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Implications of molecular characters for the phylogeny of the genus *Entyloma**

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Many species formerly listed in *Entyloma* have been removed and are now placed in several orders. This study aims to clarify the framework of the genus *Entyloma* and the phylogeny of these plant parasitic smut fungi with molecular data. Analyses of LSU and ITS sequences are presented and support the monophyly of the order *Entylomatales*. The sequences show higher similarities within the examined species of *Entyloma* than within other smut families and genera, suggesting a recent radiation. Within the *Entylomatales* a cluster of anamorphic *Tilletiopsis washingtonensis*, *T. lilacina* and *T. cremea* collections is the sister group to *Entyloma*. The phylogenetic relationships in the genus *Entyloma* are a result of joint evolution with their hosts. The analyses of the sequence data show unresolved groups on *Ranunculales* and a well-supported group on *Asteridae*.

INTRODUCTION

The genus *Entyloma* was characterized by de Bary (1874) by the formation of teliospores, the germination of these spores with *Tilletia*-like basidia, and the characteristic white dense leaf spots caused by the plant parasitic stages. Because of the life cycle, he placed the genus in the smut fungi, although the species do not produce dark coloured, loose teliospores. The systematic position was confirmed by ultrastructural and molecular data (Bauer, Oberwinkler & Vánky 1997, Begerow, Bauer & Oberwinkler 1997). Bauer *et al.* (1997) described the order *Entylomatales* characterized by simple septal pores, teliospores and a local interaction apparatus with homogeneous contents. Up to 170 species have been described in the genus *Entyloma*. They parasitize a wide range of plants that belong to different phylogenetic groups. In Fig. 1 the distribution of the *Entyloma* species on different host families is illustrated. About 85% of the species occur on asterids and ranunculoids.

Entyloma species grow and sporulate intercellularly. Sometimes an anamorphic sporulation can be observed on plant surfaces (Vánky 1994a). The saprotrophic stage is poorly understood. Morphological characters such as sorus morphology, size and colour of the spores, and thickness and surface of spore walls are hardly differentiated amongst species of the genus. As the information to be inferred from morphological characters is limited, more detailed studies were needed to recognize new genera and make new combinations. Several species have now been placed in other families and orders. *Entyloma callitrichis* (now *Doassinga callitrichis*) was transferred to the *Doassansiales* (Vánky, Bauer & Begerow 1998), *Entyloma vignae* (now *Erratomyces patelii*) to the *Tilletiales* (Piepenbring & Bauer 1997), *Entyloma fluitans* (now *Ustilentyloma fluitans*) to the *Microbotryales* (Vánky 1970), and *Entyloma sparganii* (now *Nannfeldtiomyces sparganii*) to the *Doassansiales* (Vánky 1981). Most recently, we studied the *Entyloma* species on *Poaceae* and placed them in the *Georgefischeriales* (Bauer, Begerow & Oberwinkler 2001).

Beyond that, the species concept within the genus *Entyloma* has been in discussion for a long time. The consistency of morphological characters is doubtful. For example, Ward (1887) described the variable conidiogenesis and suggested a dependency on the environment. In contrast, Plowright (1889) and Clinton (1905) discussed subgroups in the genus *Entyloma* based on presence or absence of several conidia types. Consequently, Savile (1947) proposed a morphological species concept. His species are based principally on spore size and anamorphs. He synonymized species with identical morphology that are parasitic on the same host family. An overview of European *Entyloma* species showed very similar and overlapping spore sizes within

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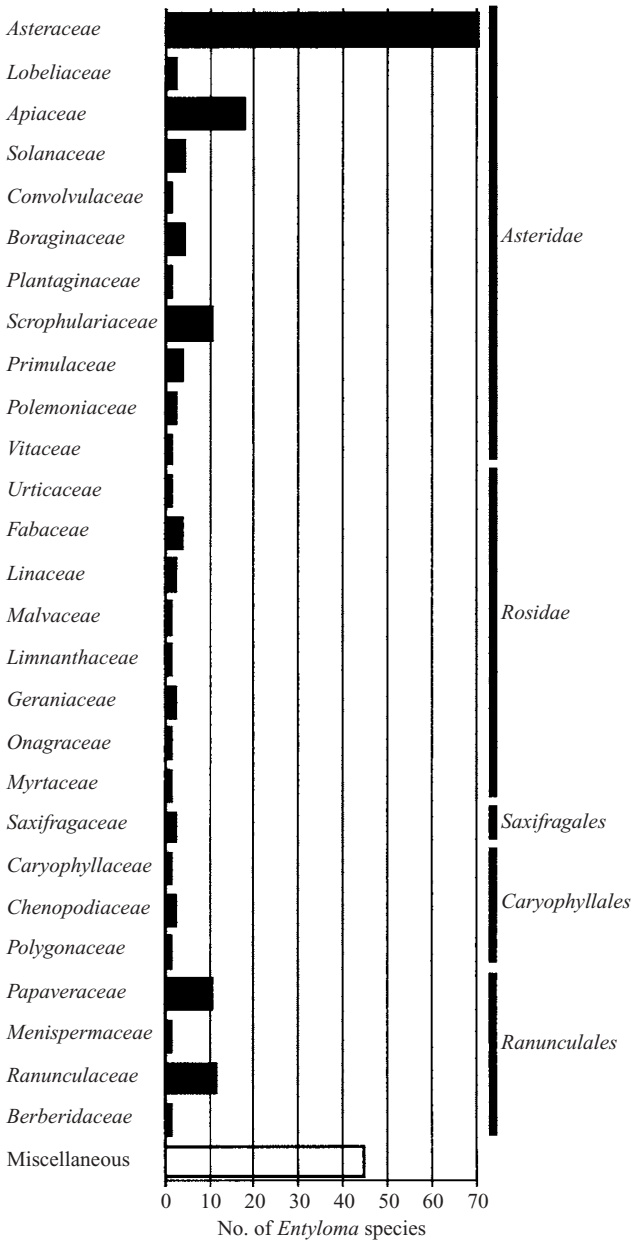

Fig. 1. Distribution of *Entyloma* species on different plant families. The higher taxonomic levels are summarized according to APG (1998) on the right hand side. Miscellaneous summarizes species of doubtful classification or taxonomy.

the described species (Vánky 1994a). Vánky used a more narrow concept based on host specificity of the *Entyloma* species and accepted 93 European species within the genus.

Molecular studies on the *Ustilaginomycetes* revealed a long common branch for the order *Entylomatales* (Begerow, Bauer & Oberwinkler 1997), suggesting that it is an old group with recent radiation. The aim of this study was to elucidate the evolution of the genus *Entyloma* by the use of molecular data.

MATERIALS AND METHODS

We isolated genomic DNA from 35 specimens (Table 1) of *Entyloma*. To extract DNA we followed the SDS method (Begerow *et al.* 1997). The 5′ region of the nuclear LSU rDNA (about 550 bp) was amplified by polymerase chain reaction (PCR) using NL1 and NL4 as primers (Boekhout, Fell & O'Donnell 1995). The ITS region (about 700 bp) localized between the 18S and 28S rRNA genes, was amplified with the primer pair ITS1-F and ITS4 (Gardes & Bruns 1993). The PCR products were purified using the QIAquick™ Kit (Qiagen, Germany) followed by an ethanol precipitation. Both strands were sequenced with the PerkinElmer ABI PRISM™ dye terminator cycle sequencing kit on an automatic sequencer (ABI 373A).

DNA sequences were aligned with the MEGALIGN module of the LASERGENE package (DNASTAR, Inc.) with some manual corrections. DNA sequences determined for this study were deposited in GenBank®, accession numbers are given in Table 1. The alignments are available upon request. PAUP* 4.0b8 (Swofford 1998) was used to perform neighbour-joining analyses and MrBayes 2.01 (Huelsenbeck & Ronquist 2001) for Bayesian inference.

After comparing the results obtained with several different analyses of LSU, ITS, and combined datasets with different methods, two sets of sequences were finally chosen. First, 31 LSU sequences of species of *Exobasidiomycetidae* were rooted with *Ustilago* species to analyse the major topology (length: 514 bp after excluding 13 ambiguous positions; 212 variable sites). Second, 33 ITS sequences of *Entyloma* species were analysed to elucidate the phylogeny within *Entyloma* (length: 604 bp after excluding 88 ambiguous positions; 120 variable sites).

Both sets were analysed with neighbour-joining analysis, carried out with the Kimura-two-parameter distance model (bootstrap values being calculated for 1000 replicates) and with a Bayesian approach (Huelsenbeck *et al.* 2001) using Monte Carlo Markov chains (MCMC) as described by Maier *et al.* (2002).[1] Four incrementally heated simultaneous Monte Carlo Markov chains were run over 1 000 000 generations using the general time reversible model (six rate classes) of DNA substitution with gamma distributed substitution rates (Swofford *et al.* 1996), random starting trees and default starting parameters of the DNA substitution model (Huelsenbeck & Ronquist 2001). Trees were sampled every 100 generations, resulting in an overall sampling of 10 000 trees. From the trees sampled after the process had reached stationarity (burnin = 1000), a 50 % majority rule consensus tree was computed to obtain estimates for the *a posteriori* probabilities. This Bayesian approach of phylogenetic analysis was repeated several times, always using random starting trees and default

[1] In contrast to the maximum-likelihood method (Felsenstein 1981), in which the probability of the DNA alignment conditional on phylogenetic trees (the 'likelihood' of the phylogenetic trees) is maximized, the Bayesian MCMC approach allows estimation of the *a posteriori* probability of phylogenetic trees, i.e. the probability that a tree is the true phylogenetic tree given the DNA alignment.

Table 1. List of species studied in molecular analyses.

Species	Host	GenBank® accession no.	Source[1]
Species newly sequenced			
Entyloma arnoseridis	*Arnoseris minima*	AY081017	ML 1001
E. atlanticum	*Geranium tuberosum*	AY081018; AY081011	Vánky Ust. Ex. 738
E. australe	*Physalis cordata*	AY081019	Vánky s.n. (H.U.P. 311)
E. bidentis	*Bidens pilosa*	AY081020	MP 991
E. browalliae	*Browallia americana*	AY081021	PS 1942
E. calceolariae	*Calceolaria chelidonioides*	AY081022	H.U.V. 907
E. calendulae	*Calendula officinalis*	AY081012	ML 1002
E. calendulae[B2]	*C. officinalis*	AY081023	ML 1223
E. chrysosplenii	*Chrysosplenium alternifolium*	AY081024	ML 1003
E. comaclinii	*Comaclinium montanum*	AY081025	MP 1762
E. compositarum	*Parthenium hysterophorus*	AY081026	Romero 197 (XAL)
E. corydalis	*Corydalis bulbosa*	AY081027	Vánky Ust. Ex. 670
E. costaricense	*Viguiera* sp.	AY081028	MP 2384
E. dahliae	*Dahlia* sp.	AY081029	MP s.n. (10.10.1993)
E. delileae	*Delilea biflora*	AY081030	MP 1004
E. diastateae	*Diastatea micrantha*	AY081031	MP 1717
E. doebbeleri	*Dahlia imperialis*	AY081032	MP 27
E. eryngii	*Eryngium campestre*	AY081033	Menge & Vánky s.n. (04.07.1992)
E. eryngii-plani	*E. planum*	AY081034	H.U.V. 507
E. ficariae	*Ranunculus ficaria*	AY081035; AY081013	ML 1013
E. fuscum	*Glaucium flavum*	AY081036; AY081014	H.U.V. 278
E. gaillardianum	*Gaillardia aristata*	AY081037	RB 2055
E. guaraniticum	*Bidens pilosa*	AY081038	MP 2264
E. hieracii	*Hieracium sylvaticum*	AY081039	MP 2351
E. holwayi	*Cosmos caudatus*	AY081040	MP 1769
E. linariae	*Linaria vulgaris*	AY081041	Vánky Ust. Ex. 671
E. lobeliae	*Lobelia laxiflora*	AY081042; AY081015	Vánky s.n. (20.01.1992)
E. madiae	*Madia gracilis*	AY081043	Vánky Ust. Ex. 742
E. matricariae	*Tripleurospermum perforatum*	AY081044	ML 766
E. microsporum	*Ranunculus repens*	AY081045	RB 1080
E. polysporum	*Ambrosia artemisifolia*	AY081046	H.U.V. 2960
E. ranunculi-repentis	*Ranunculus nemorosus*	AY081016	MP 246
E. ranunculi-repentis[B]	*Ranunculus repens*	AY081047	ML 1025
E. serotinum	*Symphytum officinale*	AY081048	ML 1026
E. zinniae	*Zinnia peruviana*	AY081049	MP 2627
Species previously sequenced			
Conidiosporomyces ayresii	*Panicum maximum*	AF 009848	Begerow *et al.* (1997)
Doassansia epilobii	*Epilobium montanum*	AF 007523	Begerow *et al.* (1997)
D. hygrophilae	*Hygrophila spinosa*	AF 007524	Begerow *et al.* (1997)
Doassinga callitrichis	*Callitriche verna*	AF 007525	Vánky *et al.* (1998)
Eballistra brachiariae	*Brachiaria distachya*	AF 009864	Begerow *et al.* (1997)
Entyloma holwayi	*Cosmos caudatus*	AF 009854	Begerow *et al.* (1997)
Erratomyces patelii	*Phaseolus vulgaris*	AF 009855	Begerow *et al.* (1997)
Exobasidium rhododendri	*Rhododendron ferrugineum*	AF 009856	Begerow *et al.* (1997)
E. rostrupii	*Vaccinium oxycoccos*	AF 009857	Begerow *et al.* (1997)
E. vaccinii	*V. vitis-idaea*	AF 009858	Begerow *et al.* (1997)
Georgefischeria riveae	*Rivea hypocrateriformis*	AF 009861	Begerow *et al.* (1997)
Graphiola phoenicis	*Phoenix canariensis*	AF 009862	Begerow *et al.* (1997)
Ingoldiomyces hyalospora	*Nassella mexicana*	AF 133576	Begerow *et al.* (2000)
Jamesdicksonia dactylidis	*Agrostis stolonifera*	AF 009853	Begerow *et al.* (1997)
Microstroma album	*Quercus robur*	AF 352052	Begerow *et al.* (2001)
Microstroma juglandis	*Juglans regia*	AF 009867	Begerow *et al.* (1997)
Nannfeldtiomyces sparganii	*Sparganium ramosum*	AF 007527	Vánky *et al.* (1998)
Rhamphospora nymphaeae	*Nymphaeae alba*	AF 007526	Begerow *et al.* (1997)
Tilletia caries	*Triticum aestivum*	AJ 235307	Boekhout *et al.* (1995)
Tilletiaria anomala	Decaying wood	AJ 235284	Boekhout *et al.* (1995)
Tilletiopsis cremea	Leaves	AJ 235279	Boekhout *et al.* (1995)
T. lilacina	Leaves	AJ 235309	Boekhout *et al.* (1995)
T. washingtonensis	Leaves	AJ 235278	Boekhout *et al.* (1995)
Tolyposporella brunkii	*Andropogon saccharoides*	AF 009875	Begerow *et al.* (1997)
Ustilago avenae	*Arrhenaterum elatius*	AF 236140	Piepenbring (1999)
U. nuda	*Hordeum leporinum*	AJ 236139	Piepenbring (1999)
U. scitaminea	*Saccharum* sp. cultivated	AJ 236138	Piepenbring (1999)
U. trichophora	*Echinochloa colonum*	AJ 236141	Piepenbring (1999)
Volvocisporium triumfetticola	*Triumfetta rhomboidea*	AF 352053	Begerow *et al.* (2001)

[1] Collection numbers of: H.U.P., herbarium *Ustilaginales* Piepenbring; H.U.V., herbarium *Ustilaginales* Vánky; ML, M. Lutz; MP, M. Piepenbring; PS, Piepenbring & Sancho; RB, R. Bauer.

[2] Superscript '*B*' denotes different specimens used for LSU and ITS sequences.

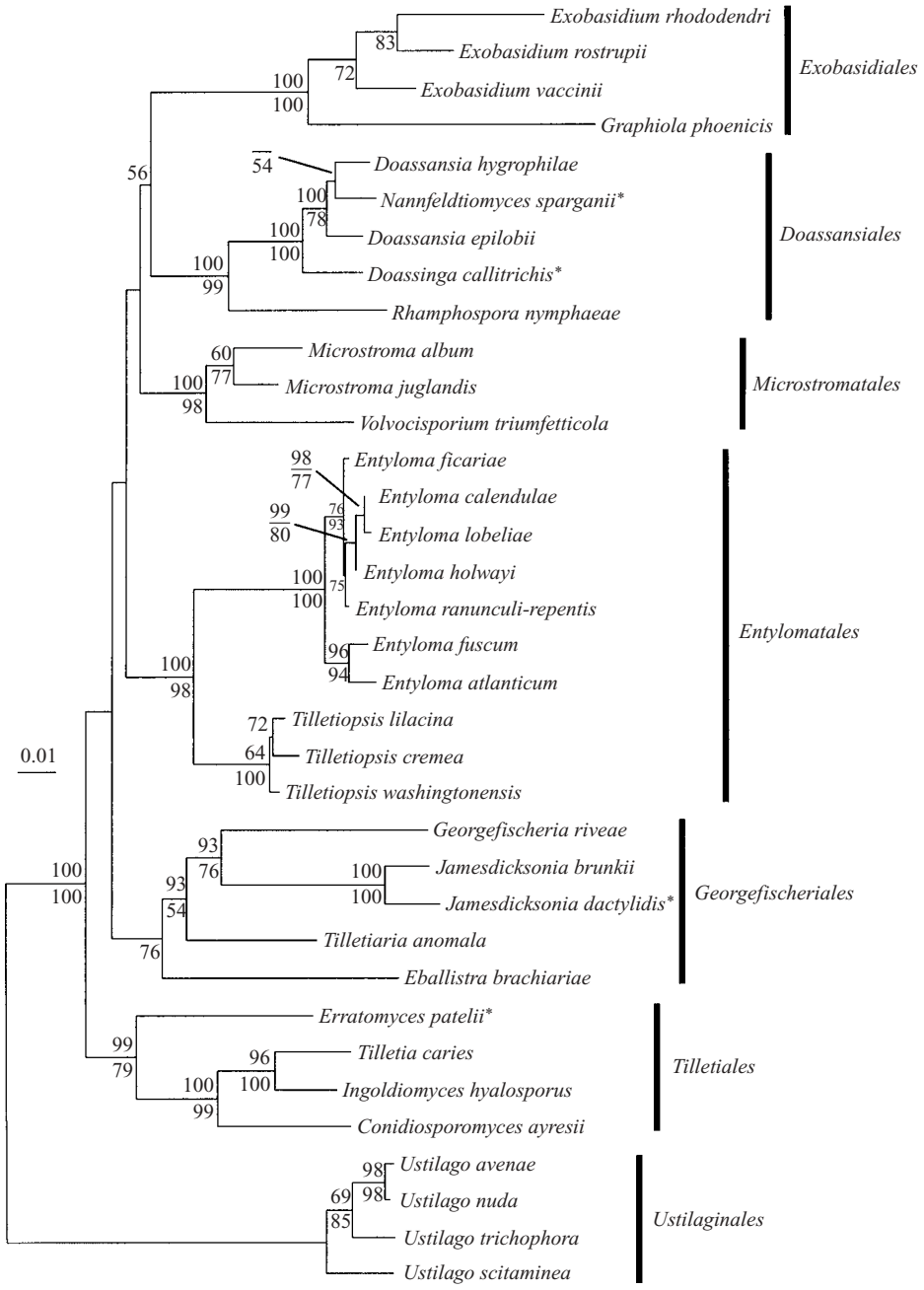

Fig. 2. Phylogram obtained by neighbour-joining analysis using Kimura-2-parameter model of LSU region sequences of 35 members of the *Ustilaginomycetes*. The topology was rooted with *Ustilago* species. Percentage bootstrap values of 1000 replicates are given at each furcation under the line. The *a posteriori* probabilities from the Bayesian inference are given above the line. Values smaller than 50% have not been taken into consideration. Branch lengths are scaled in terms of expected numbers of nucleotide substitutions per site. The stars refer to species which were listed formerly in the genus *Entyloma*.

starting values for the model parameters to test the reproducibility of the results.

RESULTS

The results of the neighbour-joining analysis of the nuclear LSU rDNA data set are illustrated in Fig. 2. The *a posteriori* probabilities are mapped into the neighbour-joining tree, as the topology was more or less the same. There is no supported resolution for the backbone in both analyses. However, the *a posteriori* probabilities are higher than the bootstrap values in general. All orders of the *Exobasidiomycetidae* (Bauer *et al.* 2001), including the *Entylomatales*, are supported by our analysis. *Tilletiopsis lilacina*, *T. washingtonensis* and *T. cremea* were supported as a sister group to *Entyloma*.

The ITS data set was analysed several times with an MCMC algorithm. As the results were consistent from the different runs, we used one to illustrate the results in Fig. 3. The bootstrap values of the neighbour-joining analysis are mapped into the tree resulting from the Bayesian inference. As the ITS sequences are not

Fig. 3. Bayesian inference of phylogenetic relationships within the genus *Entyloma*. Markov chain Monte Carlo analysis of an alignment of nuclear rDNA sequences from the ITS region using the general time reversible model of DNA substitution with gamma distributed substitution rates, random starting trees, and default starting parameters of the substitution model. Majority rule consensus tree from 9000 trees that were sampled after the process had reached stationarity. The topology was rooted with *Entyloma atlanticum und E. fuscum*. The numbers on branches are estimates for *a posteriori* probabilities (above the line). The neighbour-joining bootstrap support is given under the line. Values smaller than 50 % have not been taken into consideration. Branch lengths are mean values over the sampled trees and are scaled in terms of expected numbers of nucleotide substitutions per site.

alignable to smut fungi that do not belong to the *Entylomatales*, we used *Entyloma atlanticum* and *E. fuscum* as outgroups in the ITS analysis (Fig. 3). The basal position of *Entyloma atlanticum* and *E. fuscum*, in comparison to the other examined *Entyloma* species, is based on the LSU analysis (Fig. 2). *E. microsporum*, *E. linariae*, *E. ficariae*, *E. ranunculi-repentis* and *E. corydalis* are grouped outside a well-supported cluster of 25 *Entyloma* species infecting asterids and *Entyloma chrysosplenii* on *Chrysosplenium alternifolium* (*Saxifragaceae*). The relations within that group of *Entyloma* species are only resolved in parts. Some groups are supported by higher values such as the group of *Entyloma polysporum*, *E. hieracii*, and *E. matricariae*, or the subgroup containing *Entyloma australe*, *E. compositarum*, and *E. zinniae*. However, the asterid-infecting group (including *Entyloma chrysosplenii* on *Chrysosplenium alternifolium*) was monophyletic in all analyses.

DISCUSSION

The genus *Entyloma* appears to be monophyletic (Fig. 2). Species such as *Jamesdicksonia dactylidis*, *Erratomyces patelii*, *Doassinga callitrichis* and *Nannfeldtiomyces sparganii*, formerly treated in *Entyloma*, belong to other orders (Fig. 2). Our data confirm the description of the order by the interaction apparatus with homogeneous contents, the teliospores, and simple septal pores (Bauer *et al*. 1997). The sister group to *Entyloma* is represented by three species of *Tilletiopsis*. Boekhout *et al*. (1995) studied *Tilletiopsis* species with molecular tools and recognized the close relationship of the *T. washingtonensis* group and some species of *Entyloma*. In a broader study on anamorphs of *Ustilaginomycetes*, we demonstrated a more complex evolution of the species placed in *Tilletiopsis* (Begerow, Bauer & Boekhout 2000). Interestingly, none of the anamorphic strains clustered within *Entyloma*, although conidial stages are often observed on the leaves infected with *Entyloma*.

To describe the internal topology of the genus *Entyloma*, we used a second DNA region apart from the LSU sequences. Comparing different methods and single LSU or ITS alignments *versus* combined datasets, the highest resolution resulted from the analysis of ITS sequences with MrBayes (Fig. 3).

E. atlanticum and *E. fuscum*, on *Geranium tuberosum* and *Glaucium flavum*, respectively, are morphologically characterized by exceptionally large spores and more or less evenly thickened spore walls (Vánky 1994b). This combination is unique and not shared with other studied species. This observation is confirmed by our results showing a separation of the two species from the rest of *Entyloma* and support for their monophyly of 100 % in the ITS analysis (Fig. 3) and 76 % and 99 % in the LSU analysis for the neighbour-joining analysis and Bayesian inference respectively (Fig. 2). We used *E. atlanticum* and *E. fuscum* as outgroups for the analysis of the second set, although the relationship to the other *Entyloma* species as sister taxon is not evident from our limited dataset.

Entyloma *on* Ranunculales

Although we inferred monophyly of the *Entyloma* species parasitizing members of the *Ranunculales* (including *Entyloma linariae* on a member of *Scrophulariaceae*) in analyses with fewer taxa, we could not confirm a common ancestor of *E. microsporum*, *E. corydalis*, *E. ranunculi-repentis*, *E. ficariae*, and *E. linariae*; they do not share an obvious morphological feature. *E. corydalis* is the only species studied with warty spores. We expect *E. urocystoides* on *Corydalis bulbosa* and *E. verruculosum* on *Ranunculus* spp. to be closely related. Both possess spores with large warts and infect *Ranunculales*. An exceptionally thick spore wall is present in *E. microsporum*, but not in the other species on *Ranunculus*. The close relationship of *E. ranunculi-repentis* and *E. ficariae* is not surprising, since some authors assumed the two species on *Ranunculus* to be identical (Vánky 1994b); however, the size of the spores differs significantly and our data confirm the separation into two species. Further studies are necessary to clarify the phylogenetic position of the *Entyloma* species on different *Ranunculus* species.

Entyloma *on* Asteridae

The monophyly of the *Entyloma* species exclusively parasitizing members of the asterids (incl. *E. chrysosplenii* on *Chrysosplenium alternifolium*, a member of *Saxifragaceae*) is supported by a 100 % *a posteriori* probability and 99 % neighbour-joining bootstrap support (Fig. 3). Although most bifurcations of that subgroup are not resolved, some were observed and supported in all analyses. The groups *E. matricariae*, *E. hieracii*, and *E. polysporum*, or *E. australe*, *E. compositarum* and *E. zinniae*, were often observed in analyses of the ITS sequences. But there are no morphological characters, nor any close host relationships or ecological features that support these groups. The same could be stated for the other well-supported subgroups in the group of *Entyloma* species on asterids (including *E. chrysosplenii* on *Chrysosplenium alternifolium*).

Parallel cladogenesis as a result of cospeciation of hosts and parasites (Hafner & Nadler 1990) could not be shown within asterids infecting *Entyloma* species. For example, the species growing on members of the *Cichorioideae* (*E. arnoseridis* and *E. hieracii*) or those on members of the *Heleniinae* (*E. gaillaridianum*, *E. bidentis* and *E. guaraniticium*) cluster in different subgroups. Moreover, the distribution in the phylogenetic topology of *Entyloma australe*/*E. browalliae* on solanacean hosts, *Entyloma eryngii*/*E. eryngii-plani* on apiacean hosts, and *Entyloma diastateae*/*E. lobeliae* on lobeliacean hosts, does not suggest monophyly for the particular species. This distribution could be explained by several host shifts. In the case of *E. chrysosplenii*, the host is not even a member of the asterids but of the rosids. Furthermore, our data show that at least two morphologically distinguishable *Entyloma* species evolved independently on the same host species (*E. guaraniticum*/*E. bidentis* on *Bidens pilosa*) and the same host genus (*E. dahilae*/*E. doebbeleri* on *Dahlia*).

Evolution and the species concept in Entyloma

The topology of the ITS analysis (Fig. 3), although not resolved in parts, suggests some evolutionary trends, which could be described and interpreted in terms of some models of joint evolution of hosts and parasites. The groups of *Entyloma* species which parasitize members of the two main host groups, *Ranunculales* or asterids, seem to be separated. The support of 100 % for the monophyly of the latter group emphasizes the dependancy between phylogeny of hosts and parasites. The concept of association-by-descent (Brooks 1981, 1988, Brooks & Bandoni 1988) could aid the understanding of aspects of the phylogeny of the genus. Once

an *Entyloma* species was established on a member of the asterids, an explosive radiation on that host group might have taken place. The asteridean hosts seem to share a special character within the infection process, which made them susceptible for that lineage of *Entyloma*. As with the results of Baum & Savile (1985), our data suggest a colonization of host plant species restricted to the asterids but not other plant groups.

The genetic distances, derived from ITS sequences of *Entyloma*, are much smaller than those observed within asteridean groups (e.g. Garcia-Jacas *et al*. 2000, Schmidt & Schilling 2000). These results suggest that the radiation of *Entyloma* occurred long after the radiation of the hosts. That is reinforced by our data which exclude the possibility of parallel cladogenesis as a result of cospeciation.

Consequently, the radiation on asterids may be best described as the evolutionary process of resource tracking (Kethley & Johnston 1975). In the special case of obligate plant parasites, Roy (2001) used the term 'host tracking'. In its evolution, the genus *Entyloma* might have tracked down the susceptibility, which seems to be restricted principally to the monophyletic asterids. Within *Entyloma* the model of an association-by-colonization (Baum & Savile 1985, Pirozynski & Hawksworth 1988, Savile 1990) as well as the model of association-by-descent (Brooks 1981, 1988, Brooks & Bandoni 1988) are partially established and may help to elucidate the joint evolution of host-dependent plant parasites.

Furthermore, the evolution of the genus *Entyloma* is a good example for host shifts and host jumps (Roy 2001). The term 'host shift' could be used where parasites shift from one family to another closely related family. The evolution of *Entyloma chysosplenii* on a member of rosids could be seen as an example of a host jump in the sense of Roy (2001), since the parasite overcame the asterids and jumped to a rosidean host.

The results of our molecular analyses contradict a species concept in *Entyloma* mainly based on morphological characters, such as that of Savile (1947) but support a species concept for the genus based on host specificity. Species delimitation should, however, be complemented by a large character set of ecological and molecular features, in addition to the morphology of different stages and organs.

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