Phylogenetic placements of ustilaginomycetous anamorphs as deduced from nuclear LSU rDNA sequences*

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In order to integrate ustilaginomycetous anamorphs into the general phylogenetic system of Ustilaginomycetes, partial nuclear large subunit ribosomal DNA sequences of 56 teleomorphic and 19 anamorphic species of the Ustilaginomycetes were analysed. Maximum parsimony and neighbour joining confirm the new suprageneric system of Ustilaginomycetes and indicate that (i) the species of *Pseudosyzma* represent anamorphs of Ustilaginiales parasitizing grasses, (ii) *Pseudosyzma prolifica*, the type of *Pseudosyzma*, is very closely related to *Ustilago maydis*, (iii) *Pseudosyzma tsukubaensis* is probably synonymous with *Ustilago spernphora*, (iv) the species of *Malassezia* represent a group of its own within the Exobasidiomycetidae, (v) *Tilletiopsis cretena*, *T. lilacina* and *T. washingtonensis* belong to the Entylomatales and (vi) *T. flavus*, *T. fulvescens* and *T. minor* are members of the Geofungalesia. Like all *Tilletiopsis* species tested, *T. albescens* and *T. pallescens* are members of the Exobasidiomycetidae, but they cannot be ascribed to any of the known orders of this subclass. The description of the Malassezieas is emended.

Many basidiomycetes lack either a perfect state or it is currently unknown. Because the characters used for classifying basidiomycetes are predominantly derived from the process of sexual reproduction, these anamorphic fungi are segregated into artificial deuteromycetous taxa. In modern phylogenetic considerations, however, both teleomorphic and anamorphic fungi should be integrated where possible. Comparative morphological, ultrastructural, physiological, biochemical and molecular studies are useful for such an undertaking (e.g. Swann & Taylor, 1995).

This study was made to examine the phylogenetic relationships among a diversity of ustilaginomycetous species including both teleomorphic and anamorphic species. Partial nuclear large subunit rDNA sequences of 56 teleomorphic species of the Ustilaginomycetes were analysed together with sequences of seven species of *Pseudosyzma* (Boekhout, 1995), eight species of *Tilletiopsis* (Boekhout, Fell & O'Donnell, 1995) and four species of *Malassezia* (Guillot & Guého, 1995). It is well known that the species of *Pseudosyzma* and *Tilletiopsis* represent anamorphs of the Ustilaginomycetes. For example, species of *Ustilago* from morphologically similar colonies in the haploid state as the species of *Pseudosyzma*, whereas species of *Entyloma*, for example, develop *Tilletiopsis*-like cultures in the haploid state (Boekhout, 1987, 1991, 1995). Yeast cells of species of *Pseudosyzma* and *Tilletiopsis* are ellipsoid, ovoidal to cylindrical and usually have polar budding. Their hyphae are narrow and lack clamp connections. The species of *Pseudosyzma* differ predominantly from those of *Tilletiopsis* by the lack of ballistoconidia. *Malassezia* comprises lipophilic yeasts morphologically characterized by small cells with unipolar, enteroblastic, and repetitive budding. In *Malassezia sympodalis*, however, proliferation may be sympodial (Yarrow & Ahearn, 1984; Guého, Midgley & Guillot, 1995). Fell, Boekhout & Freshwater (1995) and Guillot, Guého & Prévost (1995) indicated the ustilaginomycetous nature of *Malassezia* using ultrastructural and molecular characteristics.

Both maximum parsimony and neighbour joining algorithms were used to determine the phylogenetic positions of the test species within the new suprageneric system of the Ustilaginomycetes, consisting of three subclasses with ten orders. This system is based predominantly on ultrastructural analysis (Bauer, Oberwinkler & Vánky, 1997) and is well supported by molecular sequence data (Begerow, Bauer & Oberwinkler, 1997).

**MATERIALS AND METHODS**

The analysed species are listed in Table 1. DNA was isolated from cultures or herbarium specimens using the SDS method as described previously (Begerow et al., 1997). The 5' region of the nuclear large subunit of the ribosomal RNA gene was amplified using the polymerase chain reaction and the primers NL1 and NL4 (O'Donnell, 1993). The PCR product was purified using the QIAquick™ protocol (QIAGEN). This dsDNA was sequenced directly using the ABI PRISM® Dye-
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<th>Collection no.</th>
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<td>H.U.V. 17816</td>
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Termination Cycle Sequencing Kit (Applied Biosystems) on an automated sequencer (ABI 373A, Applied Biosystems). An alignment of 540 bp was created using MEGALIGN of the Lasergene-package (DNASTAR, Inc. 1997) and reworked by hand. The PHYLIP package, version 3.572 (Felsenstein, 1995), was used to perform the following analyses: neighbour joining of a distance matrix (Kimura 2-parameter model, transition to transversion rate: 2.0) with 1000 bootstrap replicates and maximum parsimony (heuristic) with the jumble option turned on 10 replicates and 100 bootstrap replicates. The sequences are deposited in GenBank (see Table 1).

RESULTS

The system

Analyses of neighbour joining and maximum parsimony resulted in similar tree topologies (compare Fig. 1 and Fig. 2). Using the species of Entorrhiza as root, the species clustered into three groups. These groups were congruent to the Entorrhizomycetidae, Ustilaginomycetidae and Exobasidiomycetidae as identified by Bauer et al. (1997). In addition, the groups recognized by Bauer et al. (1997) as orders were also evident. They are the Urocystales and Ustilaginales (Ustilaginomycetidae), and the Entylomatales, Exobasidiales, Doassansiaceae, Geofischeriacaeae, Microstromatales and Tilletiales (Exobasidiomycetidae) (Figs 1–2). The two methods of analysis, however, showed some differences concerning the arrangement of the orders of the Exobasidiomycetidae (Figs 1–2).

Pseuodzyma

In both neighbour joining and maximum parsimony analyses all the anamorphic yeast species of Pseuodzyma were located in the clade representing the Ustilaginales (Figs 1–2). In both analyses the Ustilaginales occurring on grasses, Melanotiuchum pensylvanicum and Pseuodzyma species, formed a group that is statistically supported with 77 and 75% respectively (Figs 1 and 2). The sequence of P. isukuænis was identical to that of Ustilago sperrnphora, and P. prolifica, the type species of Pseuodzyma, appeared next to U. maydis.

Tilletiopsis

The species tested of Tilletiopsis were scattered among the species representing the Exobasidiomycetidae (Figs 1–2), indicating a high degree of polyphyly for this asexual genus. Both analyses placed T. crema, T. lilacina and T. washingtonensis on the clade representing the Entylomatales. They appeared as the sister-group of the Entyloma species.

Tilletiopsis minor, T. fulvescens and T. flava appeared within the clade representing the Geofischerales and the two strains of T. fulvescens appeared next to one another, although they differ in 12 bp. Within the Geofischeriales, there were three clades, representing the Tilletiariaceae, Geofischeriacaeae and the Entyloma orzyae-group (Bauer et al., 1997). T. fulvescens and T. flava were located on the clad representing the Tilletiariaceae. This group is well supported by a high bootstrap value (Figs 1–2).

T. minor appeared as sister species of Melanotanicum brachiiaceae, which represents the Entyloma orzyae-group in this study. The two methods of analysis conflicted in phylogenetic placement (compare Fig. 1 and Fig. 2) of these two species, but neither of these positions is well supported by high bootstrap values.

In both analyses T. albescens and T. pullescens were not placed in any of the groups recognised by Bauer et al. (1997) as orders (Figs 1 and 2). In addition, there were differences in phylogenetic placements of T. albescens and T. pullescens between the two algorithms and there was no statistical significance for the positions of these two species illustrated in Figs 1 and 2.
**Phylogeny of ustilaginomycetous anamorphs**

![Phylogenetic tree](image)

**Fig. 1.** Neighbor joining analysis rooted with *Entorrhiza*. Bootstrap values under 50% are not shown. Anamorphic species are marked by asterisks.

**Malassezia**

Both methods of analysis placed the four *Malassezia* species investigated together on a common well-supported branch, but the phylogenetic position within the Exobasidiomycetidae differs between the two analyses and is not statistically supported.

**DISCUSSION**

**The system**

The groups of Ustilaginomycetes reported by Bauer *et al.* (1997) and Begerow *et al.* (1997) are also evident in this molecular study with an expanded set of species. These are the *Entorrhizomycetidae* with the *Entorrhizales*, the *Ustilagino-
mycetidae with the Urocystales and Ustilaginales and the Exobasidionymyctidae with the Georgiefischerales, Tilletiales, Entylotales, Microstomatales, Doassansiales and Exobasidiales. Interestingly, the Exobasidionymyctidae are better supported by bootstrap values in the recent study than in the study of Begerow et al. (1997), possibly due to the larger number of taxa in this study. In contrast with the sequence studies, however, the ultrastructural analysis resolves the relationship among three orders of the Exobasidionymyctidae (Bauer et al., 1997). The unclear taxonomic situation of Melanotientiales and Glomosporiaceae is discussed in detail in Begerow et al. (1997).
**Pseudozyma**

Species of *Pseudozyma* have been isolated from quite different substrates (see Table 1). Our results indicate that they are aramorphs of species of Ustilaginales that parasitize grasses. These species form with *Melanopsichium pennsylvanicum* a statistically well supported subgroup of the Ustilaginales. *Pseudozyma prolifica* is most closely related to *Ustilago maydis*, as also observed by Boekhout et al. (1995). *P. tsukubaensis* may represent the yeast-form of *U. spernophora*. Certainly, the yeasts of the Ustilaginales occurring on grasses fit the concept of the anamorph genus *Pseudozyma* (Boekhout, 1987, 1995). We are not surprised by the association between *Pseudozyma* and the grass-infecting species of the Ustilaginales. About 800 from ca 1400 known species of Ustilaginomycetes are living on grasses. Most of them are members of the Ustilaginales. Furthermore, Poaceae cover about a third of the land surface with extensive populations, so yeasts of Ustilaginales occurring on grasses might be very abundant in nature.

**Tilletiopsis**

Species of *Tilletiopsis* are frequently found as epiphytes on leaves, especially those infected with powdery mildew or rust fungi (Boekhout, 1991; Urquhart & Punja, 1997). We found *Tilletiopsis* in nearly all sporeulations of *Exobasidium* spp. In fact, because of the frequent association with *Tilletiopsis* it is difficult to get a pure culture of species of *Exobasidium* Woronin (compare also Boekhout et al., 1995). Among the Ustilaginomycetes, only the Melanotaeniaceae of the Ustilaginomycetidae, the Georg fischeriaceae and Tilletiariaceae of the Georg fischeriales and the Entylomatales form *Tilletiopsis*-like pseudohyphal anamorphs that
produce ballistoconidia (Boekhout, 1987, 1991; Ingold, 1988; Bauer et al., 1997; Begerow et al., 1997). It is, therefore, not surprising that most of the species of Tilletiopsis tested belong either to the Geogheschieriales or Entylomatales. Finally, our results are in agreement with other molecular analyses (Boakhout et al., 1995; Takashima & Nakase, 1996).

Tilletiopsis cremea, T. lilacina and T. waishingtonensis are members of the Entylomatales. In terms of molecular analysis, however, these three asexual fungi are well apart from the species of Entyloma tested. They are, probably, representatives of a second family of the Entymolatales, which is possibly characterised by the loss of a phytoparasitic phase.

Our sequence analyses demonstrate that Tilletiopsis flava and T. fulvescens belong to the Tilletiaricaceae sensu Bauer et al. (1997) of the Geogheschieriales. Tilletiaria anomala forms ballistosporic phragmospadisa and is known only from cultures (Bandoni & Johri, 1972). Except for Geogheschieria, the other sexual species of the Geogheschieriales parasitize grasses (Bauer et al., 1997). The systematic position of T. minor within the Geogheschieriales is not clear. We favour a position as the sister-group of the Entyloma oryzae-group, because the species of that group, represented here by Melanotrichum brachiariae, do not form Tilletiopsis-like anamorphs (Bauer et al., 1997). We are surprised that no species of Tilletiopsis clusters with species representing that Geogheschieria. Entyloma dactyloides, for example, a member of this family with a Tilletiopsis-like anamorph is very abundant in nature, infecting nearly all individuals in a grass population in some years.

T. albescens and T. pallescens are members of the Exobasidio- mycetidae, but they cannot be ascribed to any of the known groups of this subclass. We are convinced that the ‘chlamydospore’ germination of these two species as drawn by Boekhout (1987, fig. 1A; 1991, fig. 52e) represent basidia of the exobasidioaceous type (Oberwinkler, 1978). For example, we have isolated a Tilletiopsis-like fungus (Fig. 3) from Calendula officinalis L. It developed teliospores (Fig. 4) after some months on malt-yeast-peptone-agar (Bandoni, 1972). After transferring the teliospores on water agar, they germinated with basidia of the exobasidioaceous type (hilar appendices of the basidiospores oriented abaxially, Fig. 5). These basidia are very similar to the ‘chlamydospore’ termination of T. albescens and T. pallescens drawn by Boekhout (1987, 1991). Species with this basidial type occur in the Geogheschieraceae, Tilletiales and Doassaansiales (Bauer et al., 1997). Neither T. albescens nor T. pallescens cluster with representatives of these groups. It is possible, therefore, that these species are representatives of two hitherto unknown higher groups of the Exobasidiomycetidae.

Malassezia

Malassezia comprises lipophilic yeasts that constitute part of the mycosta of the skin of warm-blooded animals (Guého et al., 1998). They have also been found associated with a variety of pathological conditions in humans including pityriasis versicolor, seborrhiec dermatitis, folliculitis and systemic infections (see Guillot & Guého, 1995; Guého et al., 1996; and the references therein). The cell wall of the Malassezia yeasts is thick, multilamellate and reveals a unique substructure with an electron- opaque, helicoidal band that corresponds to a helicoidal evagination of the plasma membrane (Takeo & Nakai, 1986; Guillot et al., 1995; Guého et al., 1996). Obviously, the band is formed at the top of the evagination of the plasma membrane.

Molecular analyses indicate that the species of Malassezia are members of the Exobasidionymyctidae, representing a group of its own—the Malasseziales (Moore, 1980). As discussed above, the separate position of the Malassezia is in agreement with morphological ultrastructural, and physiological characteristics. To accommodate the Malasseziales in the Exobasidiomycetidae (Ustilaginomycetes) the description is emended as follows as follows:

Malasseziales R. T. Moore 1980

Zoophilic members of the Exobasidionymyctidae (Ustilaginomycetes) with a monopolar budding yeast phase showing percurrent or sympodial proliferation of the buds. The yeasts are lipid-dependent or lipophilic, have a multilayered cell wall and a helicoidal evagination of the plasma membrane.

The sexual phase of Malassezia is unknown. Are the species of Malassezia phytoparasitic in the dikaryophase, or did they originate from plant parasites? We do not know the answer. On one hand, among the numerous ustilaginomycetous anamorphs we have investigated ultrastructurally, there was no yeast with the Malassezia ultrastructure. On the other hand, Ustilaginomycetes have different strategies during different phases of their life-cycle, and this may be true for Malassezia as well.

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REFERENCES


