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Flamingomyces and *Parvulago*, new genera of marine smut fungi (*Ustilaginomycotina*)☆

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ABSTRACT

Teliospore walls, teliospore germinations, hyphal septations, cellular interactions, and nucleotide sequences from the D1/D2 region of the nuLSU rRNA gene of the marine smut fungi *Melanotaenium ruppiae* and *Ustilago marina* were examined and compared with findings in other *Ustilaginomycotina*. The data show that *Melanotaenium ruppiae* belongs to the *Urocystaceae* and *Ustilago marina* to the *Ustilaginaceae*. Within the *Urocystaceae*, *Melanotaenium ruppiae* is morphologically similar to *Melanustilospora* and *Vankya*. However, according to the molecular results *Melanotaenium ruppiae* can neither be ascribed to *Melanustilospora* nor to *Vankya*. Therefore, the new genus *Flamingomyces* is proposed for *Melanotaenium ruppiae*. *Ustilago marina* differs from the other *Ustilaginaceae* in the mode of sporulation, which exclusively occurs at the base of the host plant culms. Accordingly, the new genus *Parvulago* is proposed for *Ustilago marina*.

Introduction

Studies of the new system of *Ustilaginomycetes*, now considered as the subphylum *Ustilaginomycotina* (Bauer *et al.* 2006), revealed that parallel evolution with their hosts was apparently a widespread phenomenon in the ustilaginomycotinous phylogeny (Bauer *et al.* 1997). Thus, genera with species parasitic on hosts of distantly related plant groups are possibly polyphyletic. Two of these genera are *Melanotaenium* and *Ustilago*. With the new system of *Ustilaginomycotina* it becomes clear that *Melanotaenium* species are restricted to dicotyledonous hosts and *Ustilago* species to poacean hosts (Bauer *et al.* 1997, 2001a). Accordingly, following revision of ''*Melanotaenium*'' and ''*Ustilago*'' new genera or new arrangements were described, such as the proposals of *Phragmotaenium* and *Eballistra* for some species of *Entyloma* and *Melanotaenium* on *Poaceae* (Bauer *et al.* 2001b) or *Bauerago* for some *Ustilago* species on *Commelinaceae*, *Cyperaceae*, and *Juncaceae* (Vánky 1999; Piepenbring 2002; Denchev 2003a), or the transfer of many *Ustilago* species on dicots into the genus

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Microbotryum (e.g., Bauer *et al.* 1997; Vánky 1998; Piątek 2005). However, there are still species on non-poacean hosts retained in *Ustilago* and on monocotyledonous hosts retained in *Melanotaenium*. Two of these discordant species are *Melanotaenium ruppiae* on *Ruppia maritima* (*Ruppiaceae*) and *Ustilago marina* on *Eleocharis parvula* (*Cyperaceae*). These two fungi live with their hosts in marine habitats and because of this anomalous ustilaginomycotinous habitat it was generally disputed whether these two fungi are members of the *Ustilaginomycotina* (Vánky 1994). Therefore, we have attempted to collect both species. *M. ruppiae* could not be found in its type locality of Etang du Canet, Pyrénées Orientales, France. However, in the type locality of *Ustilago marina*, the watt of the Bassin d'Arcachon, Arès, France, we found not only *U. marina*, but also *Melanotaenium ruppiae* at a distance of *ca* 10 m from each other. *E. parvula* infected with *U. marina* grew on a sandbank, whereas *R. maritima* infected with *M. ruppiae* was obviously alluvial. Accordingly, the material of *M. ruppiae* was in a bad condition for the study of the ultrastructure. In the present study we compare morphological, ultrastructural, and molecular characters of *M. ruppiae* and *U. marina* with those of the *Ustilaginomycotina* in order to estimate the phylogenetic position of these fungi.

Materials and methods

Entyloma microsporum on *Ranunculus repens* was collected in Germany, Baden-Württemberg, Nürtingen, Tiefenbachtal, 18 Aug. 1993, *R. Bauer* (TUB 012164).

Flamingomyces ruppiae (syn. *Melanotaenium ruppiae*) on *Ruppia maritima* was collected in France, Gironde, Bassin d'Arcachon, Arès, 15 June 2001, *R. Bauer & M. Lutz* (M 0124361, TUB 012165).

Melanustilospora ari on *Arum maculatum* was collected in Germany, Dillingen-Fristingen, 16 May 1997, *R. Bauer & D. Begerow* (TUB 015994).

Parvulago marina (syn. *Ustilago marina*) on *Eleocharis parvula* was collected in France, Gironde, Bassin d'Arcachon, Arès, 15 June 2001, *R. Bauer & M. Lutz* (M 0124362, TUB 012166).

Vankya ornithogali on *Gagea pratensis* was collected in Poland, Niepołomice, 20 Apr. 2005, *M. Piątek* (TUB 015993, herb. M. Piątek: HeMP-65).

Germinations were obtained from teliospores spread thinly on water agar and malt–yeast–peptone agar (Bandoni 1972) in Petri dishes at room temperature. Voucher material has been deposited in TUB and nomenclatural novelties were deposited in MycoBank (see Crous *et al.* 2004).

Electron microscopy

The ultrastructure of septa, cellular interactions, and teliospore walls of *Flamingomyces ruppiae* (syn. *Melanotaenium ruppiae*) and *Parvulago marina* (syn. *Ustilago marina*) were studied with a Zeiss EM 109 transmission electron microscope at 80 kV. Samples were fixed overnight with 2 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at room temperature. Following six transfers in 0.1 M sodium cacodylate buffer, samples were post-fixed 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 three times in 100 % acetone. Samples were embedded in Spurr's plastic and sectioned with a diamond knife. 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.

Molecular analyses

Genomic DNA was isolated from *Entyloma microsporum*, *Flamingomyces ruppiae* (syn. *Melanotaenium ruppiae*), *Melanustilospora ari*, *Parvulago marina* (syn. *Ustilago marina*), and *Vankya ornithogali*. For methods of isolation and crushing of fungal material, DNA extraction, amplification, purification of PCR products, sequencing, and processing of the raw data see Lutz *et al.* (2004). The 5′-end (about 625 bp) of the nuLSU rDNA, comprising the domains D1 and D2 (Guadet *et al.* 1989), was amplified using the primer pair NL1 and NL4 (O'Donnell 1992, 1993). DNA sequences determined for this study were deposited in GenBank, GenBank accession number are DQ185435 (*Entyloma microsporum*), DQ185436 (*Flamingomyces ruppiae*), EF517924 (*Melanustilospora ari*), DQ185437 (*Parvulago marina*), and EF210712 (*Vankya ornithogali*).

To obtain the phylogenetical position of *Flamingomyces ruppiae* (syn. *Melanotaenium ruppiae*) and *Parvulago marina* (syn. *Ustilago marina*), we analysed a dataset containing the sequences and representatives of all teleomorphic genera of *Ustilaginomycetidae sensu* Bauer *et al.* (1997), where sequences in GenBank were available (species and GenBank accession numbers are given in Fig 1). To align sequences, we used MAFFT 5.861 (Katoh *et al.* 2002, 2005) using the L-INS-i option. We avoided both manipulation of the alignment by hand and manual exclusion of any positions as recommended by Giribet & Wheeler (1999) and Gatesy *et al.* (1993), respectively.

To estimate phylogenetic relationships, we used both NJ analysis and a Bayesian approach. For NJ analysis the data were first analysed with Modeltest 3.7 (Posada & Crandall 1998) to find the most appropriate model of DNA substitution. The hierarchical likelihood ratio test proposed the TrN + I + G DNA substitution model. BS values were calculated for 1K replicates. For Bayesian analysis, we used a Bayesian approach of phylogenetic inference using an MCMC technique as implemented in the computer program MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). Four incrementally heated simultaneous Markov chains were run over 2M generations using the general time reversible model of DNA substitution with gamma distributed substitution rates (Gu *et al.* 1995; Rodriguez *et al.* 1990) and estimation of invariant sites, random starting trees, and default starting parameters of the DNA substitution model (Huelsenbeck & Ronquist 2001). Trees were sampled every 100th generation resulting in an overall sampling of 20,001 trees. From these, the first 2001 trees were discarded as burn-in. The trees sampled after the process had reached stationarity (18K trees) were used to compute a 75 % majority rule consensus tree to obtain estimates for the PPs of groups of species.

This Bayesian approach of phylogenetic analysis was repeated five times to test the independence of the results from topological priors (Huelsenbeck *et al.* 2002). The trees were rooted with three exobasidiomycetidaeous representatives, *Entyloma microsporum*, *Exobasidium pachysporum*, and *Gjaerumia ossifragi*.