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A phylogenetic hypothesis of Ustilaginomycotina based on multiple gene analyses and morphological data[1]

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Abstract: The subphylum Ustilaginomycotina comprises about 1500 species of basidiomycetous plant parasites. They are usually dimorphic, producing a saprobic haploid yeast phase and a parasitic dikaryotic hyphal phase. With only a few exceptions they occur on angiosperms and are found mainly on members of the Poaceae and Cyperaceae. Molecular methods recently have shown that anamorphic species such as members of *Malassezia* or *Tilletiopsis* should be included in this group. Here we present the most recent consensus as to the phylogeny of this group and discuss its relevant characteristics. Our morphological, ultrastructural and molecular phylogenetic data point to the existence of three lines of Ustilaginomycotina: Entorrhizomycetes, Ustilaginomycetes and Exobasidiomycetes. Entorrhizomycetes is represented by Entorrhizales, a small group of unusual teliosporic root parasites on Juncaceae and Cyperaceae. Ustilaginomycetes, to which the majority of Ustilaginomycotina belong, is a teliosporic and gastroid group characterized by the presence of enlarged interaction zones. Ustilaginomycetes is dichotomous, consisting of predominantly holobasidiate Urocystales and predominantly phragmobasidiate Ustilaginales. Exobasidiomycetes forms local interaction zones. This group is predominantly holobasidiate and consists of teliosporic Doassansiales, Entylomatales, Georgefischeriales and Tilletiales, nonteliosporic Ceraceosorales, Exobasidiales and Microstromatales, as well as the anamorphic Malasseziales. Entorrhizomycetes, Exobasidiomycetes and Ceraceosorales are proposed as new taxa, and the description of Ustilaginomycetes is emended.

Key words: molecular phylogeny, smut fungi, systematics, ultrastructure, Ustilaginomycotina

INTRODUCTION

Ustilaginomycotina is one of the best studied groups of plant parasitic fungi. *Ustilago* and *Tilletia* are well known genera, which contain economically important species (e. g. the barley, wheat, or maize smut fungi; Thomas 1989, Trione 1982, Valverde et al 1995). In addition *Ustilago maydis* is used widely as a model organism for plant pathogenesis (Kahmann and Kämper 2004) and it is the first basidiomycetous plant parasite for which the complete genome is available (MUMDB http://mips.gsf.de/genre/proj/ustilago/). Last but not least the phylogeny of the former smut fungi has been studied thoroughly in the past decade (Bauer et al 1997, 2001a, b, Begerow et al 1997, 2000, 2002a, b, 2004a, Castlebury et al 2005, Stoll et al 2003, 2005).

Beginning with Tulasne and Tulasne (1847) the smut fungi traditionally have been divided into the phragmobasidiate Ustilaginaceae or Ustilaginales and the holobasidiate Tilletiaceae or Tilletiales (e.g. Kreisel 1969, Oberwinkler 1987). The thorough investigation of ultrastructural characters ended in a complete revision of the classification of Ustilaginomycotina (Bauer et al 1997). Ustilaginomycotina not only comprises smut fungi but also nonteliosporic plant parasites such as *Graphiola*, *Exobasidium*, *Microstroma* and a few smaller genera (Bauer et al 2001a). Of interest, a group of human pathogens, Malasseziales, are placed within Ustilaginomycotina as well (Begerow et al 2000). Smut fungi of Microbotryales do not belong in Ustilaginomycotina but instead are members of Pucciniomycotina (Bauer et al 2006), which is in agreement with ultrastructural characteristics and molecular analyses (Aime et al 2006, Begerow et al 1997, Bauer et al 1997).

In contrast to Agaricomycotina and Pucciniomycotina, the septal pores of most Ustilaginomycotina are enclosed at both sides by membrane caps (FIGS. 5, 7; Bauer et al 1997). The monophyly of this group is supported further by a distinctive cellular carbohydrate composition with the dominance of glucose and absence of xylose, thus separating the taxon from Pucciniomycotina and Agaricomycotina (Prillinger et al 1993). Another important apomorphy for Ustilaginomycotina is the presence of zones of host-parasite interaction with fungal deposits resulting from exocytosis of primary interactive vesicles (FIGS. 4, 6; Bauer et al 1997). This feature of the parasitic process is unique among the basidiomycetes. Finally the mono-

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phyly of Ustilaginomycotina is supported by DNA sequence analyses (Swann and Taylor 1993, 1995, Begerow et al 1997, 2004b, Weiß et al 2004, Lutzoni et al 2004).

In contrast to Pucciniomycotina and Agaricomycotina, Ustilaginomycotina is ecologically well characterized by its plant parasitism and shares an essentially similar life cycle with a saprobic yeast-like haploid phase and a parasitic dikaryophase (Bauer et al 2001a). The haploid phase usually starts with the formation of basidiospores after meiosis of the diploid nucleus in the basidium and ends with the conjugation of compatible haploid cells to produce dikaryotic, parasitic mycelia. It has been shown that mating is essential for the infection of host plants (Kahmann and Kämper 2004). The dikaryotic phytoparasitic phase ends with the production of probasidia (teliospores). In the majority of Ustilaginomycotina the teliospore becomes thick-walled and separates itself at maturity from the sorus and functions as a dispersal agent. Almost all Ustilaginomycotina sporulates on or in parenchymatic tissues of their hosts. Depending on the fungal species the sori appear in or on different organs of the hosts (e.g. roots, stems, leaves, inflorescences, flowers, anthers, ovaries, seeds, etc.). The usually powdery, dark brown or black teliospores are the most conspicuous stage in the life cycle of these fungi, thus giving rise to their common name ''smut''.

Other than *Malassezia* species, which inhabit the skin of warm-blooded mammals including humans, and some anamorphic taxa (e.g. *Pseudozyma*, *Tilletiopsis*), the vast majority of Ustilaginomycotina parasitizes higher plants. Only two species of *Melaniella* occur on spikemosses, one species of *Exotheliospora* on ferns and two species of *Uleiella* on conifers. All other Ustilaginomycotina parasitize angiosperms with a high proportion of species on monocots, especially on Poaceae and Cyperaceae. Of the approximately 1500 species about 57% occur on Poaceae and about 12% on Cyperaceae. With few exceptions the teliospore-forming species of Ustilaginomycotina parasitize nonwoody herbs, whereas those lacking teliospores (i.e. members of Ceraceosorales, Exobasidiales and Microstromatales) prefer trees or bushes. However almost all species sporulate on parenchymatic tissues of the hosts.

The combination of structures of the cellular interaction and those of septal pores allowed the distinction of several orders of Ustilaginomycotina, whereas morphological characters of the basidia and sori are now important in the differentiation between families of some orders (Bauer et al 2001b, Begerow et al 2002a). The combination of morphological, ultrastructural and molecular data has produced a more robust classification for Ustilaginomycotina, although several problems concerning the evolution of Ustilaginomycotina still are unresolved. Examples include some discrepancies between the ultrastructural and molecular data in the interpretation of Ustilaginales, Urocystales and the families included therein (cf. Bauer et al 2001a, see below). Also, subsequent to the taxonomic revision of Ustilaginales (Bauer et al 1997), several new families have been proposed in this—still the largest—order of smut fungi (Denchev 1997, Vánky 2000, 2001, 2003), however sequence data from these taxa have not been included in molecular analyses. Moreover new species and genera of the Ustilaginomycotina have been described in recent years, which further complicate the systematics of this subphylum. In this study we aim to provide an overview of Ustilaginomycotina and its phylogeny and classification based on a comparison of new morphological data and multiple gene analysis.

MATERIALS AND METHODS

Transmission electron microscopy.—For conventional chemical fixation, samples were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at room temperature overnight. After six transfers in 0.1 M sodium cacodylate buffer, samples were postfixed in 1% osmium tetroxide in the same buffer for 1 h in the dark, washed in distilled water and stained in 1% aqueous uranyl acetate for 1 h in the dark. After five washes in distilled water samples were dehydrated in acetone, using 10 min changes at 25%, 50%, 70%, 95% and 3 times in 100% acetone. Samples were embedded in Spurr's plastic and sectioned with a diamond knife. Ultrathin serial sections were mounted on formvar-coated, single-slot copper grids, stained with lead citrate at room temperature for 5 min, and washed with distilled water. They were examined with a Zeiss transmission electron microscope operating at 80 kV.

For high-pressure freezing and freeze substitution, samples were removed with a 2 mm cork borer. To remove air from intercellular spaces samples were infiltrated with distilled water containing 6% (v/v) (2.5 M) methanol for approximately 5 min at room temperature. Single samples were placed in an aluminium holder (half with a hollow of 0.3 mm depth for the sample and the other with a flat top) and frozen immediately in the high-pressure freezer HPM 010 (Balzers Union, Lichtenstein) as described in detail by Mendgen et al (1991). Substitution medium (1.5 mL per specimen) consisted of 2% osmium tetroxide in acetone, which was dried over calcium chloride. Freeze substitution was performed at −90 C, −60 C and −30 C, 8 h for each step with a Balzers freeze substitution apparatus FSU 010. The temperature then was raised to approximately 0 C over 30 min and samples were washed in dry acetone another 30 min. Infiltration with an Epon/Araldite mixture (Welter et al 1988) was performed stepwise: 30% resin in acetone at 4 C for 7 h, 70% and 100% resin at 8 C for 20 h each and

100% resin at 18 C for approximately 12 h. Samples were transferred to fresh medium and polymerized at 60 C for 10 h. Finally samples were processed as described above for chemically fixed samples, except that the sections also were stained with 1% aqueous uranyl acetate for 1 h.

Specimens used in the transmission electron microscopy are specified in the legends with these acronyms: AN, private herbarium Apollonia Nagler; BPI, U.S. National Fungus Collection; RB, private herbarium R. Bauer.

Molecular analyses.—Taxon sampling. We used 91 LSU (large subunit ribosomal DNA) sequences, 33 SSU sequences (small subunit ribosomal DNA), 44 ITS sequences (internal transcribed spacer), 23 atp6 (ATP synthase subunit 6) sequences and 29 tub2 (beta-tubulin) sequences of which 84 are new in this study. A detailed list of specimens including accession numbers and additional information is available (SUPPLEMENTARY TABLE I). Because some of the genes could not be amplified and sequenced for all specimens, we used a supertree approach to maximize the information out of the available sequence data. With the supertree algorithms used by RadCon (Thorley and Page 2000) we were able to include specimens of which not all genes were sequenced.

Molecular data. We created alignments for each gene with MAFFT (maximum number of iterative refinements was 500, tree rebuilding 10) (Katoh et al 2002). The datasets of each gene also were combined into two single alignments, with and without the SSU sequences. The subsequent phylogenetic analyses were carried out with PAUP 4.0b10 (Swofford 2002).

Modeltest. For each alignment as well as for the combined datasets we performed a hierarchical likelihood ratio test with Modeltest 3.7 (Posada and Crandall 1998). For the NJ analyses the parameters were fixed to the values calculated by Modeltest.

NJ. Neighbor joining analyses were conducted separately for each gene with the BioNJ algorithm (Gascuel 1997) under the optimal maximum likelihood parameters chosen by Modeltest. For both combined datasets (five and four genes, respectively) bootstrap values were calculated with 10 000 replicates.

Parsimony/supertree. To integrate the data from the four BioNJ single gene topologies we followed a matrix representation with parsimony (MRP) supertree approach. We used RadCon (Thorley and Page 2000) to compute a 0/1 character matrix from the BioNJ topologies compiling the phylogenetic information of the single trees. The matrix was analyzed with a maximum parsimony ratcheting procedure (Nixon 1999). A batch file for ratcheting was computed by PaupratOSX (Sikes and Lewis 2001) using the default parameters and 10 000 iterations. A maximum parsimony ratcheting analysis (TBR, steepest descent = off) was performed with this batch file. Out of the 9730 most parsimonious trees a strict consensus tree was computed. Maximum parsimony bootstrap values (10 000 replicates) were calculated for both combined datasets with the fast step algorithm.

Bayesian analyses. We used MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) to conduct Bayesian Metropolis coupled Markov chain Monte Carlo (B-MCMCMC) analyses for both combined datasets. The analyses were run in four independent chains over 5 000 000 generations. A majority rule consensus tree was calculated from the trees that were sampled after the processes had reached stationarity.

TAXONOMY

Entorrhizomycetes Begerow, Stoll & R. Bauer class. nov. (= Entorrhizomycetidae R. Bauer & Oberw., in Bauer et al 1997)

Fungi phytoparasitici hyphis glomeratis septatis intracellularibus teliosporas terminales procreantibus.

Phytoparasitic fungi forming intracellular septate hyphal coils with terminal teliospores.

Exobasidiomycetes Begerow, Stoll & R. Bauer, class. nov. (= Exobasidiomycetidae Jülich emend. R. Bauer & Oberw., in Bauer et al 1997)

Fungi Ustilaginomycotinorum zonis interactionis localibus; hyphis glomeratis intracellularibus absentibus.

Members of Ustilaginomycotina having local interaction zones and no intracellular hyphal coils.

Ustilaginomycetes R. Bauer, Oberw. & Vánky, emend. Begerow, Stoll & R. Bauer (= Ustilaginomycetidae Jülich emend. R. Bauer & Oberw., in Bauer et al 1997)

Members of Ustilaginomycotina having enlarged interaction zones.

Ceraceosorales Begerow, Stoll & R. Bauer, ord. nov.

Fungi Exobasidiomycetum hyphis intracellularibus.

Members of Exobasidiomycetes having intracellular hyphae.

RESULTS AND DISCUSSION

The MAFFT analyses resulted in these alignments: LSU 763 bp, SSU 1,892 bp, ITS 1,154 bp, atp6 723 bp and β-tubulin 850 bp. From the ITS alignment 698 positions were excluded due to their doubtful homology, resulting in an alignment length of 453 bp. The combined alignment without SSU encompasses 2789 characters and the alignment including SSU has 4681 characters. All alignments have been deposited on TreeBASE (SN3022). The phylogenetic analyses of the single gene alignments resulted in similar topologies (data not shown). The combined analyses of the genes resulted in different topologies that fall into two categories. While the analyses of LSU, ITS, atp6 and β-tubulin resulted in

monophyletic Exobasidiomycetes (as displayed in FIG. 1) the inclusion of the SSU data often resulted in paraphyletic Exobasidiomycetes (SUPPLEMENTARY FIG. 2), which was highly sensitive to the taxon sampling. Begerow et al (1997) previously discussed the long branch of *Entorrhiza* species and based on our data we cannot decide whether the paraphyly is based on an incongruence in the analyzed genes or a result of long branch attraction of *Malassezia* and *Entorrhiza*. As long as further data are lacking we proceed with the three subgroups of Ustilaginomycotina (FIG. 1) as previously described (Bauer et al 1997, Begerow et al 1997, 2000).

Entorrhizomycetes.—*Entorrhiza* represent a rather atypical group of smut fungi that develops inside the roots of Cyperaceae and Juncaceae. In contrast with all other Ustilaginomycotina, *Entorrhiza* species form teliospores in living host cells (FIG. 2) and unlike other species of Ustilaginomycotina the pores of *Entorrhiza* are not enclosed by membrane caps (cf. FIG. 3 with FIGS. 5, 7). The germination of teliospores seems to be different from all other smuts as well because they present an internal septation and four germination tubes (Fineran 1982, Bauer et al 2001a). The genus was discussed as being a sister taxon of all other Ustilaginomycotina based on ultrastructural and molecular data (Bauer et al 1997, Begerow et al 1997).

Exobasidiomycetes.—This class represents the sister group of Ustilaginomycetes (FIG. 1; Bauer et al 1997, Begerow et al 1997). The synapomorphic character of the two classes is the presence of membrane caps at the septal pores (FIGS. 5, 7), but poreless septa have evolved in both groups independently. In different molecular analyses the statistical support for Exobasidiomycetes is different. In the molecular analyses of Begerow et al (1997) this group is only weakly supported, whereas in those of Bauer et al (2001a) and Begerow et al (2000) bootstrap values for this group are at least 56–85%. As discussed above Exobasidiomycetes appears monophyletic in most analyses of this study but paraphyletic in several analyses that include SSU data (SUPPLEMENTARY FIG. 1). Exobasidiomycetes interacts with their hosts by the formation of local interaction zones (FIG. 4; Bauer et al 1997, 2001a). In comparison to Ustilaginomycetes the ecology of Exobasidiomycetes is highly diverse. Members of Ceraceosorales, Exobasidiales and Microstromatales sporulate on woody plants and have abandoned teliospores, instead producing basidia directly on the leaf tissue. Members of Georgefischeriales, Entylomatales and Doassansiales produce teliospores inside the leaf tissue and the spores are liberated by rupture of old and decaying litter. Sori of Tilletiales are exposed by rupture of the host tissue and the species present the same biology as is seen in a large proportion of Ustilaginales. Finally, Malasseziales lacks the dikaryotic phase and are parasitic on the skin of warm-blooded animals. In the following, the orders are discussed in their alphabetical order.

Ceraceosorales.—As in Melanotaeniaceae of Ustilaginomycetes and in Microstromatales, Entylomatales, Doassansiales and Exobasidiales of Exobasidiomycetes (Bauer et al 1997) the septal pores in *Ceraceosorus bombacis* are simple and enclosed by membrane caps at both sides (SUPPLEMENTARY FIG. 5). In *Ceraceosorus* as well as in Brachybasidiaceae the basidia protrude through stomata or emerge from the disintegrated epidermis. In both they are elongate, two-sterigmate and form ballistosporic basidiospores with an adaxial orientation of the hilar appendices (Bauer et al 2001a, Begerow et al 2002a, Cunningham et al 1976). Like Brachybasidiaceae and Exobasidiomycetes in general, *Ceraceosorus* produces local interaction zones (SUPPLEMENTARY FIG. 4). Exobasidiomycetes without interaction apparatus or with simple interaction apparatus, such as Entylomatales, Georgefischeriales, Microstromatales and Tilletiales, do not form intracellular hyphae or haustoria (Bauer et al 1997, 2001a). *Ceraceosorus* however forms intracellular hyphae that do not have a consistent characteristic morphology (SUPPLEMENTARY FIGS. 2, 3). Within Exobasidiomycetes the phylogenetic position of *Ceraceosorus* is unresolved. In this first analysis this fungus appears unsupported on a common branch with Entylomatales and *Tilletiopsis albescens* (FIG. 1).

Doassansiales.—Members of this order are characterized by the presence of a complex interaction apparatus including cytoplasmic compartments (Bauer et al 1997). Most members produce large sporeballs including sterile cells germinating with sigmoid basidiospores, which are interpreted as adaptations to dispersal by water. Although they are ecologically very similar the order is morphologically highly diverse. Their host plants include some spikemosses (Selaginellaceae) and various families of angiosperms, but are all paludal or aquatic. Members of *Doassinga*, *Melaniella* and *Rhamphospora* produce solitary teliospores and group basally to the other genera of the order (Bauer et al 1999).

Entylomatales.—Presence of simple interaction apparatus at the interaction sites characterizes this order (Bauer et al 1997). So far this group comprises only species of *Entyloma* occurring on dicots and some *Tilletiopsis* species (see classification SUPPLEMENTARY TABLE II). Former *Entyloma* species occurring on monocots were transferred to several genera of

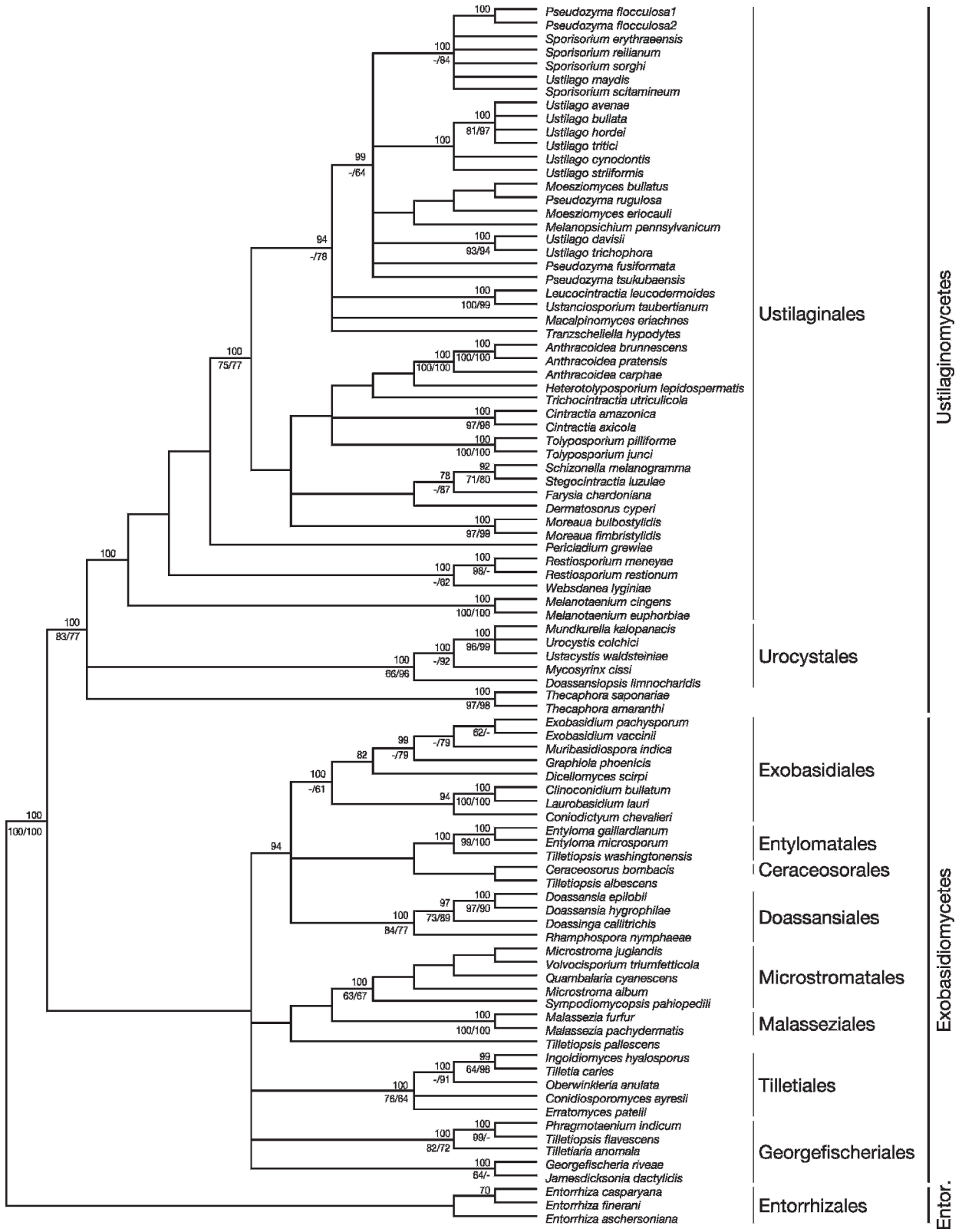


FIG. 1. Supertree topology from a parsimony ratchet analysis (10 000 iterations) of a matrix that has been generated from four neighbor joining topologies (LSU, ITS, atp6 and β-tubulin genes). Strict consensus from 9730 equally most parsimonious trees (252 steps). Posterior probabilities (20 000 trees) for the four-gene alignment of 2789 bp are shown above branches, MP and NJ bootstrap values (10 000 replicates) are given below branches. Values greater than 60% only are depicted.

Georgefischeriales, as molecular and ultrastructural analyses revealed that the genus *Entyloma* was polyphyletic (Begerow et al 1997, 2002b). The members of *Entyloma* produce their spores in leaf tissue similar to members of Doassansiales, but no sporeballs are formed. They have an anamorphic stage that produces conidia on the leaf surface and might be responsible for the mass infection of some species (e.g. *Entyloma ficariae* often infects the whole population of *Ficaria verna* within a short period of some weeks of the year).

Exobasidiales.—Presence of interaction tubes produced by a complex interaction apparatus characterizes this order (FIG. 4). The monophyly of these highly diverse species is supported by molecular analyses (FIG 1; Begerow et al 1997, Begerow et al 2002a). The members of Exobasidiales are holobasidiate and dimorphic, but do not form teliospores in the parasitic phase or ballistoconidia in the saprobic phase. Hosts are mono- and dicots and the sori appear predominantly on leaves. The different morphology and ecology of the four subgroups is reflected in the four families of Exobasidiales.

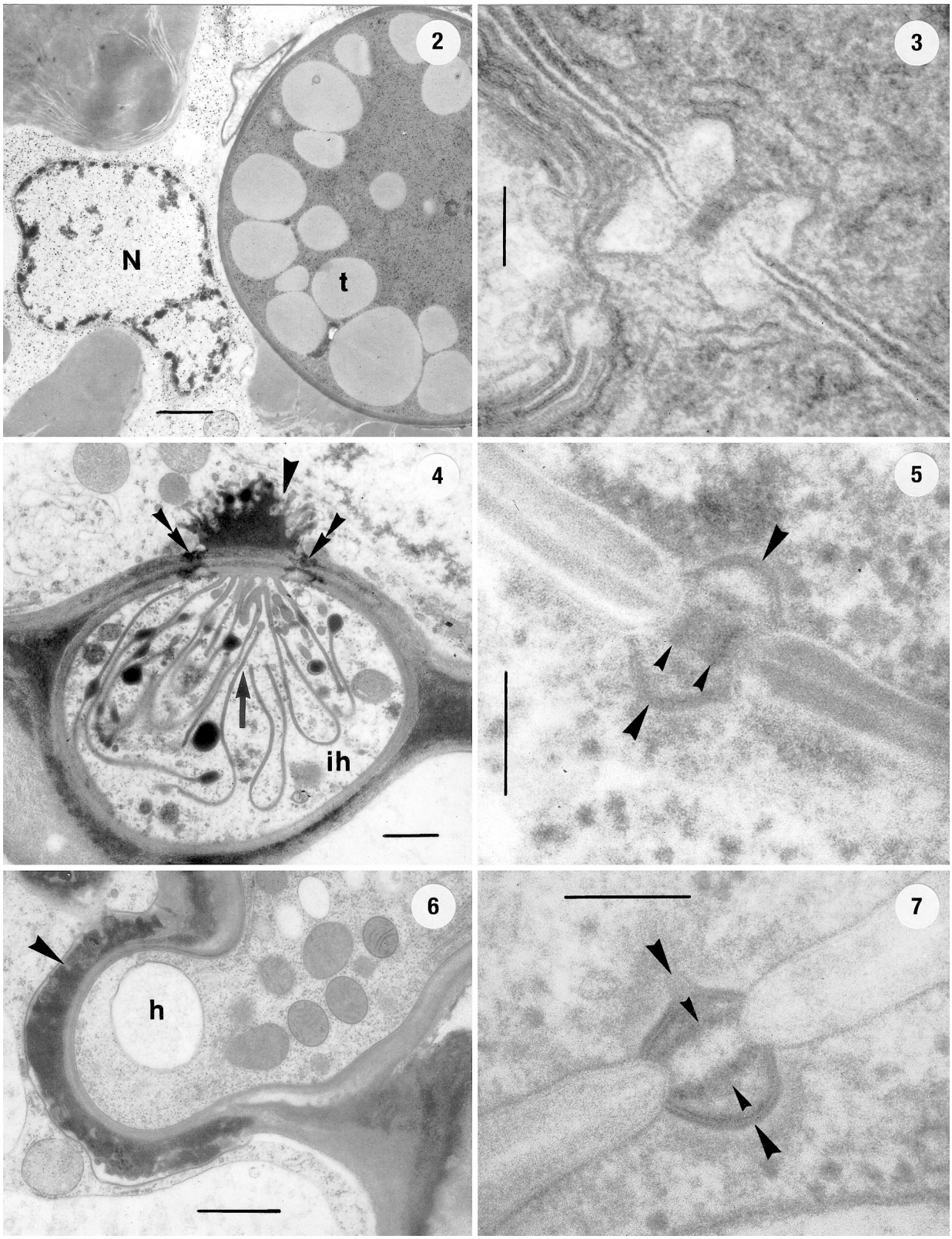
Brachybasidiaceae sporulates on the surface of the host organs of annual or perennial herbs and the elongated, ballistosporic, two-sterigmate basidia protrude through stomata or emerge from the disintegrated epidermis (Begerow et al 2002a, Cunningham et al 1976). Exobasidiaceae sporulates through stomata or from the disintegrated epidermis, the basidia are elongate and ballistosporic, and the basidiospores are thin-walled. In contrast to Brachybasidiaceae however the hilar appendices of the basidiospores are oriented abaxially at the apex of the basidia (Begerow et al 2002a). The Cryptobasidiaceae sporulates internally by producing holobasidia in peripheral lacunae of the host galls. Thus the basidia are gastroid and lack sterigmata and the basidiospores are usually thick-walled, resembling the uredospores of rust fungi or the teliospores of smut fungi (Begerow et al 2002a). Graphiolaceae are parasites of palms (Arecaceae) and the fructification of Graphiolaceae initiates between the chlorenchyma and hypodermal tissue (Cole 1983). During differentiation of the cylindrical basidiocarp the epidermis ruptures and globose basidia are produced in chains by disarticulation of sporogenous hyphae within the basidiocarps (Oberwinkler et al 1982, Begerow et al 2002a).

Georgefischeriales.—As in other molecular studies (Begerow et al 1997, 2000, Bauer et al 2001b; Weiss et al 2004) the monophyly of this order is only weakly supported (FIG. 1). Among Exobasidiomycetes, Georgefischeriales is characterized by poreless septa in mature soral hyphae. Species are dimorphic and produce light brown or dark teliospores in vegetative parts of their hosts. They interact with their respective hosts by forming local interaction zones without interaction apparatus and lack haustoria or intracellular hyphae (Bauer et al 1997). The four families are distinguished mainly by their differing basidial morphologies and might be perfect examples of ecological adaptation of basidial structures (Bauer et al 2001b, 2005). Most members of Georgefischeriales occur on monocots, but the two species of *Georgefischeria* parasitize Convolvulaceae species, which might reflect the ecological adaptation of smuts to grassland habitats like in other orders (e.g. Tilletiales, Ustilaginales).

Species of Georgefischeriaceae and Gjaerumiaceae are characterized by holobasidia and *Tilletiopsis*-like pseudohyphal anamorphs that produce ballistoconidia (Bauer et al 2001b). Species of Gjaerumiaceae have dolipores in young soral septa, in contrast to Georgefischeriaceae, which are poreless (Bauer et al 2005). Tilletiariaceae also forms a *Tilletiopsis*-like pseudohyphal anamorph that produces ballistoconidia, but they represent the only phragmobasidiate group within Exobasidiomycetes (Bandoni and Johri 1972). Members of Eballistraceae are holobasidiate and are characterized by forming a budding yeast phase without ballistoconidia from the basidiospores (Singh and Pavgi 1973).

Malasseziales.—The classification of *Malassezia* in Exobasidiomycetes is based on molecular data (Begerow et al 2000). All seven known species are isolated from the skin of warm-blooded animals and represent anamorphic, medically important, lipophylic yeasts (Guého et al 1998). It is still unclear whether *Malassezia* species are phytoparasitic in the dikaryophase or if they originated from plant parasites. They have been found associated with a variety of pathological conditions in humans including pityriasis versicolor, seborrheic dermatitis, folliculitis and systemic infections (Guého et al 1996). New complex compounds have been isolated from *Malassezia* species and their biological activity as agonists of the arylhydrocarbon receptor has been shown (Wille et al 2001).

Microstromatales.—This order is characterized by local interaction zones without interaction apparatus and their lack of teliospores (Bauer et al 1997). Only a few species have been assigned to this order so far, but all of them are inconspicuous and molecular analyses have shown that several taxa can be included (Begerow et al 2001, de Beer et al 2006). The members of Microstromatales are gastroid. Young basidia protrude through the stomata directly and



FIGS. 2–7. Ultrastructural characteristics of the Ustilaginomycotina, representative of the Entorrhizales (Entorrhizomycetes) (FIGS. 2–3), Exobasidiales (Exobasidiomycetes) (FIGS. 4–5) and Urocystales (Ustilaginomycetes) (FIGS. 6–7). 2. Young teliospore (t) of *Entorrhiza casparyana* (Magnus)Lagerh. RB 941 within a cell of *Juncus articulatus* L. The nucleus of the host cell is visible at N. 3. Dolipore without membrane caps of *Entorrhiza casparyana* (Magnus) Lagerh. RB 941. 4. Local interaction

sporulate on the leaf surface (Oberwinkler 1978, Patil 1977). Members of Volvocisporiaceae are characterized by the formation of highly septate basidiospores. In contrast to Microstromataceae, Quambalariaceae is characterized by simple dolipores (de Beer et al 2006).

Tilletiales.—This order is characterized among Exobasidiomycetes by striated dolipores in the septa (Bauer et al 1997). In contrast to all other groups of Exobasidiomycetes, Tilletiales is not dimorphic. They form local interaction zones without interaction apparatus and hyphal anamorphs with ballistoconidia (e.g. Ingold 1987, 1997). The teliospores of this order are the largest of Ustilaginomycotina. The genera of Tilletiales are similar in morphology and ecology, leading to difficulties in delineation of monophyletic genera, and results of molecular data also did not support the recognition of some genera of Tilletiaceae (Castlebury et al 2005). Except for *Erratomyces* on Fabaceae, Tilletiaceae parasitizes grasses and the sori often appear in ovaries (Piepenbring and Bauer 1997, Vánky 1994). This might indicate a convergent evolution, such as in Ustilaginales, as an adaptation to open grassland vegetation.

Exobasidianae.—Based on the existence of an interaction apparatus and its structure, Entylomatales, Doassansiales and Exobasidiales were grouped together in Exobasidianae (Bauer et al 1997). Molecular analyses neither could confirm nor reject this hypothesis with convincing support. The multiple gene analysis (FIG. 1) is the first molecular indication for this superorder.

Ustilaginomycetes.—This class in the sense of the proposed classification (SUPPLEMENTARY TABLE II) is characterized by the presence of enlarged interaction zones (FIG. 6; SUPPLEMENTARY FIGS. 6, 8, 10). All members of Ustilaginomycetes are teliosporic, gastroid and dimorphic. They are morphologically and ecologically diverse, but both ultrastructural and LSU rDNA sequence analyses confirm their monophyly (Bauer et al 1997, Begerow et al 1997). A basal dichotomy supports two orders in the class: Urocystales is characterized by the presence of pores in the septa of soral hyphae, whereas Ustilaginales lacks pores in mature septa (FIG. 7, Bauer et al 1997). The placement of Glomosporiaceae and Melanotaeniaceae in this classification however is equivocal.

Basal taxa.—Species of the three genera currently classified in Glomosporiaceae possess light brown spores or sporeballs, whose cells germinate with holobasidia (Vánky 2002). The hosts of Glomosporiaceae are dicots and recent studies have shown that the potato (Solanaceae) infecting *Thecaphora solani* Barrus may cause serious economic damage in South America (Andrade et al 2004). Glomosporiaceae was interpreted by Bauer et al (1997) as the basal family of Ustilaginales because it lacks pores in mature septa and forms intracellular hyphae (SUPPLEMENTARY FIGS. 6, 7). However (in SUPPLEMENTARY FIG. 1 and in several LSU rDNA analyses) Glomosporiaceae appears highly supported in a position at the base of Urocystales (Bauer et al 2001a, Begerow et al 1997, 2000). In the supertree topology (FIG. 1), the position of Glomosporiaceae is unclear. Thus our recent data neither can support nor reject a position of Glomosporiaceae within Ustilaginales versus Urocystales.

Melanotaeniaceae and most Urocystales have the formation of haustoria (FIG. 6, SUPPLEMENTARY FIG. 8), simple pores enclosed by membrane caps (FIG. 7, SUPPLEMENTARY FIG. 9), and holobasidia in common. In contrast to the septal pores of Urocystales however the septal pores of Melanotaniaceae have no inner nonmembranous plates (cf. FIG. 7 and SUPPLEMENTARY FIG. 9). Therefore this group was interpreted by Bauer et al (1997) as a basal taxon of Urocystales. In our molecular analyses (FIG. 1, SUPPLEMENTARY FIG. 1) as well as in other molecular analyses (Bauer et al 2001a, Begerow et al 1997, Weiß et al 2004) the representatives of Melanotaniaceae are always on a common branch with Ustilaginales with moderate to high support. Therefore we favor a classification of Melanotaeniaceae in Ustilaginales.

Urocystales.—In our proposed classification this order comprises Doassansiopsaceae, Mycosyringaceae and Urocystaceae. The species of *Doassansiopsis*, the single genus currently placed in Doassansiopsaceae, possess complex sporeballs with a central mass of

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zone of *Exobasidium pachysporum* Nannf. RB 947 on *Vaccinium uliginosum* L. Intercellular hypha (ih) in contract to a host cell showing the exocytosis profile of the interaction apparatus (arrow) with the interaction tube (double arrowheads). Note the electron-opaque deposit at the host side (arrowhead). 5. Simple pore with two membrane caps (large arrowheads) and a tube (small arrowheads) within the pore channel of *Exobasidium karstenii* Sacc. & Trotter RB 1063. 6. Enlarged interaction zone of *Ustacystis waldsteiniae* (Peck) Zundel RB 1011 on *Waldsteinia geoides* Willd. Haustorium (h) is surrounded by an electron-opaque matrix (arrowhead). 7. Simple pore with two membrane caps (large arrowheads) and two inner nonmembranous plates (small arrowheads) of *Ustacystis waldsteiniae* Willd. RB 1056. Bars = 2 μm in FIG. 2; 0.5 μm in FIGS. 4 and 6; 0.1 μm in FIGS. 3, 5, and 7. Material illustrated in FIGS. 4–7 was prepared with high-pressure freezing and freeze substitution.

pseudoparenchymatous cells surrounded by a layer of firmly adhering, lightly colored teliospores and an external cortex of sterile cells (Vánky 2002). Doassansiopsaceae and Urocystaceae have the presence of haustoria and an essentially identical septal pore apparatus in common (FIGS. 6, 7; Bauer et al 1997). Mycosyringaceae is represented by the single genus *Mycosyrinx*. *Mycosyrinx* species produce teliospores in pairs and their host range is restricted to Vitaceae (Vánky 1996). *Mycosyrinx* lacks pores in mature septa (SUPPLEMENTARY FIG. 11) like Ustilaginales, but in our molecular analysis *Mycosyrinx* appears within Urocystales (FIG. 1). Thus poreless septa at maturity seem to be a convergent feature, which appears often in other groups as well (e.g. Georgefischeriales or even in Microbotryales of Pucciniomycotina).

Urocystaceae is composed of morphologically diverse species with colored teliospores. Most of the species develop their teliospores in balls, which is arguably a common character in Urocystales. Holobasidia as well as phragmobasidia are known in this family and their broad host range covers monocots and dicots.

Ustilaginales.—This order comprises the majority of smut fungi including the large genera *Ustilago* and *Sporisorium*. Most species of this group sporulate in the reproductive parts of their hosts. The anamorphs fit the concept of *Pseudozyma sensu* Boekhout (1995). Several families recently have been proposed that would divide Ustilaginaceae into smaller groups (Denchev 1997, Vánky 2000, 2001, 2003). A comprehensive molecular study dealing with an internal classification of Ustilaginaceae *sensu* Bauer et al (1997) is lacking so far. Next we discuss a proposal to accommodate some of the hitherto published names.

Ustilaginales have darkly colored teliospores and germinate with usually four-celled phragmobasidia. The first family, which was excluded from Ustilaginaceae *sensu* Bauer et al (1997) was Anthracoideaceae (Denchev 1997). Species of *Anthracoidea* present a unique type of two-celled basidia and parasitize species of *Carex* almost exclusively. They exhibit an expanding element in the LSU rDNA, which complicates their alignment with other smut species. In our molecular tree *Anthracoidea* species appear on a common branch with *Cintractia*-like smuts but with weak support (FIG. 1). Vánky (2001) included the genera *Cintractia*, *Heterotolyposporium*, *Leucocintractia*, *Testicularia*, *Tolyposporium*, *Trichocintractia*, and *Ustanciosporium* in Cintractiaceae. In our molecular analyses a group containing species of *Anthracoidea*, *Cintractia*, *Dermatosorus*, *Farysia*, *Heterotolyposporium*, *Moreaua*, *Planetella*, *Schizonella*, *Stegocintractia*, *Tolyposporium* and *Trichocintractia* form a monophyletic clade. Therefore we accept Anthracoideaceae and consider this family for Ustilaginales that occur on Cyperaceae and Juncaceae. Consequently we reject Cintractiaceae, Dermatosoraceae and Farysiaceae (Vánky 2001) because they are not monophyletic but interspersed in Anthracoideaceae. Websdaneaeceae includes *Websdanea* and *Restiosporium*, which share the host range of Restionaceae. This group is well supported in our analyses (FIG. 1). Ustilaginaceae is composed of the large genera *Ustilago* and *Sporisorium* and several smaller genera. Except *Melanopsichium* all species parasitize Poaceae species. *Melanopsichium pennsylvanicum* is embedded in Ustilaginaceae (FIG. 1), and consequently we reject Melanopsichiaceae (Vánky 2001). Several molecular studies have shown that the separation of *Ustilago* and *Sporisorium* is difficult based on previously used characters (Stoll et al 2003, 2005). To our astonishment we discovered in our molecular analysis (FIG. 1) that *Macalpinomyces* and *Tranzscheliella*, which both occur on Poaceae, are not members of the clade representing Ustilaginaceae. An analysis of cospeciation revealed a relative high degree of jumps for members of *Sporisorium* and *Ustilago*, but the jumps always were restricted to a monophyletic host group (Begerow et al 2004a). Recent reclassifications of the Ustilaginales are consistent with host range as being more phylogenetically informative than previously appreciated. Nearly all groups of smut fungi that have been analyzed to date revealed a high degree of cospeciation and host specificity (Begerow et al 2002a, 2004a, Bauer et al 2005). Vánky (2001) also created Clintamraceae for *Clintamra*, Geminaginaceae for *Geminago* and Uleiellaceae for *Uleiella* mainly based on host relationships. Molecular data unfortunately are not available for these genera and it is not clear whether these genera represent recent or ancient host jumps; thus we currently do not recognize these families.

The systematics of Ustilaginomycotina is far from being understood and fixed in all details and the discrepancies between morphological and molecular data could not be resolved in every case. The simple addition of more genes and multiple loci analyses did not necessarily result in a better phylogenetic resolution for all taxa. However the integration of all available data lets us summarize the state of the knowledge and present the most comprehensive phylogenetic classification to date (supplement).

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