly delineated from the healthy host tissue, at first yellow-greenish, later brownish, usually closed but sometimes old pustules cracked. *Spores* embedded in the leaf tissue, single, very densely crowded in the intercellular space between the mesophyll cells, which, in older pustules are destroyed; spores subhyaline (in young sori), pale yellow to yellow (in mature sori), very variable in shape and size, globose, subglobose, broadly ellipsoidal, rarely elongated, usually more or less polyangular, (11.5–)15.0–21.5(–26.5) × (10.5–)12.0–16.5(–19.5) µm (av. ± SD, 18.1 ± 2.9 × 14.9 ± 1.8 µm, *n* = 150/5), with smooth surface; teliospore wall 2-layered, 2.5–7.0(–8.0) µm thick (including inner layer c. 0.8–1.0 µm thick), layers well visible in LM, often with angles, inner layer evenly thickened, outer layer unevenly thickened, spore surface rough or superficially cracked, rarely smooth. *Asexual morph* not found.

Diagnostic bases — Within the *E. microsporum* complex there are 19 diagnostic bases across all four loci (Fig. 3, Table 2).

Host plant — Parasitic on *Ranunculus paludosus*.

Additional specimens examined. Greece, Rhodes, c. 2.8 km NW of Lindos, Phrygana, way up Mountain, hiking path, N36°05'48" E28°03'13", elevation c. 145 m a.s.l., on *Ranunculus paludosus*, 10 Mar. 2016, *J. Kruse* (GLM-F107634); eastern coast, c. 3.5 km NE of Archangelos, Tsambika, way up to the monastery, northern slope, phrygana, N36°14'16" E28°09'16", elevation c. 160 m a.s.l., on *Ranunculus paludosus*, 11 Mar. 2016, *J. Kruse* (GLM-F107635); c. 1 km S of Salakos, way up to Mt Profitis Ilias, phrygana, N36°17'03" E27°56'38", elevation c. 275 m a.s.l., on *Ranunculus paludosus*, 13 Mar. 2016, *J. Kruse* (GLM-F107636); c. 1 km NW of Siana, way up to Akramitis, open phrygana, plateau, N36°09'23" E27°45'59", elevation c. 650 m a.s.l., on *Ranunculus paludosus*, 15 Mar. 2016, *J. Kruse* (GLM-F107637).

Notes — The smut specimens with swollen pustules on the leaves of *Ranunculus paludosus* are usually assigned to *Entyloma microsporum* (Vánky 2012), but the molecular analyses in the present study reveal that they form a distinct lineage, for which the name *Caeoma bullosum* is available. This species was described by Saccardo (1915) from leaves of *Ranunculus*

Table 2 The diagnostic bases within the *Entyloma microsporum* complex and the *Entyloma ranunculi-repentis* complex, apart from the type host of the respective complex.

	Gene Loci							
	atp2		*map*		*ssc1*		ITS	
	Position	Base	Position	Base	Position	Base	Position	Base
***E. microsporum* complex**								
on *Ranunculus paludosus*	232	T / G	28	G / A	36, 171	G / A	196	T / C
	364, 388, 440, 467	G / A	115, 289	A / G	68	T / C		
	375	C / T	256	T / G	237	T / G		
	382	A / G	322, 343	T / C	255	C / T		
					282	C / G		
***E. ranunculi-repentis* complex**								
on *Ranunculus acris*	x	x	x	x	27, 72, 96	A / G	x	x
					57	T / C		
on *Ranunculus auricomus*	142	C / G	x	x	x	x	168	G / A
	358	T / A o. G						
	437	G / C						
	440	G / A						
	473	A / G						
on *Ficaria verna* (*E. ficariae*)	x	x	22, 169	A / G	142	C / T	43	T / –
			364	T / G			44	G / –
							209	G / A
on *Ficaria verna* (*E. majewskii*)	328, 336, 428	A / G	4	T / G o. C	1, 261	G / A	226, 413	G / T
			19, 172	A / G	2	A / T		
					111	T / G o. A		
					258, 389	A / G		
on *Ranunculus lanuginosus*	389	T / C	x	x	144	A / G	462	C / A
on *Ranunculus marginatus*	458	C / G	226, 235	G / A	345	A / G	x	x
			253	A / G o. R				
			274	C / T				
on *Ranunculus montanus*	22, 28, 220, 241	G / A	1, 211	A / C o. G	75, 267	A / G o. T	130, 143, 171, 202, 207	A / G
	139	A / T	28, 58	G / T o. A	81, 108, 264, 267	A / G	47, 123,162, 429, 461	C / T
	274	C / A	37, 92, 175, 208, 220	A / G	189	T / C	55, 124, 169, 172, 444	G / A
	313, 327, 337, 352, 378	C / T	96	T / C	309	G / C	181	C / G
	325, 391	– /A o. G	166	G / T			457, 480	G / T
	354	T / G o. C	352	G / A			158	T / G
	339	C / A o. G	427	G / T o. C			161	A / T
	355	T / G					164	– / G
	362	G / T					211	G / T o. C
	372	C / T o. A					392	– / A
	380, 461	T / A						
	384	A / G o. C						
	392	– / T						
	422, 433	A / G						
on *Ranunculus oreophilus*	295, 374	A / G	31	A / G	192	A / G	447	A / T
	330	C / G			194	C / T		
on *Ranunculus paludosus*	333	T / G o. –	x	x	207	A / G o. C	395	G / T
							464	C / T
on *Ranunculus sceleratus*	1, 169	A / G	25, 82	C / G	51	A / G	448, 466	A / G
	34, 357	T / C	40	T / G	174	C / T	453	– / G
	349	G / C	94	A / G o. C	180, 336	G / A		
	354	C / G o. T	157	T / C	210, 303	C / A		
	358	G / A o. T	265	G / T o. C				
	371	T / G	406	A / G				
	384	C / A o. G						
	393	T / C o. –						

* / = instead of; x = no diagnostic bases; o. = or.

chaerophyllos collected in Malta. Sydow (1924) considered that *Caeoma bullosum* was identical with *E. microsporum*. In the protologue, Saccardo (1915) did not provide the author of the name *Ranunculus chaerophyllos*, and in the current usage this name can be applied to three species, namely *R. chaerophyllos*, *R. gracilis*, and *R. paludosus*, of which only the latter occurs in Malta (Euro+Med 2006–onwards). Thus, the host plant for *Caeoma bullosum* is assumed to have been *Ranunculus paludosus*. No authentic material of this species is currently preserved in the herbarium of P.A. Saccardo deposited in PAD (R. Marcucci, pers. comm.) or in the herbarium of H. Sydow in B (R. Lücking, pers. comm.). The morphological characters provided in the protologue (swollen sori, 1–2 mm diam, yellow, angular, globose, as well as ellipsoidal spores 20–23 × 18–20 µm, spore wall of 3–5 µm thickness with a more or less warty surface; – excerpt from the Latin description) agree well with the morphology of the specimens analysed in the current study. Therefore, a neotype was designated from among the sequenced specimens to fix the application of this name. *Entyloma bullosum* differs from the other currently recognized species in the *E. microsporum* complex by a larger mean spore size and thicker spore walls.

Entyloma microsporum (Unger) J. Schröt., in Rabenhorst, Fungi Europ. no. 1872. 1874 — Fig. 5

Basionym. *Protomyces microsporus* Unger, Die Exantheme der Pflanzen, etc.: 343. 1833.

Synonym. *Entyloma ungerianum* de Bary, Bot. Zeitung (Berlin) 32: 101. 1874, nom. nov. superfl. pro *P. microsporus*.

Type. Austria, Tirol, Kitzbühel, on *Ranunculus repens*, *F. Unger* (type could not be located, probably lost). – Germany, Hesse, county Groß-Gerau, Ginsheim-Gustavsburg, bikeway to Mainspitzdreieck, wayside, N49°59'37" E08°17'46", on *Ranunculus repens*, 17 Nov. 2013, *J. Kruse* (GLM-F107661 neotype designated here; KRAM F-59043 isoneotype; MycoBank MBT380061; ex-type sequences available in GenBank: MF924636 (ITS), MH022760 (*atp*2), MF939279 (*ssc*1), MF939213 (*map*)).

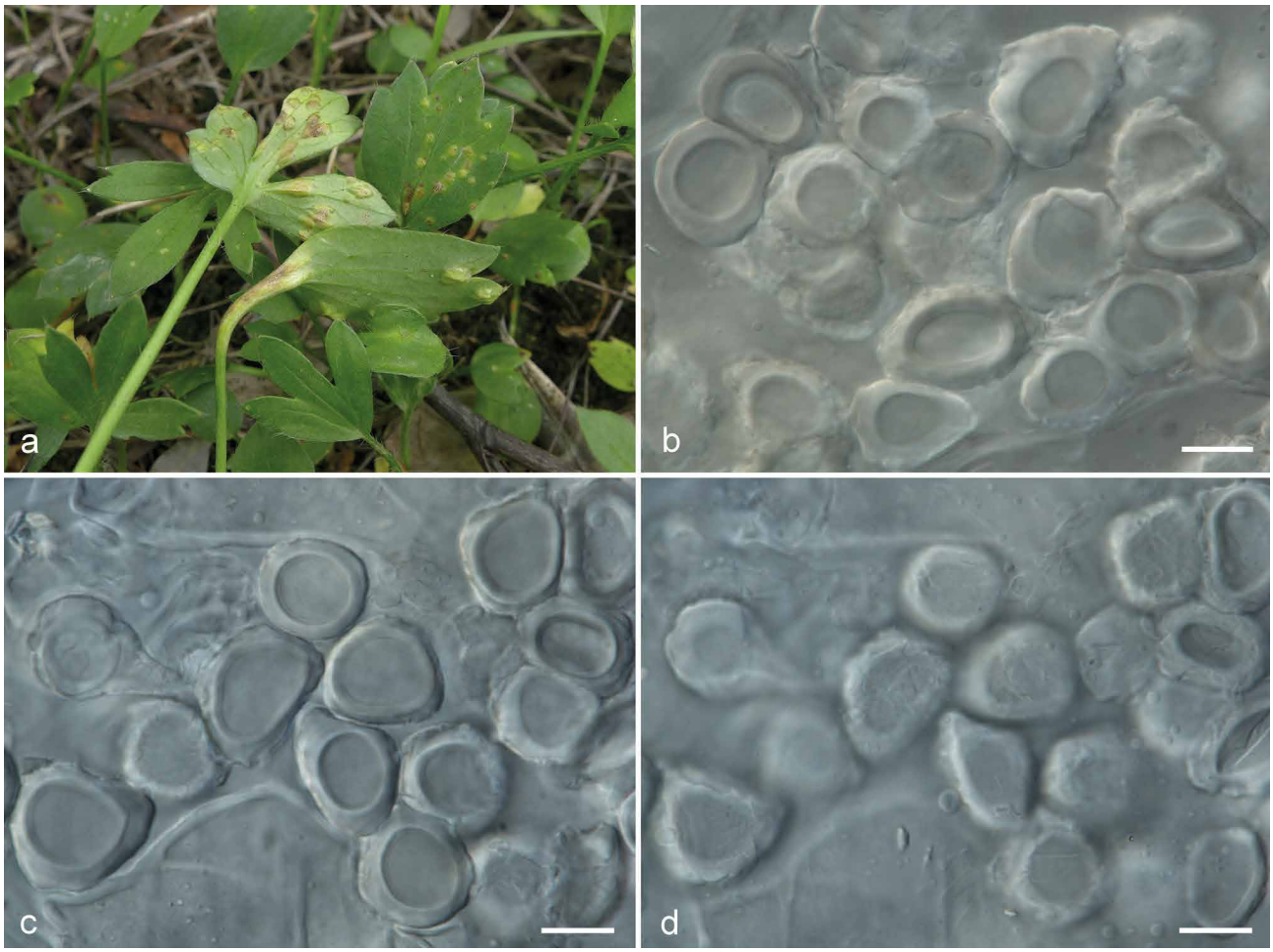

Fig. 4 *Entyloma bullosum* on *Ranunculus paludosus*. a. Macroscopic symptoms of infection; b–d. spores, as seen in light microscopy, median and superficial views (from neotype). — Scale bars = 10 µm.

Sori in the leaves, rarely leaf petioles, on the leaves forming distinct, rounded or elongated, hard, swollen pustules, 1–6 mm diam, markedly delineated from the healthy host tissue, at first yellow-cream, later brownish, pustules at first closed but at the maturity cracked. *Spores* embedded in the leaf tissue, single, very densely crowded in the intercellular space between the mesophyll cells, which in mature pustules are totally destroyed; spores subhyaline or rarely pale yellow, variable in shape and size, globose, subglobose, broadly ellipsoidal, rarely elongated, often more or less irregular, 10.0–18.5(–24.0) × (9.5–)10.0–13.5(–17.5) µm (av. ± SD, 14.6 ± 2.8 × 12.2 ± 1.7 µm, *n* = 150/5), with smooth or granular context; wall 2-layered, (1.5–)2.0–4.5 µm, occasionally 7.0 µm thick (including inner layer c. 0.5–1.0 µm thick), sometimes with angles, layers well visible in LM, inner layer evenly thickened, outer layer evenly or unevenly thickened, spore surface rough or superficially cracked, rarely smooth. *Asexual morph* not found.

Host plants — Parasitic on *Ranunculus acris* and *R. repens*.

Additional specimens examined. Germany, Baden-Württemberg, county Konstanz, Hegau, W of Singen, way up to Mt Hohentwiel, wayside, elevation c. 600 m a.s.l., on *Ranunculus repens*, 29 May 2013, *J. Kruse* (GLM-F107672); Bavaria, county Rottal-Inn, Simbach, road St 2112, grassland at roundabout, N48°16'23" E13°00'53", elevation c. 370 m a.s.l., on *Ranunculus acris*, 14 Aug. 2014, *J. Kruse* (GLM-F107663); Lower Saxony, county Hildesheim, Brüggen, Kirschweg, Sieben Bergen, Mt Hohe Tafel, wayside, elevation c. 395 m a.s.l., on *Ranunculus repens*, 8 May 2011, *J. Kruse* (GLM-F107667). – Poland, Małopolska Province, Tatra Mts, Hala Gąsienicowa glade (near Murowaniec cabin), elevation c. 1510 m a.s.l., on *Ranunculus repens*, 24 Sept. 2005, *J. Piątek & M. Piątek* (KRAM F-59041).

Notes — This species has been first described as *Protomyces microsporus*. In the protologue, Unger (1833) contrasted it with *Protomyces macrosporus* (*Ascomycota*, *Taphrinales*) as a species forming pustules on stems and leaf veins of *Ranunculus repens* and having very small, rounded and pale sporidia (= spores). De Bary (1874) obtained spore germination of this species and concluded that it is not a member of *Protomyces* but a smut fungus, for which he described the distinct genus, *Entyloma*. He introduced the new name *Entyloma ungerianum* for this species. However, this was superfluous and Schröter (in Rabenhorst 1874) combined the species in *Entyloma* as *E. microsporum*. The original material probably does not exist anymore. Piepenbring (2003) could not locate it in BPI, GJO, M, and W. The current species concept of *E. microsporum* is based on a long tradition of application of this name to any specimen of *Ranunculus* displaying the characters reported by Unger (1833). However, spore sizes were not reported in the protologue (Unger 1833). Also De Bary (1874) did not provide spore sizes for material examined by him. Schröter (1887) finally measured the spores of this species, reporting the following values: spores 15–24 µm long and 12–17 µm wide, wall up to 7 µm thick. Similar counts were reported more recently, e.g., Vánky (1994, 2012: spores 11–23 × 10–16 µm, wall 1–9 µm thick), Scholz & Scholz (1988: spores 10–25 µm diam, wall 1–9 µm thick), but Kochman (1936) reported that spores were 10–20 µm diam (with mean 14 µm) and the wall thickness was reported as 1.5–5 µm. The latter observations are in agreement with our observations, and it seems possible that the larger spore sizes reported by other authors result from the presentation of extreme values without indicating which values predominated in the overall spore counts.

In the phylogenetic analyses the specimens forming swollen pustules on *Ranunculus repens* clustered in two lineages: one

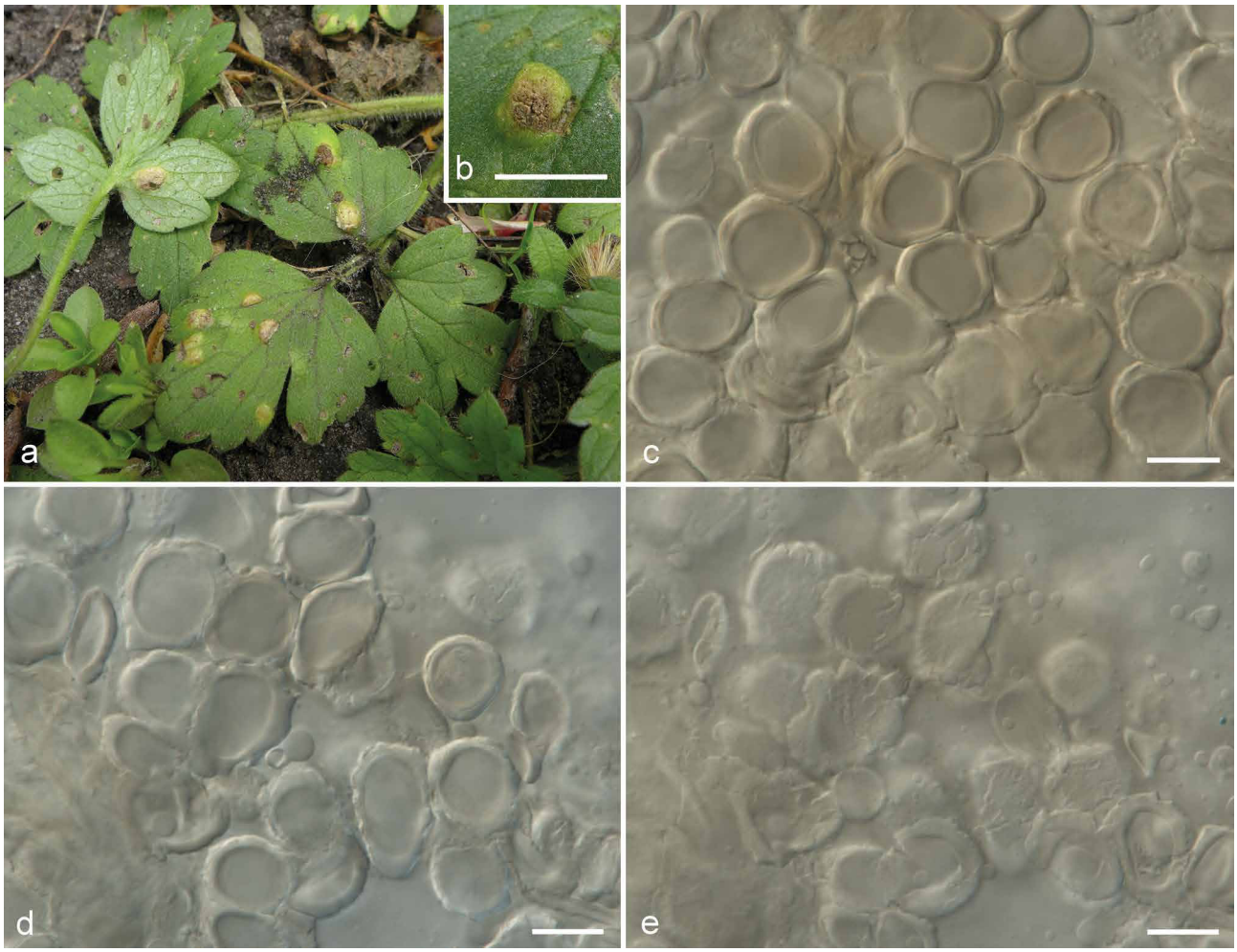

Fig. 5 *Entyloma microsporum* on *Ranunculus repens*. a–b. Macroscopic symptoms of infection; c–e. spores, as seen in light microscopy, median and superficial views (from neotype). — Scale bars: b = 5 mm; c–e = 10 µm.

containing the majority of accessions on *R. repens* and two accessions on *R. acris*, and the other containing the minority of accessions on *R. repens* with predominance of accessions on *R. polyanthemos* subsp. *nemorosus*. The specimens in both lineages were morphologically similar, and it is not clear to which of the two lineages the name *E. microsporum* could be applied. Therefore, to stabilize this fungus name we designate a neotype from specimens from the lineage where most accessions on *R. repens* were placed. The specimens on *R. acris* were inseparable morphologically and only very weakly separated genetically, and are therefore currently remain in *E. microsporum*. The specimens forming the second lineage are accommodated in the novel species, *E. piepenbringiae*.

Entyloma piepenbringiae J. Kruse, M. Lutz, Piątek & Thines, *sp. nov.* — MycoBank MB824511; Fig. 6

Etymology. Named in honour of Prof. dr Meike Piepenbring (Frankfurt a. Main), for her contributions to the knowledge of temperate and tropical smut fungi.

Type. Germany, Bavaria, Oberallgäu, Einödsbach, Allgäu Alps, hiking path from Black cabin to Rappensee cabin, meadow W Rappensee cabin, N47°17'24" E10°14'40", elevation c. 1900 m a.s.l., on *Ranunculus polyanthemos* subsp. *nemorosus*, 26 July 2015, *J. Kruse* (GLM-F107687 holotype; ex-type sequences available in GenBank: MF924664 (ITS), MH022788 (*atp*2), MF939302 (*ssc*1), MF939236 (*map*)).

Sori in the leaves, rarely leaf petioles, on the leaves forming distinct, rounded or elongated, hard, swollen pustules, 1–5 mm diam, markedly delineated from the healthy host tissue, at first creamy yellow, later brownish, usually closed but sometimes old pustules cracked. *Spores* embedded in the leaf tissue, single, very densely crowded in the intercellular space between the mesophyll cells, which in older pustules are totally destroyed; spores subhyaline or rarely pale yellow, variable in shape and size, globose, subglobose, broadly ellipsoidal, rarely elongated, often more or less irregular, (10.5–)12.0–17.5(–21.0) × (9.0–)10.0–15.5(–16.0) µm (av. ± SD, 14.5 ± 2.4 × 12.5 ± 1.4 µm, *n* = 150/5), with smooth context; wall 2-layered, (1.5–)2.5–4.0(–6.0) µm thick (including inner layer c. 0.7–1.0 µm thick), sometimes with angles, layers well visible in LM, inner layer evenly thickened, outer layer evenly or unevenly thickened, spore surface rough or superficially cracked, rarely smooth. *Asexual morph* not found.

Host plants — Parasitic on *Ranunculus polyanthemos* subsp. *nemorosus* and *R. repens*.

Additional specimens examined. Germany, Baden-Württemberg, county Konstanz, communal Moos, S of Weiler, near Grey Reed, wayside, elevation c. 445 m a.s.l., on *Ranunculus repens*, 30 May 2013, *J. Kruse* (GLM-F107694); Bavaria, Upper Bavaria, county Garmisch-Partenkirchen, c. 2.8 km SE of Mittenwald, Karwendel mountains, meadow around Brunnstein cabin, N47°24'49" E11°16'41", elevation c. 1475 m a.s.l., on *Ranunculus polyanthemos* subsp. *nemorosus*, 8 July 2016, *J. Kruse* (GLM-F107690); hiking path 290 towards Brunnstein cabin, serpentines, open mixed mountainous forest, N47°24'44" E11°16'23", elevation c. 1260 m a.s.l., on *Ranunculus polyanthemos* subsp. *nemorosus*, 6 July 2016, *J. Kruse* (GLM-F107693); c. 3.2 km SE of Mittenwald, Karwendel mountains, hiking path 291 from Brunnstein cabin towards Mt Brunnsteinspitze, scree, N47°24'33" E11°16'59", elevation c. 1760 m a.s.l., on *Ranunculus polyanthemos* subsp. *nemorosus*, 7 July 2016, *J. Kruse* (GLM-F107691).

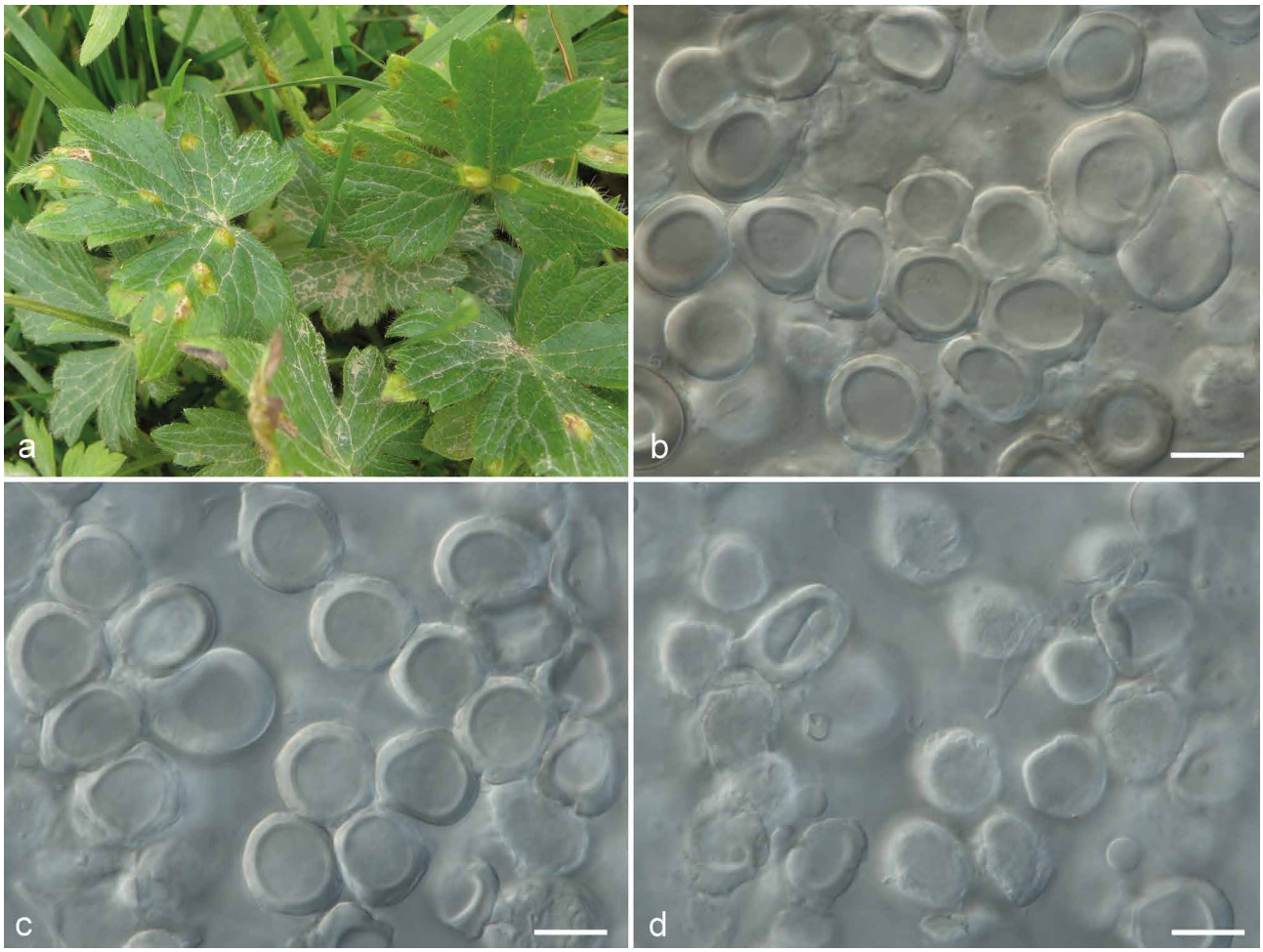

Fig. 6 *Entyloma piepenbringiae* on *Ranunculus polyanthemos* subsp. *nemorosus*. a. Macroscopic symptoms of infection; b–d. spores, as seen in light microscopy, median and superficial views (from holotype). — Scale bars = 10 µm.

Notes — The specimens on *Ranunculus polyanthemos* subsp. *nemorosus* and *R. repens* were morphologically similar and clustered together, and are therefore considered as belonging to the same species.

Entyloma ranunculi-repentis complex

Entyloma eburneum (J. Schröt.) J. Kruse, M. Lutz, Piątek & Thines, *comb. nov.* — MycoBank MB824512; Fig. 7

Basionym. *Fusidium eburneum* J. Schröt., Beitr. Biol. Pflanzen 2 (3): 373. 1877.

Type. On *Ranunculus repens*, (further details not included in the protologue, but probably the material was collected in Silesia, now in Poland, by J. Schröter, before 1877 (type could not be located, probably lost). – Poland, Małopolska Province, Kraków-Pleszów, at Suchy Jar street, on *Ranunculus repens*, 20 Nov. 2010, *M. Piątek* (KRAM F-59037 neotype designated here; MycoBank MBT380062; ex-type sequences available in GenBank: MF924689 (ITS), MH022813 (*atp2*)).

Synonyms. *Ramularia repentis* Oudem., Beih. Bot. Centralbl.: 15. 1902.
Type. The Netherlands, Valkenberg, on *Ranunculus repens*, 1900, *C.A.J.A. Oudemans* (L, see Braun 1998).

Entyloma ranunculi-repentis Sternon, L'hétérogenité du genre Ramularia, These, Nancy: 34, 45. 1925.
Type. Belgium, Gembloux, Virton and Rochefort, on *Ranunculus repens*, 1917, *F. Sternon* (no type designated, see Vánky 2012).

Entyloma wroblewskii Kochman, Acta Soc. Bot. Poloniae 11 (Suppl.): 291. 1934.
Type. Poland, Anin near Warszawa, on *Ranunculus polyanthemos*, 15 Sept. 1933, *J. Kochman* (KRAM F-2658 holotype; KRAM F-2656 and KRAM F-2657 isotypes).

Sori in the leaves, forming very distinct, flat, rounded, polyangular or irregular spots, 0.5–4 mm long, 0.5–2 mm wide, usually partly delineated by the leaf veins of the host, at first whitish or cream-coloured due to the presence of the conidiophores and conidia of the asexual morph, later pale brown on both sides of the leaf. *Spores* embedded in the leaf tissue, single, loosely scattered or moderately densely crowded in the intercellular space between the mesophyll cells; spores pale yellow to yellow, globose, subglobose or rarely broadly ellipsoidal, regular in shape, (9.5–)11.0–13.5(–16.0) × (9.0–)9.5–13.5(–14.5) µm (av. ± SD, 12.3 ± 1.4 × 11.3 ± 1.3 µm, *n* = 200/6), with smooth context; wall 2-layered, 1.0–1.5(–2.0) µm thick (including inner layer c. 0.5–0.8 µm thick), without angles, layers well visible in LM, both layers evenly thickened, spore surface smooth. *Asexual morph* entylomella-like, very well developed. *Caespituli* both hypophyllous and epiphyllous, conidiophores in dense, agglutinated fascicles, emerging through stomata, hyaline, conidiogenous loci inconspicuous. *Conidia* solitary, hyaline, dimorphic, cylindrical, straight or somewhat curved, 15–22 × 2.5–4.0 µm, and acicular, straight or somewhat curved, 30.0–45.0(–60.0) × (2.0–)2.5–3.5 µm, non-septate, hilum inconspicuous, not darkened.

Host plants — Parasitic on *Ranunculus bulbosus*, *R. polyanthemos*, and *R. repens*.

Additional specimens examined. Germany, Baden-Württemberg, Swabian Alps, county Sigmaringen, Leibertingen-Wildenstein, S of Beuron, ascent to castle Wildenstein, mixed forest, wayside, N48°02'49" E08°58'17", elevation c. 682 m a.s.l., on *Ranunculus repens*, 6 June 2014, *J. Kruse* (GLM-F107638); Bavaria, Upper Bavaria, county Garmisch-Partenkirchen, c. 4.9 km NE of Mittenwald, Karwendel mountains, hiking path 266 from Rehbergalm to

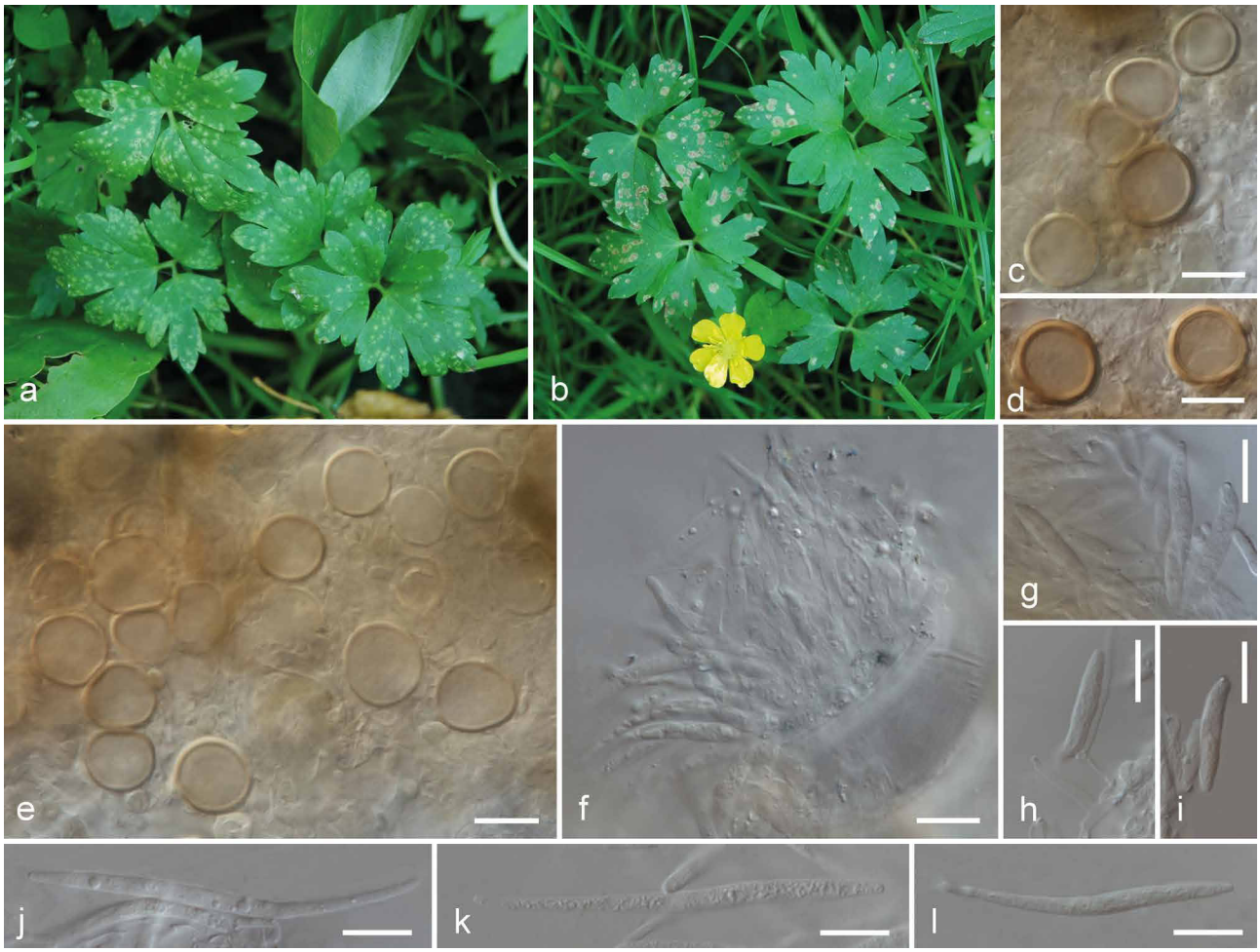

Fig. 7 *Entyloma eburneum* on *Ranunculus repens*. a–b. Macroscopic symptoms of infection; c–e. spores, as seen in light microscopy; f. conidiophores emerging through the stoma, as seen in light microscopy; g–i. cylindrical conidia (with one conidiophore on 'h'), as seen in light microscopy; j–l. acicular conidia, as seen in light microscopy (from neotype). — Scale bars = 10 µm.

Hochland cabin, mixed mountainous forest, N47°27'37" E11°18'36", elevation c. 1575 m a.s.l., on *Ranunculus polyanthemos* subsp. *nemorosus*, 11 July 2016, *J. Kruse* (GLM-F107647); Hesse, Main-Taunus-county, Hattersheim on Main, grassland at Welschenstream, Kuckuckspfad, wayside, N50°03'54" E08°30'03", elevation c. 90 m a.s.l., on *Ranunculus bulbosus*, 30 Apr. 2016, *J. Kruse* (GLM-F107644); Lower Saxony, county Northeim, at the bottom of the Katlencastle, wayside near river, elevation c. 110 m a.s.l., on *Ranunculus repens*, 23 Apr. 2010, *J. Kruse* (GLM-F107648). – Italy, Liguria, Lower Varavalley, c. 1.5 km SW Tavarone, circular path, Monte Alpe from Agriturismo Giandriale, east slope, meadow, N44°18'28" E09°31'58", elevation c. 725 m a.s.l., on *Ranunculus bulbosus*, 10 May 2016, *J. Kruse* (GLM-F107645).

Notes — The *Entyloma* species on *Ranunculus repens* causing flat spots is usually referred to as *Entyloma ranunculi-repentis*, which is the earliest available name for the teleomorph (Vánky 2012). *Ramularia gibba* (= *Entylomella gibba*) was considered to be the earliest name for the asexual morph (Braun 1998, Vánky 2012), which is an earlier name than *Entyloma ranunculi-repentis*, and following the 'one fungus, one name rule' (Hawksworth et al. 2011), Rossman & Castlebury (in Rossman et al. 2016) proposed the new combination *Entyloma gibbum*. However, they were apparently not aware that the original description and type material of *Ramularia gibba* were based on mixed elements of two fungi: the entylomella-like asexual morph of *E. eburneum*, and the sexual morph of *E. microsporum*. Due to the inseparable chimeric description and material, Kruse & Thines (2017) proposed the rejection of *Ramularia gibba*. The oldest available name for a flat-spotting *Entyloma* species on *Ranunculus repens* is *Fusidium eburneum*. This species has been described by Schröter (1877) for a conidial fungus on *Ranunculus repens* resembling the conidial state of *Entyloma ranunculi* (= *Entyloma ficariae*), producing whitish or yellowish spots, 1.5–2 mm diam and having hyaline, filamentous conidia 40–50 µm long and 2.5–3.0 µm wide. This morphological characterisation agrees well with the morphology of the asexual state in the holomorphic specimens analysed in the current study. Schröter (1877) did not observe corresponding *Entyloma*-like spores in the leaves. He thus might have analysed a young infection in which leaf spots and conidia are prominently developed, but teliospores are lacking. *Fusidium eburneum* is an earlier name than *Entyloma ranunculi-repentis*, and in line with the current International Code of Nomenclature for algae, fungi, and plants (McNeill et al. 2012) should be applied for the holomorph. In the protologue, Schröter (1877) did not provide a specific localization of the collected material, but in the monograph dealing with Silesian fungi (Schröter 1887), he enumerated several collections from Silesia. Authentic material of *Fusidium eburneum* is not preserved in the herbarium of J. Schröter deposited in WRSL (M. Halama, pers. comm.). Likewise, we could not locate any original material in other herbaria where some specimens of J. Schröter might have been deposited (e.g., in HBG; T. Feuerer, pers. comm.). Therefore, we are designating a neotype from among the specimens that were sequenced in this study. The neotype represents a holomorphic specimen with an asexual morph having characters that perfectly fit with the description in the protologue.

The present molecular and morphological analyses suggest that *Entyloma* specimens on *Ranunculus bulbosus*, *R. polyanthemos* subsp. *nemorosus*, and *R. repens* p.p. represent a single species. *Entyloma* on *Ranunculus polyanthemos* was

previously described as a distinct species, *Entyloma wroblewskii* (Kochman 1934), which is considered as synonym with *Entyloma eburneum*, here. In the protologue of *E. wroblewskii*, Kochman (1934) reported one collection on *Ranunculus polyanthemos* collected in September 1933 in Anin near Warszawa (now within the borders of Warszawa) in Poland. In the herbarium KRAM F there are three specimens of *E. wroblewskii* having labels matching all information from the protologue, with the exception that the date of collection is given precisely as 15 September 1933 – these specimens apparently represent one original gathering. The label on one of these specimens is written in Latin and the species name is given as '*Entyloma Wróblewskii* n. sp. Kochman' – this specimen should be considered as holotype. The labels on two remaining specimens are written in Polish and lack 'n. sp.' next to the species name – these specimens should be considered as isotypes. Vánky (2012) mentioned that type of *E. wroblewskii* is deposited in the herbarium WA. However, the corresponding herbarium specimen in WA was apparently collected in Anin a year later, on 15 September 1934 (M. Graniszewska, pers. comm.) and distributed in Kochman's exsiccates, Ustilaginales Poloniae no. 28 – therefore, this specimen does not represent the original gathering.

Entyloma eburneum is morphologically distinct from most other *Entyloma* species infecting *Ranunculus* spp. in having prominently developed leaf spots, relatively large spores and dimorphic conidia (cylindrical and acicular). *Entyloma ranunculi-sclerati* is the most similar species, but differs in having somewhat smaller spores and longer, predominantly acicular conidia.

Entyloma jolantae J. Kruse, M. Lutz, Piątek & Thines, *sp. nov.* — MycoBank MB824513; Fig. 8

Etymology. Named after Jolanta Piątek (Kraków, Poland), Polish phycologist, who together with the second author of this work collected this smut and many other smut fungi during joint field trips in Europe and Africa.

Type. Poland, Małopolska Province, Tatra Mts, Mała Dolinka valley – northern slopes of Giewont Mt, elevation c. 1230 m a.s.l., on *Ranunculus oreophilus*, 25 Aug. 2008, *J. Piątek & M. Piątek* (KRAM F-59030 holotype; ex-type sequences available in GenBank: MF924688 (ITS), MH022812 (*atp*2), MF939316 (*ssc*1), MF939250 (*map*)).

Sori in the leaves, forming distinct flat spots, 0.5–3 mm long, 0.5–2 mm wide, rounded or more or less polyangular – usually well delineated by the leaf veins of the host, at first cream-coloured, later brownish on both sides of the leaf, finally necrotic. *Spores* embedded in the leaf tissue, single, densely crowded in the intercellular space between the mesophyll cells; spores subhyaline to pale yellow, globose, subglobose or broadly ellipsoidal and often somewhat irregular due to mutual pressure, 10.5–15.5(–16.5) × 10.0–13.5(–14.5) µm (av. ± SD, 13.2 ± 1.4 × 11.6 ± 1.1 µm, *n* = 60/2), with smooth context; wall 2-layered, 1.5–2.0 µm thick (including inner layer c. 0.5–0.8 µm thick), layers well visible in LM, inner layer evenly thickened, outer layer unevenly thickened, spore surface smooth. *Asexual morph* not found.

Diagnostic bases — Within the *E. ranunculi-repentis* complex there are seven diagnostic bases distributed among all loci (Fig. 3, Table 2).

Host plant — Parasitic on *Ranunculus oreophilus*.

Additional specimen examined. Poland, Małopolska Province, Tatra Mts, Mała Dolinka valley – northern slopes of Giewont Mt, elevation c. 1260 m a.s.l., on *Ranunculus oreophilus*, 25 Aug. 2008, *J. Piątek & M. Piątek* (KRAM F-59031).

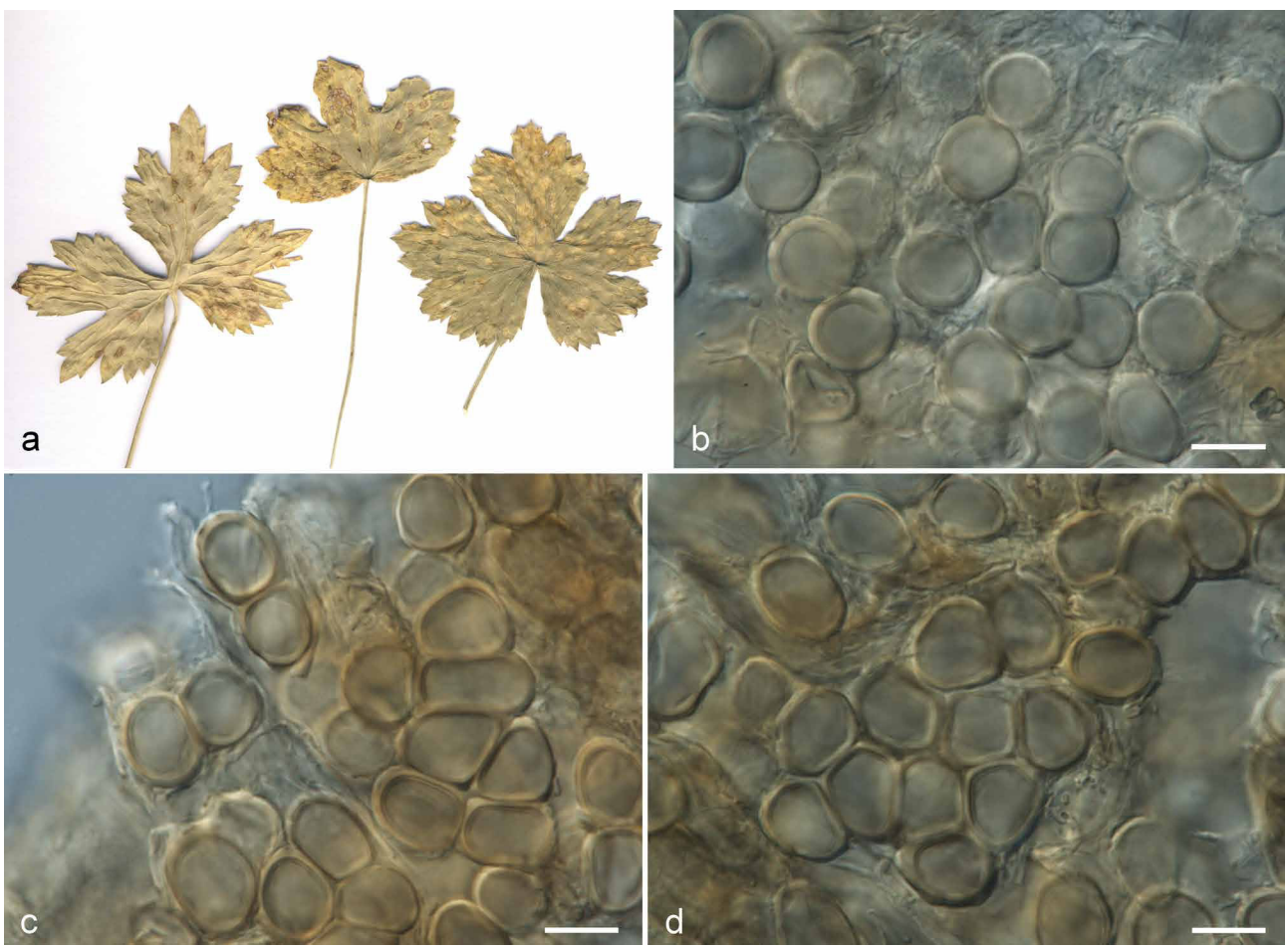

Fig. 8 *Entyloma jolantae* on *Ranunculus oreophilus*. a. Macroscopic symptoms of infection (two leaves to the left from holotype, one leaf to the right from paratype); b–d. spores as seen in light microscopy (from holotype). — Scale bars = 10 µm.

Notes — This species differs from most other species in the *E. ranunculi-repentis* complex by having larger spores with larger mean spore sizes, somewhat thicker spore walls, and lacking the asexual morph. *Entyloma savchenkoi* is the most similar species that differs in having an asexual morph.

Entyloma klenkei J. Kruse, M. Lutz, Piątek & Thines, *sp. nov.* — MycoBank MB824514; Fig. 9

Etymology. Named after Friedemann Klenke (Naundorf, Germany), for his eminent contributions as field mycologist to the knowledge on plant pathogenic fungi, e.g. as the lead author of the reference work *Pflanzenparasitische Kleinpilze* (Klenke & Scholler 2015).

Type. Greece, Rhodes, c. 0.7 km W of Archipoli, Eparchiaki Odos Pastidas-Mesanagrou, field beneath street, N36°15'58" E28°03'11", elevation c. 185 m a.s.l., on *Ranunculus marginatus*, 13 Mar. 2016, *J. Kruse & V. Kummer* (GLM-F107659 holotype; ex-type sequences available in GenBank: MF924663 (ITS), MH022787 (*atp*2), MF939301 (*ssc*1), MF939235 (*map*)).

Sori in the leaves, forming indistinct, flat, polyangularly rounded spots, 1.5–2 mm diam, dirty yellow in colour. *Spores* embedded in the leaf tissue, single, loosely scattered in the intercellular space between the mesophyll cells; spores subhyaline to pale yellow, globose or subglobose, regular in shape, 10.5–13.0 × 10.0–12.5 µm (av. ± SD, 11.7 ± 0.9 × 11.1 ± 0.7 µm, *n* = 30/1), with smooth context; wall 2-layered, 1.0–1.8 µm thick (including inner layer c. 0.5 µm thick), without angles, layers hardly visible in LM, both layers evenly thickened, spore surface smooth. *Asexual morph* not found.

Diagnostic bases — Within the *E. ranunculi-repentis* complex there are six diagnostic bases distributed among all loci except ITS (Fig. 3, Table 2).

Host plant — Parasitic on *Ranunculus marginatus*.

Notes — This species differs from most other species in the *E. ranunculi-repentis* complex by the combination of small spores (with thin walls), which are loosely scattered between leaf mesophyll cells, and lacking an asexual morph. The most similar species is *Entyloma thielii*, which differs in having densely crowded spores, often in compact groups, in the intercellular space between the mesophyll cells.

Entyloma kochmanii J. Kruse, M. Lutz, Piątek & Thines, *sp. nov.* — MycoBank MB824515; Fig. 10

Etymology. Dedicated to the memory of Józef Kochman (1903–1995), Polish smut taxonomist, who first challenged the view that *Entyloma* specimens on *Ranunculus* represent just three species.

Type. Italy, Liguria, Varavalley, c. 2 km NE of Caranza, Strada Provinciale from Caranza to Passo della Cappelletta, alluvial canyon forest, N44°23'33" E09°38'44", elevation c. 840 m a.s.l., on *Ranunculus lanuginosus*, 9 May 2016, *J. Kruse* (GLM-F107660 holotype; ex-type sequences available in GenBank: MF924678 (ITS), MH022802 (*atp*2), MF939309 (*ssc*1), MF939243 (*map*)).

Sori in the leaves, forming small, moderately distinct, flat, rounded or somewhat polyangular spots, 0.5–1 mm diam, usually delineated by the leaf veins of the host, yellow or cream-coloured on the upper side of the leaf, whitish on the lower side of the leaf due to the presence of the conidiophores and conidia of the asexual morph. *Spores* embedded in the leaf tissue, single, loosely scattered in the intercellular space between the mesophyll cells; spores pale yellow, globose or subglobose, regular in shape, (9.0–)11.0–13.0 × (9.0–)10.0–12.5 µm (av. ± SD, 11.7 ± 0.9 × 10.9 ± 0.8 µm, *n* = 30/1), with smooth context; wall 2-layered, 0.5–1.5 µm thick (including inner layer c. 0.2–0.5 µm thick), without angles but sometimes with hyaline appen-

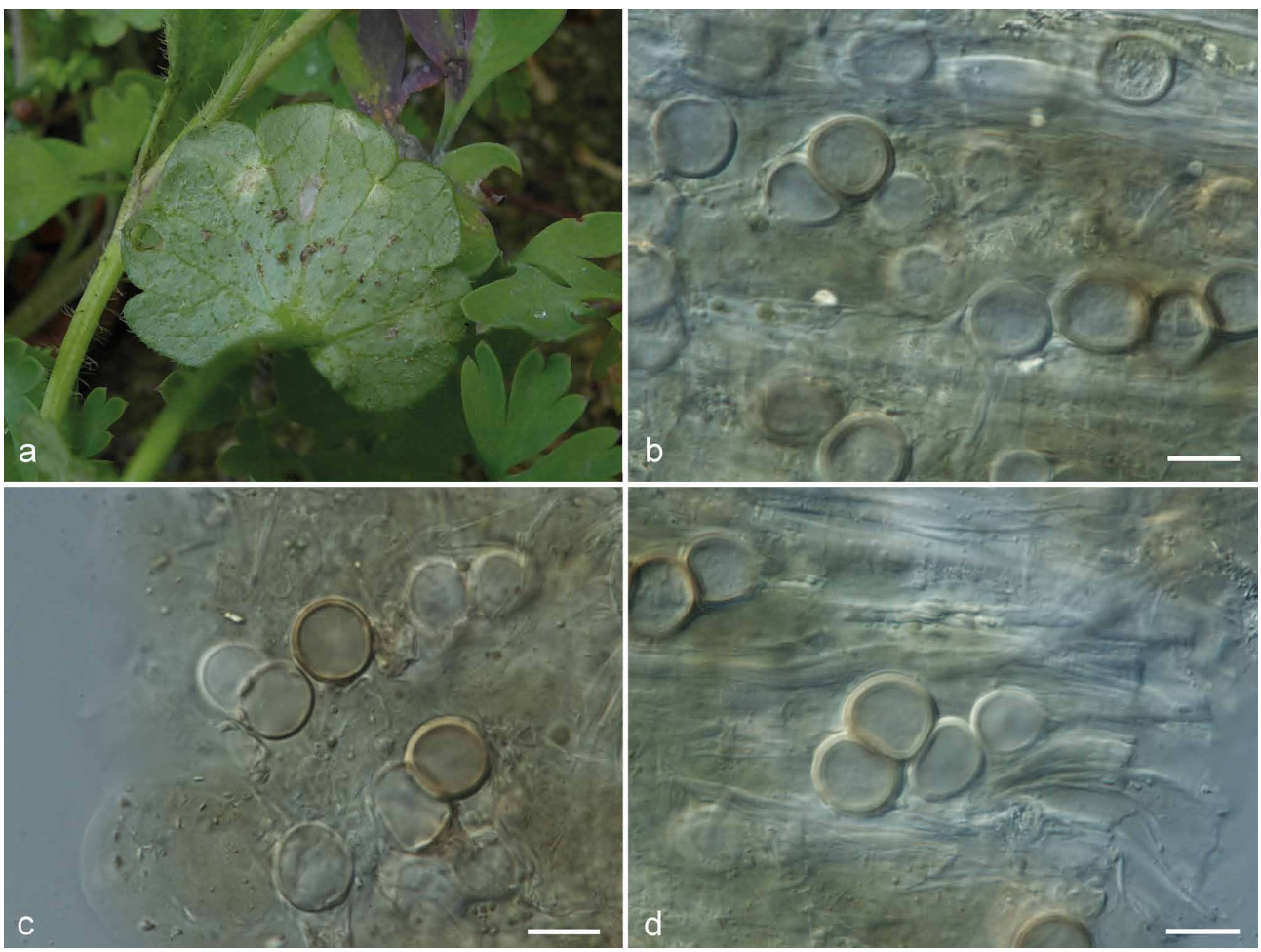

Fig. 9 *Entyloma klenkei* on *Ranunculus marginatus*. a. Macroscopic symptoms of infection; b–d. spores, as seen in light microscopy (from holotype). — Scale bars = 10 µm.

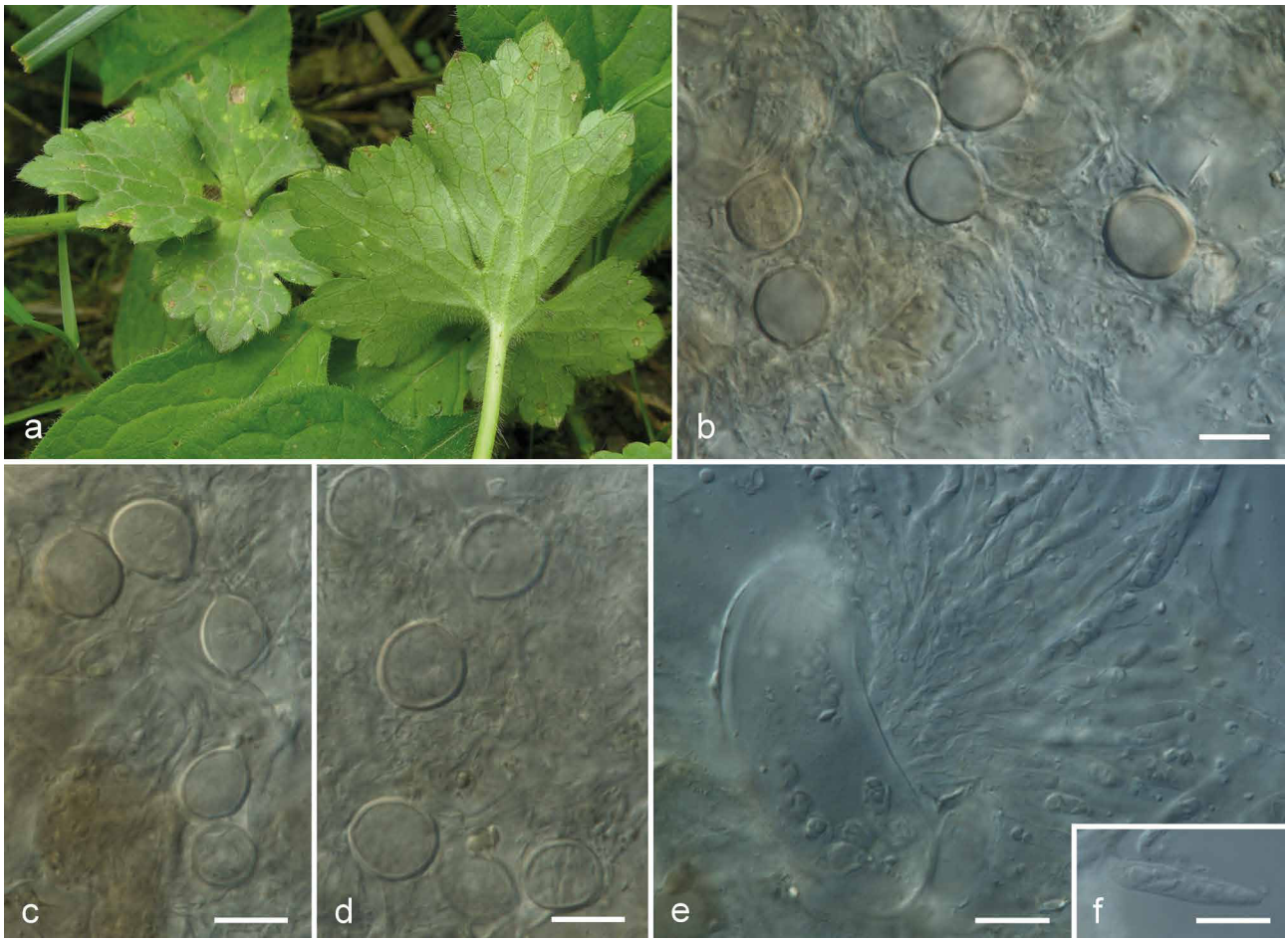

Fig. 10 *Entyloma kochmanii* on *Ranunculus lanuginosus*. a. Macroscopic symptoms of infection; b–d. spores, as seen in light microscopy; e. conidiophores emerging through the stoma, as seen in light microscopy; f. conidium, as seen in light microscopy (from holotype). — Scale bars = 10 µm.

dage, layers hardly visible in LM, both layers evenly thickened, spore surface smooth. *Asexual morph* entylomella-like, weakly developed. *Caespituli* hypophyllous, conidiophores in dense fascicles, emerging through stomata, hyaline, conidiogenous loci inconspicuous. *Conidia* solitary, hyaline, cylindrical, 20–24 × 3.0–3.5(–4.0) µm, non-septate, hilum inconspicuous, not darkened.

Diagnostic bases — Within the *E. ranunculi-repentis* complex there are three diagnostic bases distributed among all loci, except *map* (Fig. 3, Table 2).

Host plant — Parasitic on *Ranunculus lanuginosus*.

Notes — This species differs from most other species in the *E. ranunculi-repentis* complex by the combination of small spores (with thin walls) and cylindrical conidia. *Entyloma ranunculacearum* differs in having shorter conidia, while *E. ranunculorum* differs in having longer conidia.

Entyloma ranunculacearum Kochman, Pl. Polon. 4: 105. 1936 — Fig. 11

Type. Ukraine, district Mościska, Krukienice, on *Ranunculus acris*, 17 Aug. 1935, *J. Kochman* (KRAM F-2606 lectotype indicated by Lindeberg 1959: 41, but precisely designated here; MycoBank MBT380645).

Reference specimen. Germany, Saxony-Anhalt, county Wittenberg, Kemberg, district Rotta-Gniest, Heidestreet, wayside, N51°45'04" E12°35'33", elevation c. 105 m a.s.l., on *Ranunculus acris*, 13 Nov. 2013, *J. Kruse* (GLM-F107680 reference specimen designated here; ex-reference specimen sequences available in GenBank: MF924637 (ITS), MH022761 (*atp*2), MF939280 (*ssc*1), MF939214 (*map*)).

Sori in the leaves, forming distinct, flat, rounded or somewhat irregular spots, 0.5–4 mm diam, usually partly delineated by the leaf veins of the host, yellowish on the upper side of the leaf, whitish on the lower side of the leaf due to the presence of the conidiophores and conidia of the asexual morph. *Spores* embedded in the leaf tissue, single, loosely scattered in the intercellular space between the mesophyll cells; spores subhyaline to pale yellow, globose, subglobose or rarely broadly ellipsoidal, regular in shape, 10.0–13.5(–14.5) × (9.0–)10.0–12.5(–13.5) µm (av. ± SD, 11.8 ± 1.1 × 10.9 ± 0.8 µm, *n* = 150/5), with smooth context; wall 2-layered, 0.8–1.5 µm thick (including inner layer c. 0.3–0.5(–0.8) µm thick), without angles, layers hardly visible in LM, both layers evenly thickened, spore surface smooth. *Asexual morph* entylomella-like, prominently developed. *Caespituli* hypophyllous, conidiophores in dense, agglutinated fascicles, emerging through stomata, hyaline, conidiogenous loci inconspicuous. *Conidia* solitary, hyaline, cylindrical, straight, sometimes slightly curved, (10–)15–19(–25) × 2.5–3.5(–4.0) µm, non-septate, hilum inconspicuous, not darkened.

Diagnostic bases — Within the *E. ranunculi-repentis* complex there are four diagnostic bases within the *ssc*1 locus (Fig. 3, Table 2).

Host plant — Parasitic on *Ranunculus acris*.

Additional specimens examined. Germany, Baden-Württemberg, county Konstanz, Lake Constance, Radolfzell, SE of Möggingen, Mindelsee, circular path around lake, littoral and wayside, elevation c. 420 m a.s.l., on *Ranunculus acris*, 30 May 2013, *J. Kruse* (GLM-F107678); Bavaria, Oberfranken, county Bamberg, SE of Sandhof, Mönchsweiher, mixed forest on Keuper-Sandstone, elevation c. 290 m a.s.l., on *Ranunculus acris*, 5 May 2012, *J. Kruse* (GLM-F107676); Hesse, Rheingau-Taunus-county, Eltville on Rhine, Rheinsteig, direction to forest-restaurant Rausch, N50°02'46" E08°05'44", elevation c. 160 m a.s.l., on *Ranunculus acris*, 8 Mar. 2014, *J. Kruse* (GLM-F107679); Saarland, Mettlach-Orscholz, county Merzig-Wadern, Cloef-Street, surround-

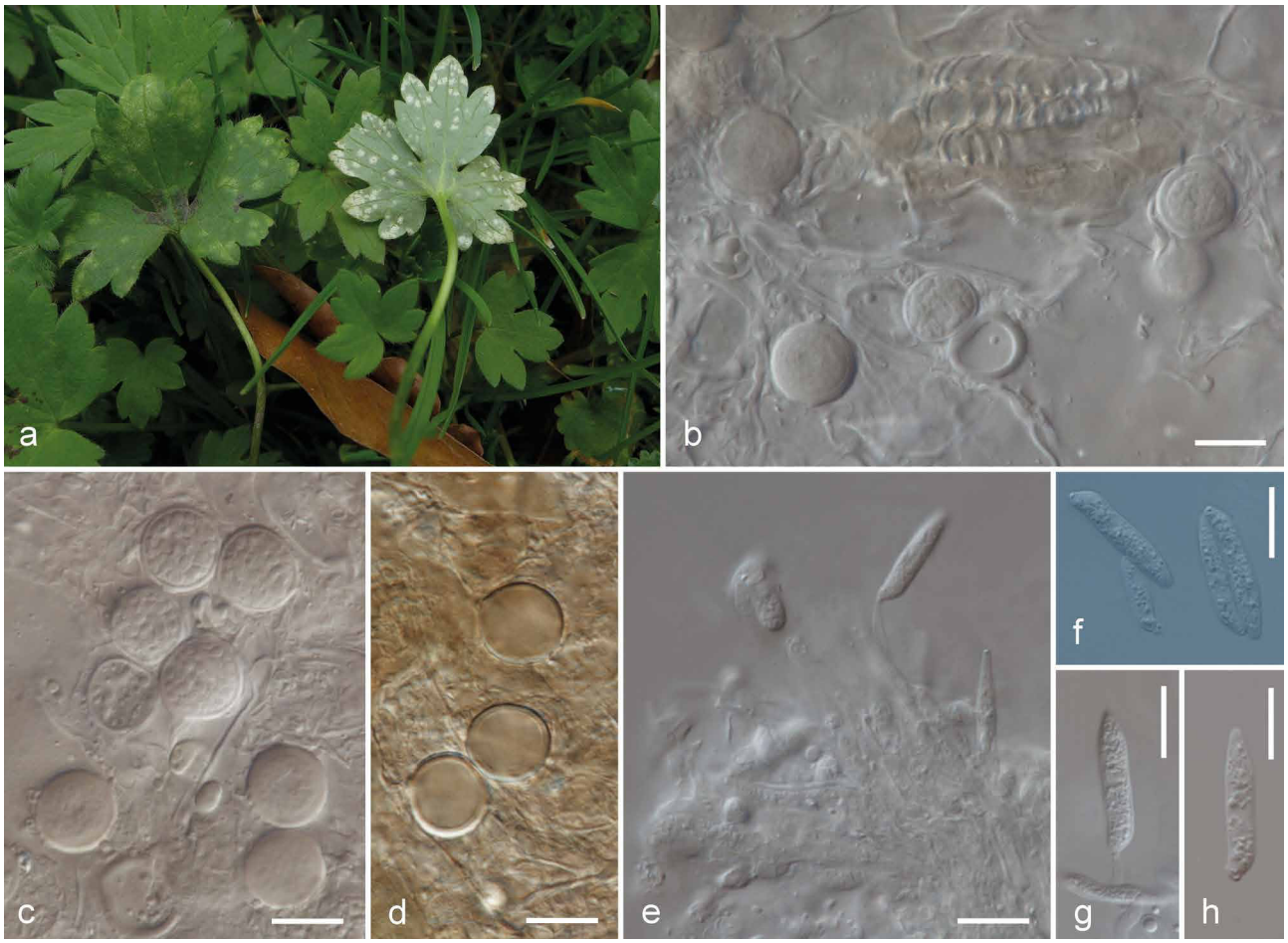

Fig. 11 *Entyloma ranunculacearum* on *Ranunculus acris*. a. Macroscopic symptoms of infection; b–d. spores, as seen in light microscopy; e. conidiophores emerging through the stoma (with one conidium attached to the conidiophore), as seen in light microscopy; f–h. conidia, as seen in light microscopy. Note conidium attached to the conidiophore seen on 'g' (from reference specimen). — Scale bars = 10 µm.

ings of Cloef-Atrium and Varadeser Park, N49°30'20" E06°32'06", elevation c. 395 m a.s.l., on *Ranunculus acris*, 29 Sept. 2014, *J. Kruse* (GLM-F107684).

Notes — When describing this species, Kochman (1936) reported three collections: two on *R. acris* and one on *R. lanuginosus*. In the Polish text he wrote that the typical form of this species infects *R. acris* and an additional host is *R. lanuginosus*. In the Latin diagnosis, Kochman (1936) reported only *R. acris* as type host without an indication of the specific collection. Lindeberg (1959) designated the lectotype from one of the two collections on *R. acris* (collected in 1935 in Krukienice, district Mościska, then in Poland but now in Ukraine), but she did not mention where the specimen was deposited. This specimen is currently preserved in the herbarium KRAM F. Kochman (1936) reported the date of collection as 1935, but on the lectotype specimen the exact date is given as 17 August 1935. *Entyloma* sp. on *R. lanuginosus* belongs to a distinct species, described here as *E. kochmanii*, which is phylogenetically closely related but distinct from *E. ranunculacearum* in having longer conidia.

Entyloma ranunculi-sceleratii Kochman, Pl. Polon. 4: 104. 1936 — Fig. 12

Type. Poland, Skierniewice-Glinianki, on *Ranunculus sceleratus*, 2 July 1927, *W. Konopacka* (BRIP: HUV 974 lectotype, isolectotypes in Kochman, Ust. Pol. no. 29; lectotype designated by Lindeberg 1959: 41, corrected and narrowed by Vánky 1985: 66).

Reference specimen. Poland, Mazowieckie Province, Warszawa-Wesoła, on *Ranunculus sceleratus*, 17 July 2015, *P. Mędykowski* (KRAM F-59032 reference specimen designated here; ex-reference specimen sequences available in GenBank: MF924691 (ITS), MH022815 (*atp2*)).

Sori in the leaves, forming distinct, flat, rounded spots, 1–4 mm diam, yellow or light brown on the upper side of the leaf, whitish or cream coloured on the lower side of the leaf due to the presence of the conidiophores and conidia of the asexual morph, surrounded by brownish rim, finally necrotic – starting from the centre of the sori. *Spores* embedded in the leaf tissue, single, loosely scattered in the intercellular space between the mesophyll cells; spores subhyaline, pale yellow or yellow, globose or subglobose, regular in shape, (9.5–)10.0–12.5(–13.5) × (9.0–)10.0–12.5(–13.0) µm (av. ± SD, 11.7 ± 0.9 × 11.0 ± 0.9 µm, *n* = 150/5), with smooth context; wall 2-layered, 1.0–1.5 µm thick (including inner layer c. 0.3–0.8 µm thick), without angles, layers hardly visible in LM, both layers evenly thickened, spore surface smooth. *Asexual morph* entylomella-like, prominently developed. *Caespituli* both hypophyllous and epiphyllous, conidiophores in dense fascicles, emerging through stomata, hyaline, conidiogenous loci inconspicuous. *Conidia* solitary, hyaline, acicular, rarely cylindrical, usually straight, 20–60 × (2.0–)2.5–3.5(–4.0) µm, non-septate, hilum inconspicuous, not darkened.

Diagnostic bases — Within the *E. ranunculi-repentis* complex there are 26 diagnostic bases distributed among all loci (Fig. 3, Table 2).

Host plant — Parasitic on *Ranunculus sceleratus*.

Additional specimens examined. Germany, Saxony-Anhalt, SE of Allstedt, Ziegelrodaer forest (N-part), airport Allstedt (NW-side), on *Ranunculus sceleratus*, 23 Oct. 2005, *H. Jage* (GLM-F076138); Lodersleben, near castle, in Querne, on *Ranunculus sceleratus*, 6 May 2005, *H. John & H. Jage* (GLM-F076186); Friedersdorf near Lohsa S, WSW Neuhof, near Ballackmühle, Maxsee (part of Ballacksee), on *Ranunculus sceleratus*, 26 May 2006,

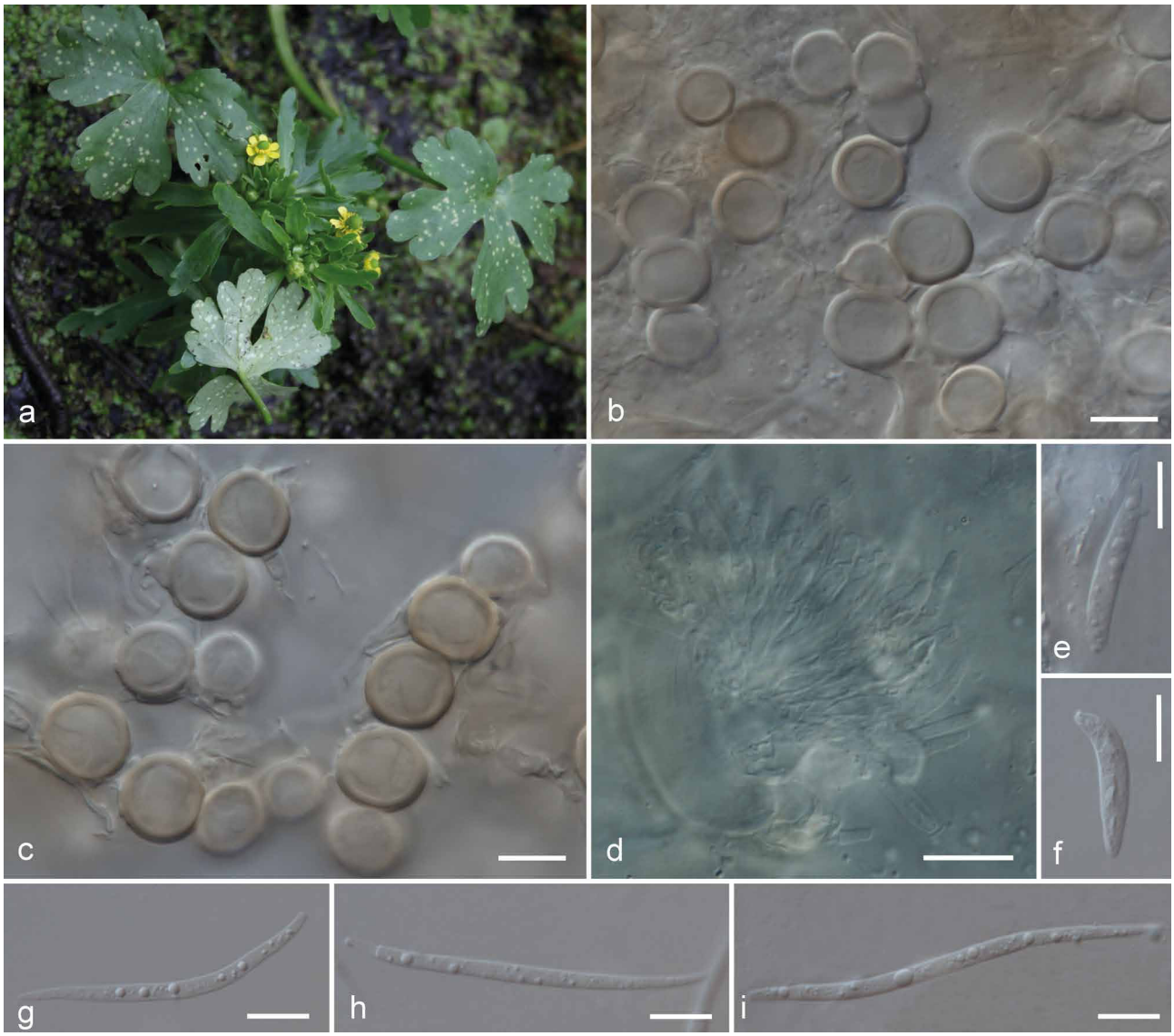

Fig. 12 *Entyloma ranunculi-scelerati* on *Ranunculus sceleratus*. a. Macroscopic symptoms of infection; b–c. spores, as seen in light microscopy; d. conidiophores emerging through the stoma, as seen in light microscopy; e–f. cylindrical conidia, as seen in light microscopy; g–i. acicular conidia, as seen in light microscopy (from reference specimen). — Scale bars = 10 µm.

H. Jage (GLM-F086008); Bavaria, Oberpfalz, county Grafenwöhr, E of Hütten, littoral of lake, N49°40'52" E11°58'42", elevation c. 410 m a.s.l., on *Ranunculus sceleratus*, 1 May 2016, *G. Hübner* (GLM-F107685).

Notes — In the protologue, Kochman (1936) reported two collections on *Ranunculus sceleratus*: one collected in Skierniewice-Glinianki in 1927 by W. Konopacka, and another, collected in Skierniewice-Zwierzyniec in 1925 by W. Siemaszko, both in Poland. Lindeberg (1959) designated the collection in Skierniewice-Glinianki as the lectotype, but erroneously wrote that the material was collected in 1925 by W. Siemaszko – apparently mixing data from both original collections. Also, Lindeberg (1959) did not mention where the specimen is deposited. Vánky (1985) corrected her mistake and narrowed the lectotype to the specimen in HUV. Kochman (1936) reported the date of collection as 1927 but on the lectotype specimen the exact date is given as 2 July 1927. KRAM F-2628 is a specimen labelled as *Entyloma ranunculi-scelerati* collected on *Ranunculus sceleratus* in Skierniewice-Glinianki by W. Konopacka on 28 May 1927. This specimen may represent authentic material but in the light of Vánky's (1985) lectotypification its status remains unclear.

Entyloma ranunculi-scelerati is most similar to *E. eburneum*, which differs in having somewhat larger spores and shorter conidia.

Entyloma ranunculorum Liro, Mycoth. Fennic. Die Etiketten. No. 301–600: 25. 1939 — Fig. 13

Synonym. *Entyloma ranunculorum* Liro, Ann. Acad. Sci. Fenn., Ser. A, 42 (1): 111. 1938, invalid name, no Latin description or diagnosis.

Type. Sweden, Härjedalen, Fjellnäs, on *Ranunculus auricomus*, July 1897, *G. Lagerheim* (BRIP: HUV 894 lectotype, isolectotypes in Sydow, Ust. no. 233, as *Entyloma ranunculi*; lectotype designated by Vánky 1985: 66).

Reference specimen. Germany, Bavaria, Oberfranken, county Kulmbach, Lindau, Mountain chain Rough Mt, wayside, elevation c. 410 m a.s.l., on *Ranunculus auricomus* agg., 12 May 2012, *J. Kruse* (GLM-F107686 reference specimen designated here, ex-reference specimen sequences available in GenBank: MF924629 (ITS), MH022753 (*atp*2)).

Sori in the leaves, forming distinct, flat, rounded or somewhat polyangular spots, 1–4 mm diam, whitish or cream coloured on both sides of the leaf. *Spores* embedded in the leaf tissue, single, loosely scattered or moderately densely crowded in the intercellular space between the mesophyll cells; spores subhyaline, pale yellow to yellow, globose, subglobose or broadly ellipsoidal, usually regular but sometimes somewhat irregular due

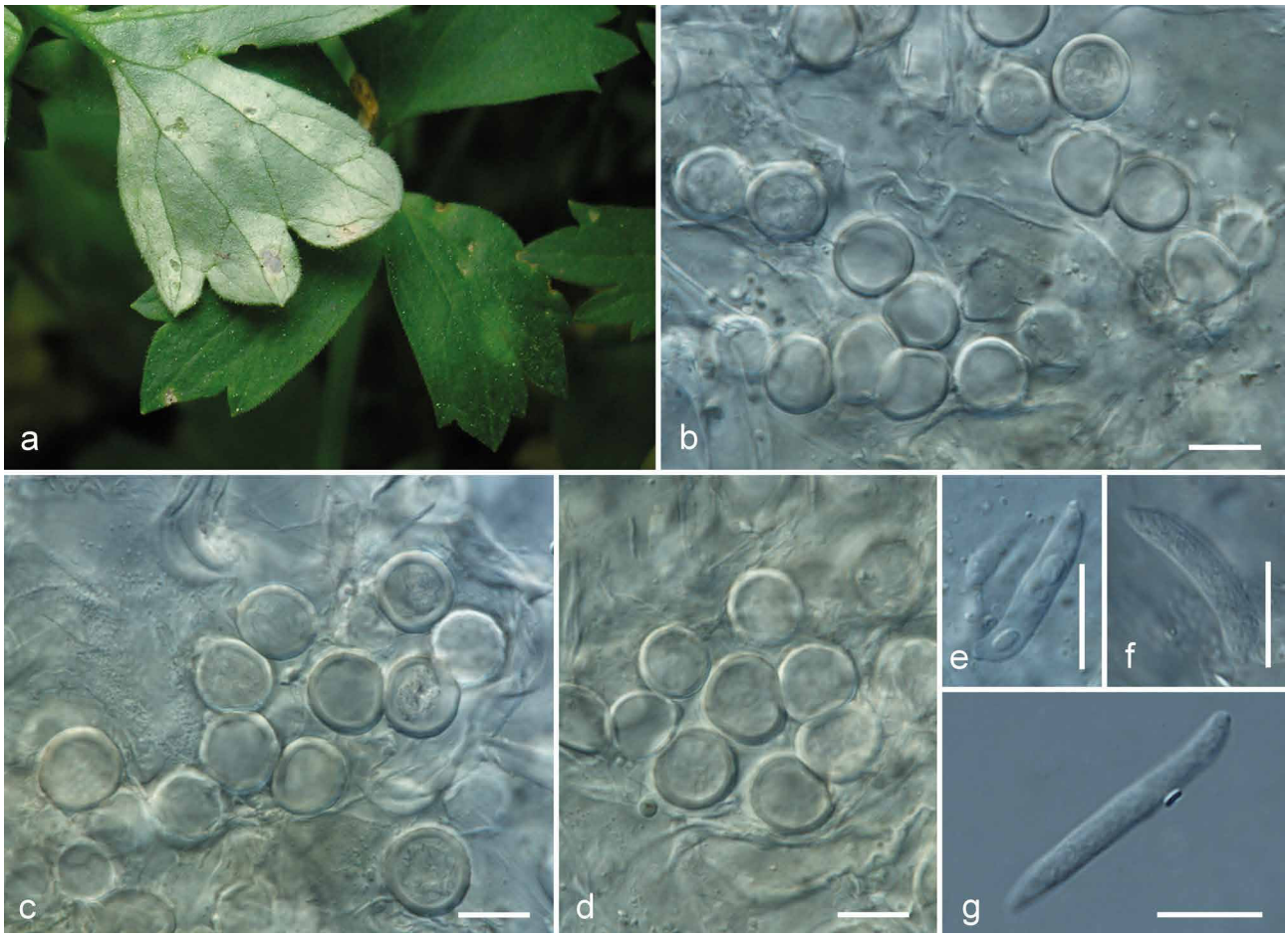

Fig. 13 *Entyloma ranunculorum* on *Ranunculus auricomus*. a. Macroscopic symptoms of infection; b–d. spores, as seen in light microscopy; e–g. conidia, as seen in light microscopy (from reference specimen). — Scale bars = 10 µm.

to mutual pressure, 10.0–12.5(–14.5) × (9.0–)10.0–12.5(–13.0) µm (av. ± SD, 11.8 ± 0.9 × 10.9 ± 0.9 µm, *n* = 60/2), with smooth context; wall 2-layered, 1.0–1.5(–1.8) µm thick (including inner layer c. 0.5–0.8 µm thick), without angles, layers hardly visible in LM, both layers evenly thickened, spore surface smooth. *Asexual morph* entylomella-like, well-developed. *Caespituli* hypophyllous, conidiophores in densely agglutinated fascicles, emerging through stomata, hyaline, conidiogenous loci inconspicuous. *Conidia* solitary, hyaline, cylindrical, usually curved, rarely almost straight, 16–28 × 2.5–3.5(–4.0) µm, non-septate, hilum inconspicuous, not darkened.

Diagnostic bases — Within the *E. ranunculi-repentis* complex there are six diagnostic bases within ITS and the *atp*2 locus (Fig. 3, Table 2).

Host plant — Parasitic on *Ranunculus auricomus*.

Additional specimen examined. Germany, Saxony-Anhalt, E of Dölkau, Burgholz (E-part) Jagen 29, alluvial forest, elevation c. 25 m a.s.l., on *Ranunculus auricomus*, 19 Apr. 1998, *H. Jage* (GLM-F048093).

Notes — The most similar species are *Entyloma kochmanii* and *E. ranunculacearum*, which differ in having shorter conidia.

Entyloma savchenkoi J. Kruse, M. Lutz, Piątek & Thines, *sp. nov.* — MycoBank MB824516; Fig. 14

Etymology. Named after Kyrylo G. Savchenko (Pullman, United States), Ukrainian mycologist, for his contributions to *Entyloma* phylogeny and taxonomy.

Type. Greece, Rhodes, eastern coast, c. 2.5 km N of Kalathos, street towards Masari, wayside, olive grove, N36°08'47" E28°03'33", elevation c. 15 m a.s.l., on *Ranunculus paludosus*, 20 Mar. 2016, *J. Kruse* (GLM-F107699 holotype; ex-type sequences available in GenBank: MF924662 (ITS), MH022786 (*atp*2), MF939300 (*ssc*1), MF939234 (*map*)).

Sori in the leaves, forming rather indistinct, flat, rounded or somewhat polyangular spots, 1–3 mm long, 1–2 mm wide, yellow or light brown on the upper side of the leaf, whitish or cream coloured on the lower side of the leaf. *Spores* embedded in the leaf tissue, single, loosely scattered or moderately densely crowded in the intercellular space between the mesophyll cells; spores subhyaline, pale yellow to yellow, globose, subglobose or broadly ellipsoidal, usually regular but sometimes somewhat irregular due to mutual pressure, (10.0–)12.0–16.5(–18.0) × (9.0–)11.0–14.5(–15.0) µm (av. ± SD, 13.9 ± 1.4 × 12.3 ± 1.2 µm, *n* = 120/4), with smooth context; wall 2-layered, 1.5–2.5 (–3.0) µm thick (including inner layer c. 0.5–1.0 µm thick), without angles, layers well visible in LM, both layers evenly thickened, spore surface smooth. *Asexual morph* entylomella-like, weakly developed. *Caespituli* hypophyllous, conidiophores in densely agglutinated fascicles, emerging through stomata, hyaline, conidiogenous loci inconspicuous. *Conidia* solitary, hyaline, acicular-cylindrical, straight, 25–40 × 2.5–3.0(–3.5) µm, non-septate, hilum inconspicuous, not darkened.

Diagnostic bases — Within the *E. ranunculi-repentis* complex there are four diagnostic bases distributed among all loci, except *map* (Fig. 3, Table 2).

Host plant — Parasitic on *Ranunculus paludosus*.

Additional specimens examined. Greece, Rhodes, c. 1 km S of Salakos, way up to Mt Profitis Ilias, *Quercus coccifera* forest, N36°16'59" E27°56'42", elevation c. 320 m a.s.l., on *Ranunculus paludosus*, 13 Mar. 2016, *J. Kruse* (GLM-F107696); c. 1 km NW of Siana, way up to Akramitis, open Phrygana, plateau, N36°09'23" E27°45'59", elevation c. 650 m a.s.l., on *Ranunculus*

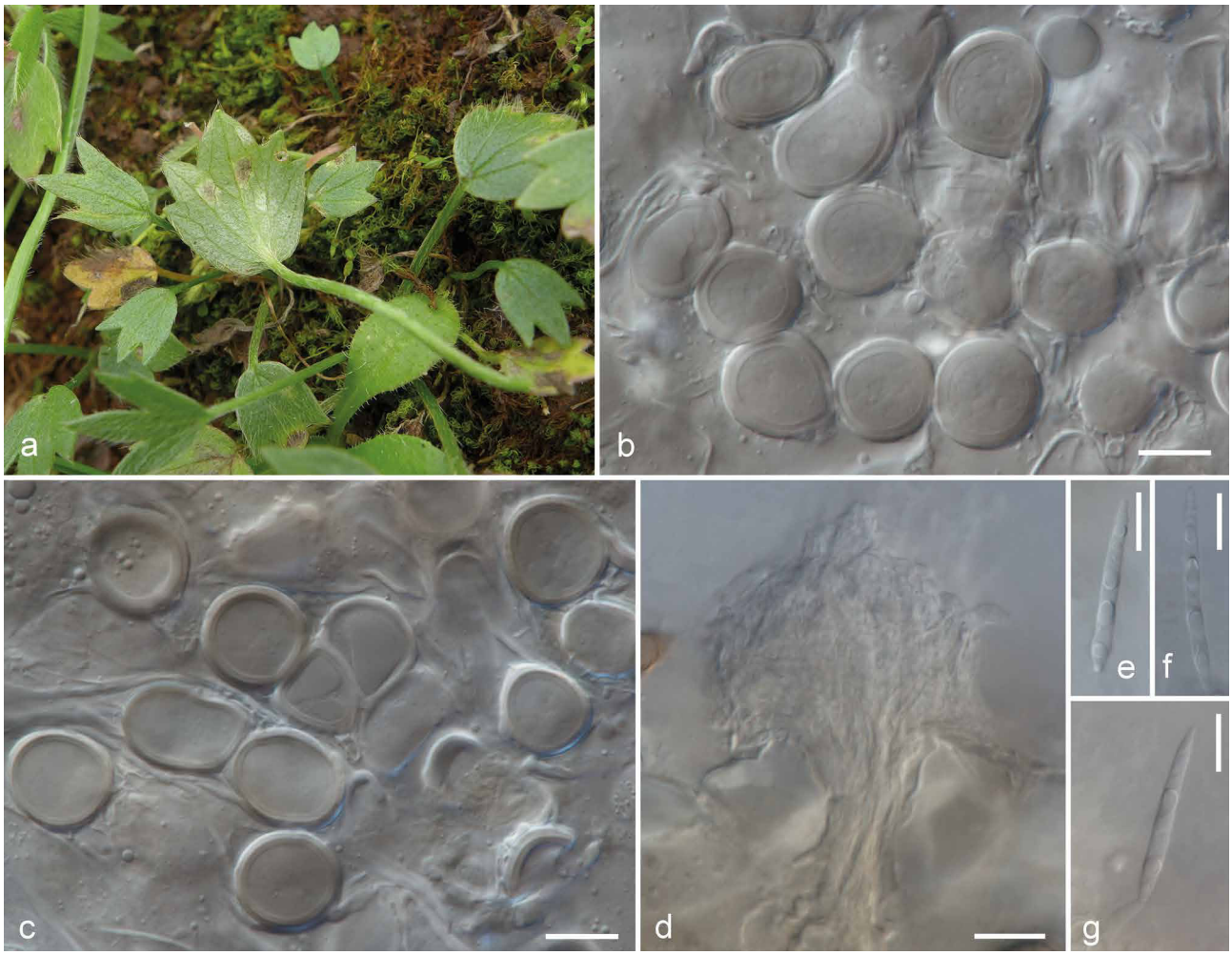

Fig. 14 *Entyloma savchenkoi* on *Ranunculus paludosus*. a. Macroscopic symptoms of infection; b–c. spores, as seen in light microscopy; d. conidiophores emerging through the stoma, as seen in light microscopy; e–g. conidia, as seen in light microscopy. Note conidium attached to the conidiophore seen on 'g' (from reference specimen). — Scale bars = 10 µm.

paludosus, 15 Mar. 2016, *J. Kruse* (GLM-F107697); c. 1.2 km SE of Theologos, olive grove, N36°22'00" E28°02'45", elevation c. 40 m a.s.l., on *Ranunculus paludosus*, 16 Mar. 2016, *J. Kruse* (GLM-F107698).

Notes — This species is most similar to *Entyloma jolantae*, which differs in lacking an asexual morph.

Entyloma thielii J. Kruse, M. Lutz, Piątek & Thines, *sp. nov.* — MycoBank MB824517; Fig. 15

Etymology. Named after Hjalmar Thiel from Jameln (Germany), for his contributions to the knowledge of phytopathogenic fungi and for enabling well-sampled phylogenetic investigations in various plant pathogen groups by his collections.

Type. Germany, Bavaria, Upper Bavaria, county Garmisch-Partenkirchen, c. 2.8 km SE of Mittenwald, Karwendel mountains, meadows around Brunnstein cabin, N47°24'49" E11°16'41", elevation c. 1475 m a.s.l., on *Ranunculus montanus*, 8 July 2016, *J. Kruse* (GLM-F107702 holotype; ex-type sequences available in GenBank: MF924694 (ITS), MH022818 (*atp*2), MF939319 (*ssc*1), MF939253 (*map*)).

Sori in the leaves, forming indistinct, flat, polyangular spots, 1–3 mm long, 0.5–2 mm wide, partly delineated by the leaf veins of the host, yellow or light brown on the upper side of the leaf, greyish or cream coloured on the lower side of the leaf. *Spores* embedded in the leaf tissue, single, densely crowded, often in compact groups, in the intercellular space between the mesophyll cells; spores hyaline, subhyaline to pale yellow, globose, subglobose or broadly ellipsoidal, often somewhat irregular due to mutual pressure, (9.5–)11.0–14.5(–16.5) × 9.0–12.5(–13.0) µm (av. ± SD, 12.5 ± 1.5 × 10.8 ± 1.0 µm, *n* = 150/5), with smooth context; wall 2-layered, 0.8–1.5 µm thick (including inner layer c. 0.3–0.5 µm thick), without angles, layers very hardly visible in LM, both layers evenly thickened, spore surface smooth. *Asexual morph* not found.

Diagnostic bases — Within the *E. ranunculi-repentis* complex there are 68 diagnostic bases distributed among all loci (Fig. 3, Table 2).

Host plant — Parasitic on *Ranunculus montanus*.

Additional specimen examined. Germany, Bavaria, Upper Bavaria, county Garmisch-Partenkirchen, c. 2.8 km SE of Mittenwald, Karwendel mountains, hiking path 290 from Brunnstein cabin towards Mittenwald, serpentines, sparse mixed mountainous forest, N47°24'48" E11°16'33", elevation c. 1380 m a.s.l., on *Ranunculus montanus*, 8 July 2016, *J. Kruse* (GLM-F107700); meadows around Brunnstein cabin, N47°24'49" E11°16'41", elevation c. 1475 m a.s.l., on *Ranunculus montanus*, 8 July 2016, *J. Kruse* (GLM-F107701); c. 4.9 km NE of Mittenwald, Karwendel mountains, hiking path 266 from Rehbergalm to Hochland cabin, mixed mountainous forest, N47°27'37" E11°18'36", elevation c. 1575 m a.s.l., on *Ranunculus montanus*, 11 July 2016, *J. Kruse* (GLM-F107704); Oberallgäu, Einödsbach, Rappensee cabin, near Rappensee, wayside, N47°17'11" E10°15'19", elevation c. 2080 m a.s.l., on *Ranunculus montanus*, 29 July 2015, *J. Kruse* (GLM-F107705).

Notes — This species differs from the other species in the *Entyloma ranunculi-repentis* complex in having densely crowded spores, often in compact groups, in the intercellular space between the mesophyll cells.

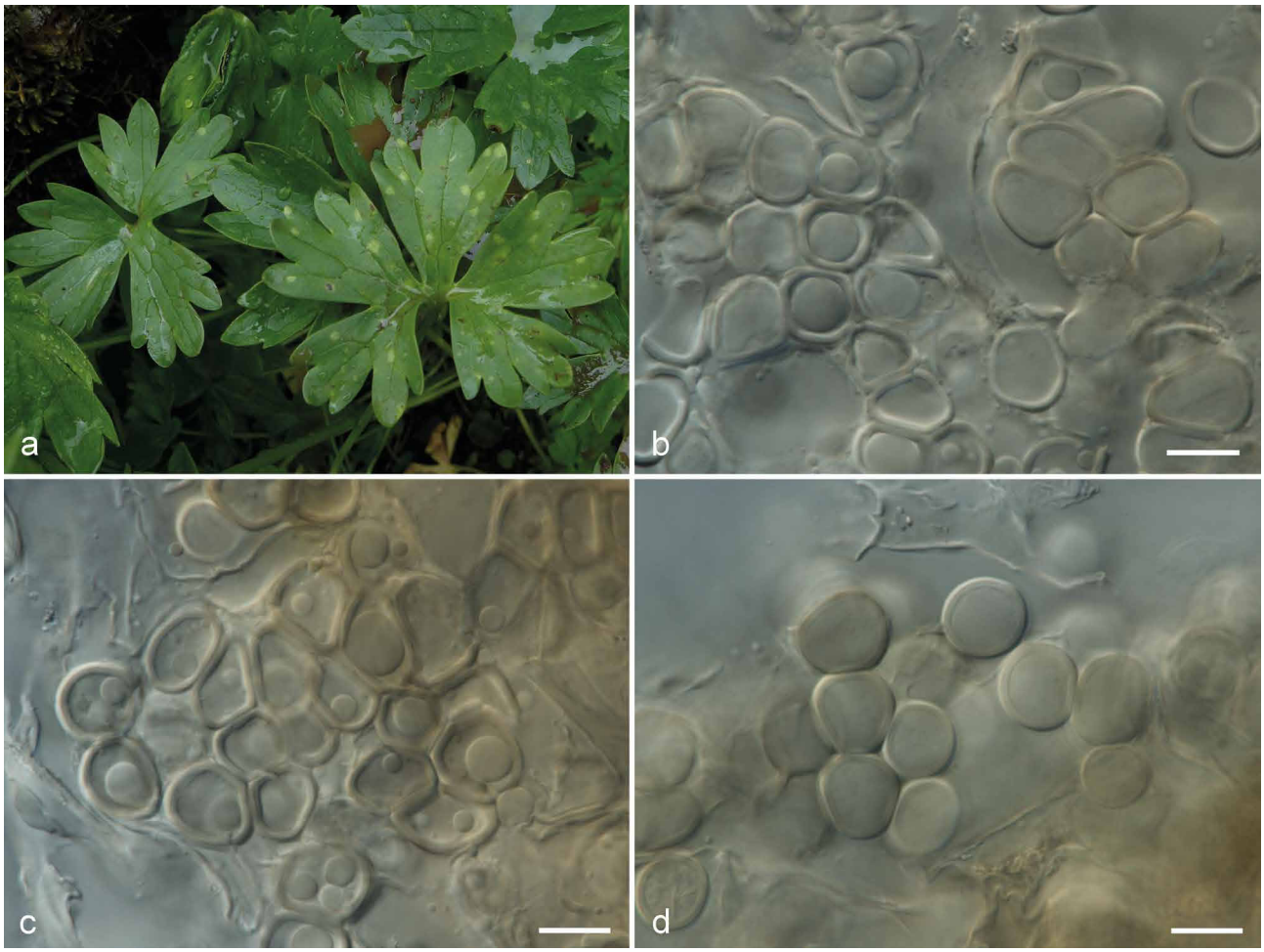

Fig. 15 *Entyloma thielii* on *Ranunculus montanus*. a. Macroscopic symptoms of infection; b–d. spores, as seen in light microscopy (from holotype). — Scale bars = 10 µm.

Entyloma verruculosum

Entyloma verruculosum Pass., Nuovo Giorn. Bot. Ital. 9: 239. 1877; in Rabenhorst, Fungi Europ. no. 2253. 1877; in Fischer von Waldheim, Bull. Soc. Imp. Naturalistes Moscou 52: 310. 1877 — Fig. 16

Type. Italy, Parma, on *Ranunculus velutinus*, May 1873, *G. Passerini* (BRIP: HUV 1307 lectotype, isolectotypes in Rabenhorst, Fungi Europ. no. 2253; lectotype designated by Vánky 1985: 80).

Sori in the leaves, forming indistinct, flat, polyangular spots, 1–5 mm long, 1–3 mm wide, partly delineated by the leaf veins of the host, yellow or light brown on the upper side of the leaf, cream coloured on the lower side of the leaf. *Spores* embedded in the leaf tissue, single, densely crowded in the intercellular space between the mesophyll cells; spores subhyaline to pale yellow, globose or subglobose, regular in shape, (11.0–)12.0–14.5(–16.0) × (10.5–)11.0–14.5(–15.0) µm (av. ± SD, 13.4 ± 1.4 × 13.1 ± 1.4 µm, *n* = 30/1), with granular context; wall apparently 1-layered, 1.5–2.5 µm thick, without angles, spore surface distinctly tuberculate. *Asexual morph* not found.

Host plant — Parasitic on *Ranunculus* spp.

Specimen examined. Italy, Apulia, Monte Sant'Angelo, Provinz Foggia, c. 12 km N of Monte Sant'Angelo, road SP52b, Foresta Umbra, beech forest, N41°47'52" E15°58'44", elevation c. 720 m a.s.l., on *Ranunculus lanuginosus*, 19 Apr. 2016, *J. Kruse* (GLM-F107706).

Notes — The specimens of *Entyloma verruculosum* on the type host (*Ranunculus velutinus*) were not available for molecular analyses, and the morphological description is based on the sequenced specimen on *R. lanuginosus*. The smut species was additionally reported on *Ranunculus acris*, *R. repens*, and *R. sceleratus* (Vánky 2012), which indicates that *E. verruculosum* may represent a species complex, too, to be resolved in future studies.

DISCUSSION

The analyses of the morphology and molecular phylogenetics presented in this study indicate that most of the *Entyloma* species on *Ranunculus* spp. are specific at the host species level. This provides evidence for two more assumed broad-range biotrophic pathogens to be species complexes, rather than single species, similar to the situation observed in other pathogens (e.g., Lutz et al. 2005, Beenken et al. 2012, Choi et al. 2015, Scholler et al. 2016, Kruse et al. 2018, Ziegler et al. 2018). The three major lineages found within *Entyloma* (the *E. microsporum* complex, the *E. ranunculi-repentis* complex, and *E. verruculosum*) are readily distinguished by teliospore surface ornamentation. Species in the *E. microsporum* complex have cracked spore surfaces, those in the *E. ranunculi-repentis* complex are smooth, and spores of *E. verruculosum* are verrucose. In addition, species in the *E. microsporum* complex cause swollen galls readily distinguishing them from the other two lineages. *Entyloma verruculosum*, for which we examined only a single specimen, may represent yet another complex to be resolved in future studies, as it has been reported on five different *Ranunculus* species (Vánky 2012).

For the *Entyloma ranunculi-repentis* complex the four-gene dataset (with ITS, *atp*2, *ssc*1, and *map* sequences) recovered 11 mostly highly supported host-specific lineages (nine on

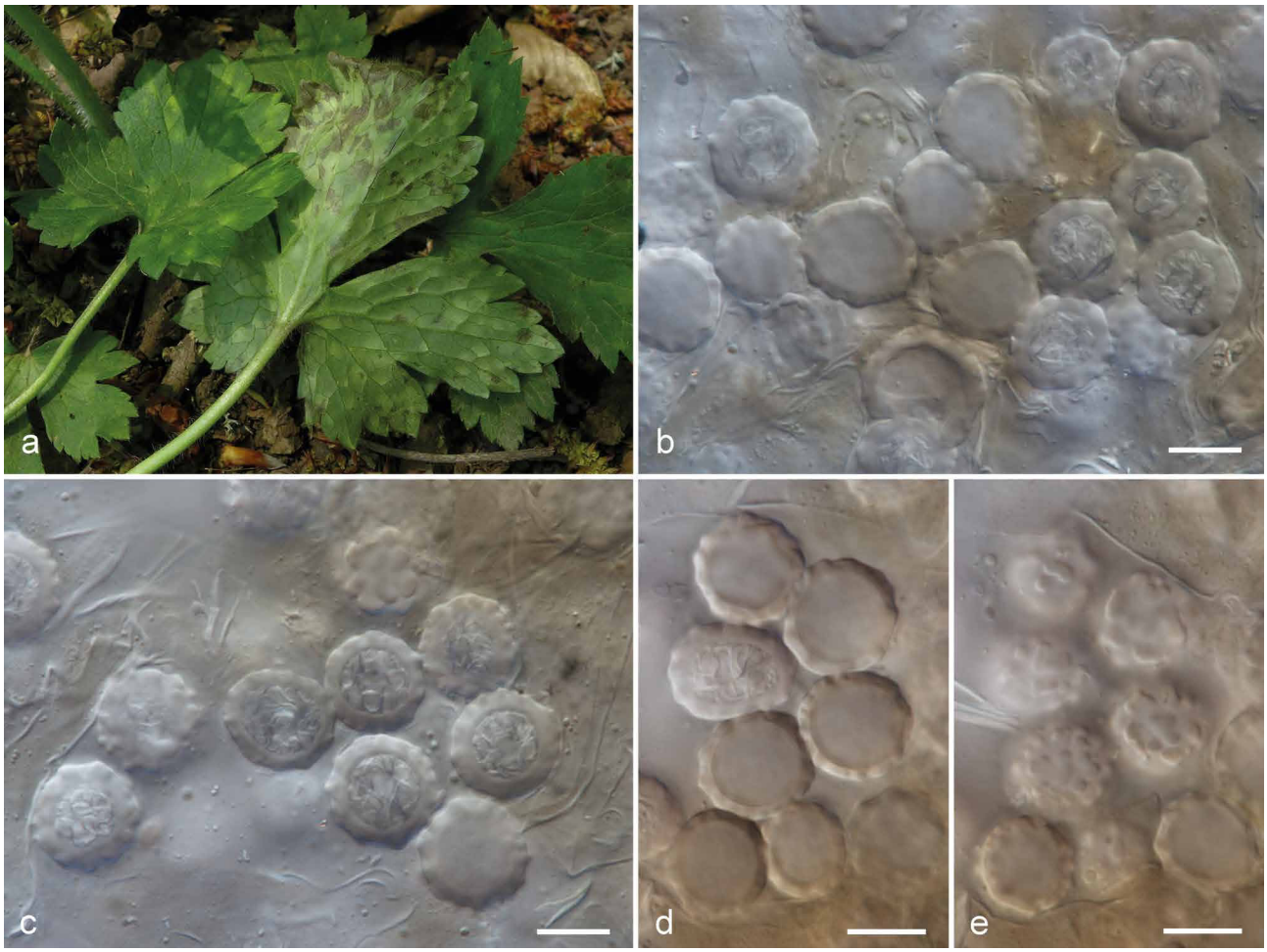

Fig. 16 *Entyloma verruculosum* on *Ranunculus lanuginosus*. a. Macroscopic symptoms of infection; b–e. spores as seen in light microscopy, median and superficial views (from GLM-F107706). — Scale bars = 10 µm.

Ranunculus spp. and two on *Ficaria verna*). These lineages are also correlated with (sometimes subtle) morphological characters. The most informative morphological and biological characters were the arrangement of spores within the leaf spot; size of spores; mean size of spores; spore wall thickness; presence of an asexual morph; and the shape and size of conidia (see Table 3).

For some of the lineages in the *E. ranunculi-repentis* complex validly published names are available, previously often listed as synonyms of *E. ranunculi-repentis* s.lat. (Vánky 2012). The results of this study support *E. ranunculacearum* (on *R. acris*), *E. ranunculi-scelerati* (on *R. sceleratus*), and *E. ranunculorum* (on *R. auricomus*) as distinct species (Kochman 1936, Liro 1938). For six other lineages, each associated with a single host plant species, new species were introduced.

In addition to these host-specific *Entyloma* species, one additional clade with specimens from related species (Paun et al. 2005), *R. bulbosus*, *R. polyanthemos* subsp. *nemorosus*, and *R. repens*, has been assigned to a new combination in *Entyloma* for *Fusidium eburneum*. Further study is needed to determine if this clade represents a recently-differentiated species complex. If it contained distinct species, the name *Entyloma wroblewskii* (Kochman 1934) could be adopted for the *Entyloma* pathogen on *Ranunculus polyanthemos*. As even more loci or microsatellites would be needed to resolve this question, we have taken a conservative approach, considering the whole clade to represent *E. eburneum*.

The species *Ramularia gibba*, which was thought to be connected with the asexual morph-forming species of *Entyloma* on *Ranunculus repens* (Braun 1998), is a chimera that contains the diagnostic features of both the *E. microsporum* and the *E. ranunculi-repentis* species complexes (De Bary 1874). An inspection of the type specimen revealed a dual infection was present on the leaves, explaining the chimeric nature of the description. Consequently, the name cannot be applied to a species in either group and has been proposed for rejection (Kruse & Thines 2017).

There was less resolution of species in the *E. microsporum* complex than in the *E. ranunculi-repentis* complex with the four loci used in the present study. However, as specimens from *Ranunculus paludosus* were clearly distinct, the name *Caeoma bullosum* should be reinstated in its combination in *Entyloma*. The additional two clades found in the *E. microsporum* complex were each represented by specimens from different host species. Both lineages include morphologically similar specimens and both include specimens from *Ranunculus repens*, the type host of *Entyloma microsporum*. To fix the application of the name *E. microsporum*, a neotype was selected from among the specimens in the clade containing most accessions on *Ranunculus repens*, and a new species is introduced for the specimens of the other clade. Both clades with specimens from *Ranunculus repens* showed some internal differentiation according to the host species and thus might be revealed to be species complexes in future studies.

The relationships of the *Entyloma* species covered in this study do not correspond to the relationships of the respective hosts (Paun et al. 2005). It is, thus, conceivable that, similar to the situation in obligate biotrophic downy mildews (Choi & Thines 2015), species of *Entyloma* do not diversify by long-term co-evolution, but rather by host jumps, subsequent radiation, and

Table 3 Main diagnostic ecological (host species) and morphological characters for *Entyloma* species on *Ranunculus*. *E.* = *Entyloma*, *R.* = *Ranunculus*.

Species	Host plant	Arrangement of spores in the sori (between the leaf mesophyll cells)	Spore sizes (µm)	Mean spore sizes and standard deviation (µm)	Spore wall thickness (µm)	Asexual morph	Conidia
***Entyloma microsporum* complex** (sori forming swollen pustules and spores with cracked surface)							
E. bullosum	*R. paludosus*	very densely crowded	(11.5–)15.0–21.5(–26.5) × (10.5–)12.0–16.5(–19.5)	18.1 ± 2.9 × 14.9 ± 1.8	2.5–7.0(–8.0)	absent	absent
E. microsporum	*R. acris*, *R. repens* (type host)	very densely crowded	10.0–18.5(–24.0) × (9.5–)10.0–13.5(–17.5)	14.6 ± 2.8 × 12.2 ± 1.7	(1.5–)2.0–4.5	absent	absent
E. piepenbringiae	*R. polyanthemos* subsp. *nemorosus* (type host), *R. repens*	very densely crowded	(10.5–)12.0–17.5(–21.0) × (9.0–)10.0–15.5(–16.0)	14.5 ± 2.4 × 12.5 ± 1.4	(1.5–)2.5–4.0(–6.0)	absent	absent
***Entyloma ranunculi-repentis* complex** (sori forming flat leaf spots and smooth spores)							
E. eburneum	*R. bulbosus*, *R. polyanthemos*, *R. repens*	loosely scattered or moderately densely crowded	(9.5–)11.0–13.5(–16.0) × (9.0–)9.5–13.5(–14.5)	12.3 ± 1.4 × 11.3 ± 1.3	1.0–1.5(–2.0)	present	dimorphic, cylindrical, 15–22 × 2.5–4.0 µm, and acicular, 30.0–45.0(–60.0) × (2.0–)2.5–3.5 µm
E. jolantae	*R. oreophilus*	densely crowded	10.5–15.5(–16.5) × 10.0–13.5(–14.5)	13.2 ± 1.4 × 11.6 ± 1.1	1.5–2.0	absent	absent
E. klenkei	*R. marginatus*	loosely scattered	10.5–13.0 × 10.0–12.5	11.7 ± 0.9 × 11.1 ± 0.7	1.0–1.8	absent	absent
E. kochmanii	*R. lanuginosus*	loosely scattered	(9.0–)11.0–13.0 × (9.0–)10.0–12.5	11.7 ± 0.9 × 10.9 ± 0.8	0.5–1.5	present	cylindrical, 20–24 × 3.0–3.5(–4.0) µm
E. ranunculacearum	*R. acris*	loosely scattered	10.0–13.5(–14.5) × (9.0–)10.0–12.5(–13.5)	11.8 ± 1.1 × 10.9 ± 0.8	0.8–1.5	present	cylindrical, (10–)15–19(–25) × 2.5–3.5(–4.0) µm
E. ranunculi-scelerati	*R. sceleratus*	loosely scattered	(9.5–)10.0–12.5(–13.5) × (9.0–)10.0–12.5(–13.0)	11.7 ± 0.9 × 11.0 ± 0.9	1.0–1.5	present	dimorphic, acicular, rarely cylindrical, 20–60 × (2.0–)2.5–3.5(–4.0) µm
E. ranunculorum	*R. auricomus*	loosely scattered or moderately densely crowded	10.0–12.5(–14.5) × (9.0–)10.0–12.5(–13.0)	11.8 ± 0.9 × 10.9 ± 0.9	1.0–1.5(–1.8)	present	cylindrical, 16–28 × 2.5–3.5(–4.0) µm
E. savchenkoi	*R. paludosus*	loosely scattered or moderately densely crowded	(10.0–)12.0–16.5(–18.0) × (9.0–)11.0–14.5(–15.0)	13.9 ± 1.4 × 12.3 ± 1.2	1.5–2.5(–3.0)	present	acicular-cylindrical, 25–40 × 2.5–3.0(–3.5) µm
E. thielii	*R. montanus*	densely crowded, often in compact groups	(9.5–)11.0–14.5(–16.5) × 9.0–12.5(–13.0)	12.5 ± 1.5 × 10.8 ± 1.0	0.8–1.5	absent	absent
Entyloma verruculosum (indistinct sori and distinctly tuberculate spores)							
E. verruculosum	*Ranunculus* spp.	densely crowded in the intercellular spaces	(11.0–)12.0–14.5(–16.0) × (10.5–)11.0–14.5(–15.0)	13.4 ± 1.4 × 13.1 ± 1.4	1.5–2.5	absent	absent

finally specific adaptation, leading to diversification into distinct species. As there are numerous additional hosts for *Entyloma* in the genus *Ranunculus* (Vánky 2012) that could not be included in the current study, it seems likely that additional species await discovery and more detailed patterns regarding the evolution of *Entyloma* await revelation.

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