Fungal Genetics and Biology **24,** 325–334 (1998)
Article No. FG981063

Relationships of the Insect-Pathogenic Order Entomophthorales (Zygomycota, Fungi) Based on Phylogenetic Analyses of Nuclear Small Subunit Ribosomal DNA Sequences (SSU rDNA)

Annette Bruun Jensen,*,[1] Andrea Gargas,* Jørgen Eilenberg,† and Søren Rosendahl*

**Department of Mycology, Botanical Institute, University of Copenhagen, Øster Farimagsgade 2D, 1353 Copenhagen K, Denmark; and* †*Department of Ecology and Molecular Biology, Royal Veterinary and Agricultural University, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark*

Accepted for publication May 10, 1998

Jensen, A. B., Gargas, A., Eilenberg, J. and Rosendahl, S. 1998. Relationships of the insect-pathogenic order Entomophthorales (Zygomycota, Fungi) based on phylogenetic analyses of nuclear small subunit ribosomal DNA sequences (SSU rDNA). *Fungal Genetics and Biology* 24, 325–334. We sequenced the nuclear small subunit of ribosomal DNA (SSU rDNA) from seven species within the insect-pathogenic order Entomophthorales. These sequences were aligned with other published SSU rDNA sequences and phylogenies were inferred using phenetic and cladistic methods. Based on three different phylogenetic methods the Entomophthorales (excluding *Basidiobolus ranarum*) is monophyletic; *B. ranarum* was more closely related to chytrids from Chytridiales and Neocallimasticales than to Entomophthorales, as was proposed by Nagahama *et al.* (*Mycologia* 87: 203–209, 1995). Nuclear characters (large nuclei containing conspicuous condensed chromatin and lack of a prominent nucleolus) were of predictive value for the monophyly of the family Entomophthoraceae. Conidial characters separate the Entomophthoraceae, which only includes obligate pathogens, into at least two lineages: one lineage with uninucleate conidia and another with multinucleate conidia. The two species of *Conidiobolus* studied were paraphyletic in our analyses and only distantly related to each other. This information may prove to be important in the use of these fungi as biocontrol agents.

Index Descriptors: fungi; Zygomycota; Entomophthorales; insect-pathogens; biological control; SSU rDNA, small subunit ribosomal DNA; molecular evolution; phylogeny.

Entomophthorales, true to their name "insect destroyer," are potent pathogens of insects. Signs of one species, *Entomophthora muscae,* are commonly seen on window panes as a halo of ejected spores around dead house flies, *Musca domestica.* Other species of Entomophthorales also cause epidemics in certain insect species, including important pests. Because this pathogenicity has the potential to be used for biological control (Carruthers and Hural, 1990), assessing the relationships of these fungi is important in planning future biocontrol strategies (Hajek, 1997).

Based on the presence of zygospores and a coenocytic mycelium, Entomophthorales is placed in the phylum Zygomycota (Waterhouse, 1973), which is separated from the Chytridiomycota by the presence of uniflagellate

[1] To whom correspondence should be addressed at present address: Department of Ecology and Molecular Biology, Royal Veterinary and Agricultural University, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark. E-mail: abj@kvl.dk.

1087-1845/98 $25.00

zoospores in the latter. It has been suggested that Zygomycota and Chytridiomycota are polyphyletic with flagellae having been lost several times during fungal evolution (Nagahama *et al.*, 1995). Therefore, to understand the relationship of Entomophthorales within the Eumycota and among the Zygomycota it is essential to include representatives of the chytrids.

Within the zygomycetes, members of the Entomophthorales are the only group that repetitively discharge conidia, a character that has been used to segregate the order (Waterhouse, 1973). Families within the Entomophthorales are distinguished primarily on the basis of nuclear characters (Humber, 1981, 1984a).

The Entomophthoraceae, which includes only obligate pathogens (meaning that they only proliferate in nature by attacking living hosts), have large nuclei (5–15 μm). These nuclei contain conspicuous amounts of condensed chromatin which stains readily with orcein and lack a prominent nucleolus during interphase (Humber, 1989). Among the Zygomycota these nuclear characters are only present in the Entomophthoraceae, suggesting that it is a monophyletic lineage. Entomophthoraceae has been further separated into genera with uninucleate and multinucleate conidia, suggesting that these characters are phylogenetically significant (Humber, 1989; Keller, 1987, 1991).

Ancylistaceae, another important entomophthoralean family, includes the genus *Conidiobolus* whose members live as saprotrophs and facultative insect pathogens (Humber, 1989). Their smaller nuclei, which resemble most other fungal nuclei, together with their saprotrophic lifestyles have been used as evidence that they are a basal group within the Entomophthorales (Humber, 1984a). Several different morphological conidial types are found within the genus *Conidiobolus* (Bałazy, 1993), however, which suggests that this genus may be polyphyletic.

Data from sequences of ribosomal genes have been used to reconstruct phylogenetic relationships among fungi where the evolutionary interpretation of morphological features is ambiguous (Taylor, 1993). We sequenced the nuclear small subunit ribosomal DNA (SSU rDNA) gene from seven members of Entomophthorales representing two families and several genera within these. These sequences were analyzed with others obtained from GenBank (Table 1) to assess phylogenetic relationships of Entomophthorales within the fungi. From refined phylogenetic hypotheses we sought to elucidate the evolution of selected morphological and ecological characters.

MATERIALS AND METHODS

Cultures

The taxa used in this study and their GenBank accession numbers are given in Table 1. Data on the isolates supplied by the Royal Veterinary and Agricultural University, Department of Ecology and Molecular Biology, Denmark, are as follows: *Entomophthora schizophorae* KVL 2 (ARSEF 2541) isolated from *Psila rosae* (Diptera), *Eryniopsis ptycopterae* KVL 48 (ARSEF 2671) isolated from *Ptycoptera contaminata* (Diptera), *Zoophthora radicans* KVL 610 isolated from *Epinotia aporema* (Lepidoptera), *Pandora neoaphidis* (synonym: *Erynia neoaphidis*) KVL 633 (ARSEF 5403) isolated from *Sitobion avenae* (Hemiptera), and *Strongwellsea castrans* resting spores from *Delia radicum* (Diptera). Data on isolates supplied by the Collection of Entomopathogenic Fungal Cultures (ARSEF), USDA-ARS Plant Protection Research Unit, US Plant, Soil and Nutrition Laboratory (Ithaca, NY) are as follows: *Conidiobolus thromboides* ARSEF 115 isolated from *Therioaphis maculata* (Hemiptera), *Macrobiotophthora vermicola* ARSEF 650 isolated from *Cruznema lambdiense* (Nematoda, Rhabditida).

DNA Extraction and Amplification

Cultures were grown in GLEN liquid medium (Beauvais and Latgé, 1988), and total genomic DNA was extracted by the method of Lee and Taylor (1990). Isolated DNA was used for PCR-amplification of the SSU rDNA, except for *S. castrans* where resting spores were used directly for PCR.[2]

Fungal universal primers nu-SSU-0021-5′ (Gargas and DePriest, 1996) and nu-SSU-1780-3′ (DePriest, 1993) were used for the PCR reactions with an initial denaturation for 1 min at 96°C, followed by 35 cycles with denaturation 1 min at 95°C, annealing 1 min at 48–50°C, extension 2 min at 72°C, and a final extension of 7 min at 72°C.

DNA Sequencing

PCR products were purified with Millipore filters (Millipore Corporation, Bedford, MA) and used in cycle-sequencing reactions (ABI Dye Terminator Cycle Sequenc-

[2] Abbreviation used: PCR, polymerase chain reaction.

TABLE 1
List of the Species Used in This Study, with GenBank Accession Numbers of Their SSU rDNA Sequences and References

Major group	Species	Accession no.	Reference
Basidiomycota	*Boletus satanas*	M94337	Bruns *et al.* (1992)
Basidiomycota	*Schizophyllum commune*	X54865	Bruns *et al.* (1992)
Basidiomycota	*Tremella foliacea*	L22262	Swann and Taylor (1993)
Basidiomycota	*Dacrymyces stillatus*	L22258	Swann and Taylor (1993)
Basidiomycota	*Tilletia caries*	U00972	Berbee and Taylor (1993)
Ascomycota	*Arthonia radiata*	U23537	Gargas *et al.* (1995)
Ascomycota	*Morchella elata*	L37537	Gargas and Taylor (1995)
Ascomycota	*Neurospora crassa*	X04971	Sogin *et al.* (1986)
Ascomycota	*Saccharomyces cerevisiae*	J01353	Mankin *et al.* (1986)
Ascomycota	*Schizosaccharomyces pombe*	X54866	Sogin and Elwood (Unpub.)
Zygomycota			
Zygomycetes			
Glomales	*Gigaspora albida*	Z14009	Simon *et al.* (1993)
Glomales	*Glomus versiforme*	X86687	Gehrig *et al.* (1996)
Glomales	*Geosiphon pyriforme*	X86686	Gehrig *et al.* (1996)
Endogonales	*Endogone pisiformis*	X58724	Simon *et al.* (1992)
Mucorales	*Mortierella polycephala*	X89436	Gehrig *et al.* (1996)
Mucorales	*Mucor mucedo*	X89434	Gehrig *et al.* (1996)
Mucorales	*Rhizomucor racemosus*	X54863	Sogin and Elwood (Unpub.)
Mucorales	*Syncephalastrum racemosum*	X89437	O'Donnell (Unpub.)
Entomophthorales	*Conidiobolus coronatus*	D29947	Nagahama *et al.* (1995)
Entomophthorales	*Conidiobolus thromboides*	AF052401	This study
Entomophthorales	*Entomophaga aulicae*	U35394	Silver (Unpub.)
Entomophthorales	*Entomophthora muscae*	D29948	Nagahama *et al.* (1995)
Entomophthorales	*Entomophthora schizophorae*	AF052402	This study
Entomophthorales	*Eryniopsis ptycopterae*	AF052403	This study
Entomophthorales	*Macrobiotophthora vermicola*	AF052400	This study
Entomophthorales	*Pandora neoaphidis*	AF052405	This study
Entomophthorales	*Strongwellsea castrans*	AF052406	This study
Entomophthorales	*Zoophthora radicans* is1[a]	D61381	Nagahama *et al.* 1995)
Entomophthorales	*Zoophthora radicans* is2[a]	AF052404	This study
Trichomycetes			
Harpellales	*Smittium culisetae*	D29950	Nagahama *et al.* (1995)
Chytridiomycota			
Blastocladiales	*Allomyces macrogynus*	U23936	Paquin *et al.* (1995)
Blastocladiales	*Blastocladiella emersonii*	X54264	Förster *et al.* (1990)
Neocallimasticales	*Neocallimastix joyonii*	M62705	Dore and Stahl (Unpub.)
Chytridiales	*Chytridium confervae*	M59758	Bowman *et al.* (1992)
Entomophthorales	*Basidiobolus ranarum*	D29946	Nagahama *et al.* (1995)
Choanoflagellida	*Diaphanoeca grandis*	L10824	Wainright *et al.* (1993)
Choanoflagellida	*Acanthocoepsis unguiculata*	L10823	Wainright *et al.* (1993)
Metazoa	*Scypha ciliata*	L10827	Wainright *et al.* (1993)
Metazoa	*Microciona prolifera*	L10825	Wainright *et al.* (1993)
Protozoa	*Apusomonas proboscidea*	L37037	Cavalier-Smith and Chao (Unpub.)

[a] Is1 and 2 indicate the two isolates of *Zoophthora radicans* from different sources.

ing Ready Reactions Kit) together with specific sequencing primers (Table 2). Reactions were run on an automatic sequencer (ABI Prism 377 DNA Sequencer), and chromatograms of these sequences were checked using Sequencer 3.1. (Gene Codes Corporation, Inc., Ann Arbor, MI). Both strands of the SSU rDNA gene were sequenced except near the 3′-end.

Sequence Alignment

Seven new sequences from this study were aligned with 33 published SSU rDNA sequences (Table 1) using the computer program Pileup (Genetics Computer Group, Madison, WI), and the alignment was adjusted manually, taking the SSU secondary structure into account (Van de

TABLE 2
List of the Primers Used in This Study Listed 5′ → 3′ from Left to Right

Primer name	Primer sequence	Reference
nu-SSU-0021-5′	CTGGTTGATTCTGCCAGT	Gargas and DePriest (1996)
nu-SSU-0305-3′	TCGAAAGTTGATAGGGCAG	Gargas *et al.* (1995)
nu-SSU-0553-3′	GGCTGCTGGCACCAGACTTGC	White *et al.* (1990)
nu-SSU-0573-5′	GCAAGTCTGGTGCCAGCAGCC	White *et al.* (1990)
nu-SSU-0807-3′	AATACAATTAGCATGGAATAA	This study
nu-SSU-1150-5′	AACTTAAAGGAATTGACGGAAG	White *et al.* (1990)
nu-SSU-1184-3′	GAGTTTCCCCGTGTTGAGTC	Gargas *et al.* (1995)
nu-SSU-1380-5′	AGTTGGTGGAGTATTTGTCTGG	This study
nu-SSU-1475-3′	CAGTGTAGCGCGCGTGCGGCC	This study
nu-SSU-1497-5′	GGGCCGCACGCGCGCTACACT	This study
nu-SSU-1780-3′	AATGATCCTTCCGCAGGT	DePriest (1993)

Peer *et al.*, 1997). Choanoflagellates and animals, based on their sister group relationships to the fungi (Wainright *et al.*, 1993; Kumar and Rzhetsky, 1996), and a protozoan were chosen as outgroups.

Phylogenetic analysis

Maximum parsimony analyses were performed with the software PAUP 3.1.1 (Swofford, 1993). Heuristic searches with 100 random replications were used to search for the shortest tree as the data set was too large for branch-and-bound or exhaustive searches within a realistic time frame. All characters were equally weighted and invariant characters ignored. All branch lengths equal to 0 were collapsed to polytomies. Support for internal branches in the most parsimonious trees was assessed with 200 bootstrap replications.

Topologically constrained analyses were used to evaluate the hypotheses that either Zygomycota or Chytridiomycota are monophyletic. Parsimony analyses were performed using these two constraints with the same settings as above. The most parsimonious trees resulting from each of the three searches were evaluated by the Kishino-Hasagawa test using maximum likelihood (Kishino and Hasagawa, 1989). In this test, trees are compared with the most likely tree and are considered significantly worse in explaining the data set, if the log likelihood difference is more than 1.96× the standard deviation.

Maximum likelihood analyses were performed with the program fastDNAml (Olsen *et al.*, 1994); the transition/tranversion ratio was set to 1.6 to 1, the ratio found to give the highest likelihood for analyses of nuclear SSU rDNA for Ascomycetes (Gargas and DePriest, 1997). The maximum likelihood analyses were based on the evolutionary model of Felsenstein (1981), except for the allowance of different transition and tranversion rates and different evolutionary rates between sites.

Neighbor-joining analysis were performed with the software package TREECON for Windows (Van de Peer and De Wachter, 1994) using the Jukes-Cantor evolutionary model. Support for internal branches was assessed with 200 bootstrap replications.

RESULTS

Nearly complete sequences from the nuclear SSU rDNA gene of seven representatives of Entomophthorales were obtained, though about 260 nucleotides between helix 19 and helix E23-8 (Van de Peer *et al.*, 1997) were missing for *M. vermicola.* Phylogenetic relationships of Entomophthorales were inferred using an alignment of 40 taxa with 1581 unambiguously aligned positions.

Maximum parsimony (MP; Fig. 1), neighbor-joining (NJ; Fig. 2) and maximum likelihood (ML; data not shown) analyses produced trees with nearly identical topologies. In each of the trees the Ascomycota and the Basidiomycota had monophyletic origins, with Glomales as their sister group. These findings were consistent with other fungal nuclear SSU rDNA sequence analyses (Bruns *et al.*, 1992; Simon *et al.*, 1992; Berbee and Taylor, 1993; Nagahama *et al.*, 1995; Gerhig *et al.*, 1996).

In none of the three phylogenetic analyses were Chytridiomycota or Zygomycota monophyletic; as a group Chytridiomycota plus Zygomycota was paraphyletic to the Ascomycota and the Basidiomycota (Figs. 1 and 2). Given the low bootstrap values in the basal nodes, the hypotheses that Chytridiomycota or Zygomycota were monophyletic was evaluated. Alternate tree topologies in which Chytridiomycota representatives were constrained to form a monophyletic group were 199 steps longer (9.3%) and those in which Zygomycota representatives were constrained to form a monophyletic group were 207 steps longer (9.6%). All of the constrained trees were significantly less likely than the nonconstrained trees using the Kishino–Hasagawa test (Table 3), suggesting that neither the Chytridiomycota nor the Zygomycota are monophyletic.

Eight equally parsimonious trees were found in the maximum parsimony analysis. The major difference between these was whether the chytrids *Neocallimastix, Chytridium,* and *Basidiobolus* and the zygomycetes *Endogone* and *Mortierella* clustered together or not. In the

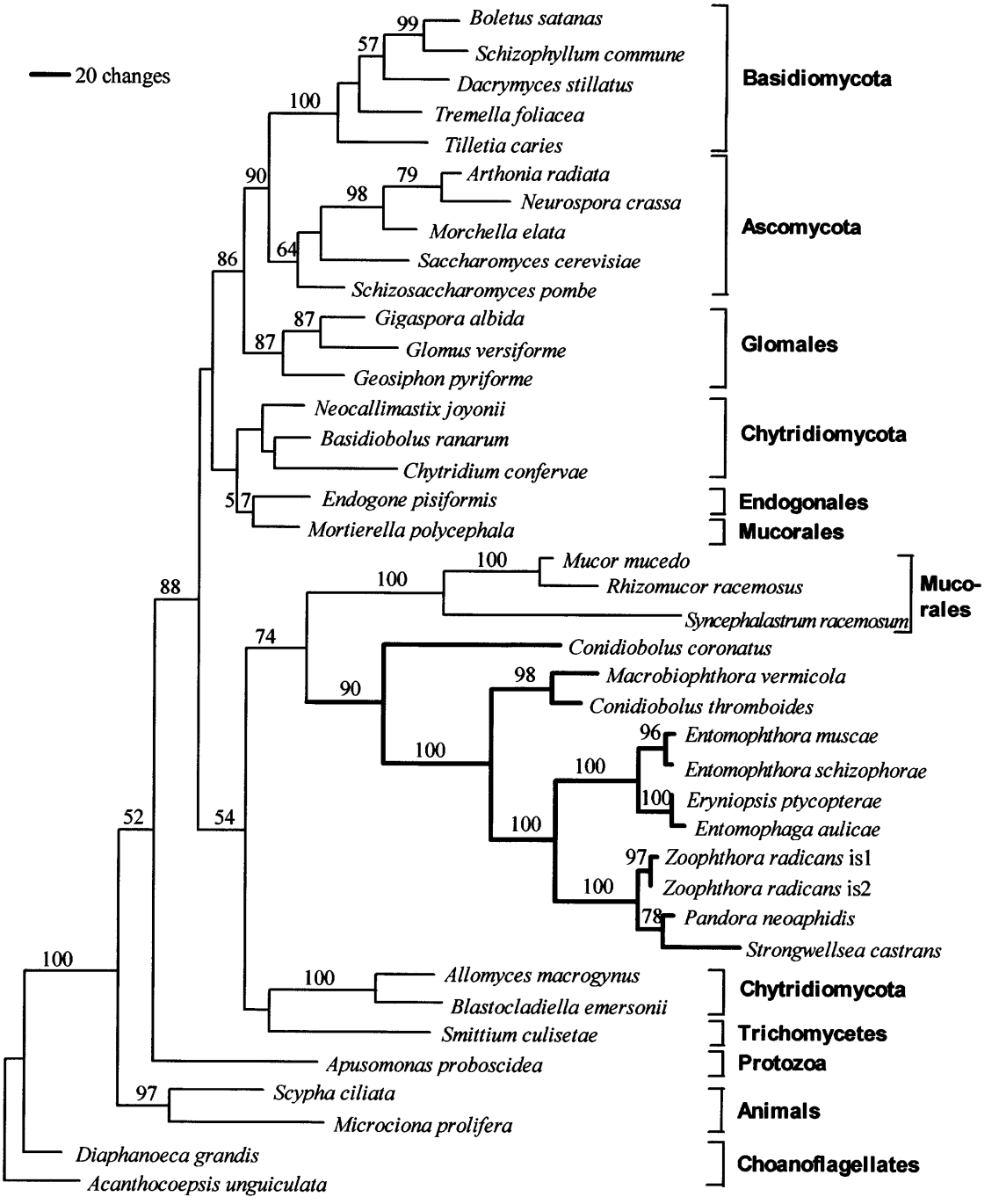

FIG. 1. Phylogenetic relationships within Entomophthorales inferred from parsimony analysis of 1581 nucleotides of nuclear SSU rDNA. Bootstrap percentages over 50% from 200 replicates are shown above each supported branch. The branches connecting Entomophthorales are shown with bolder lines. One of eight equally parsimonious phylograms requiring 2146 steps (CI = 0.4660 and RI = 0.6355) is shown. The scale bar corresponds to 20 nucleotide changes. The major difference in the phylograms was whether the chytrids *Neocallimastix, Chytridium,* and *Basidiobolus* and the zygomycetes *Endogone* and *Mortierella* clustered together or not.

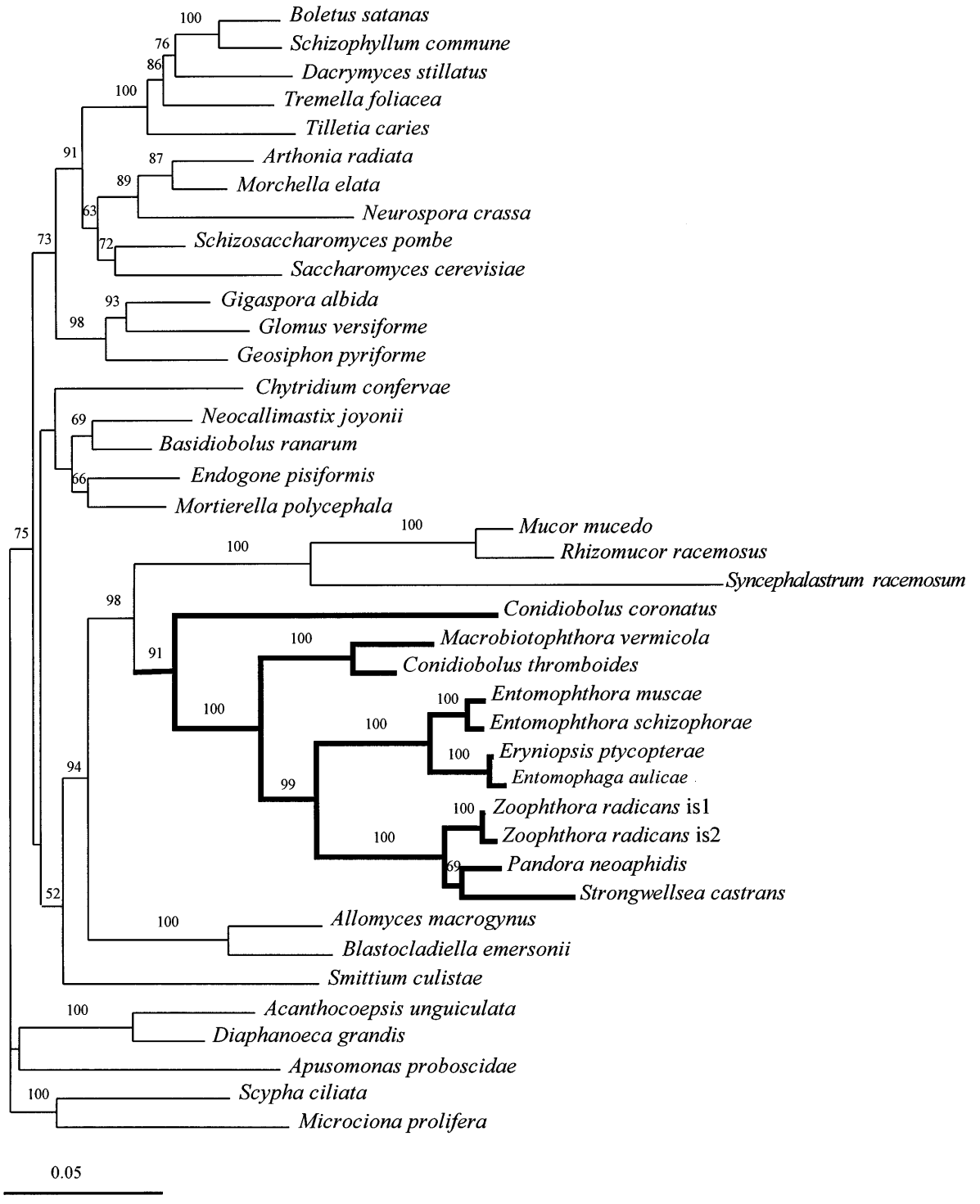

FIG. 2. Phylogenetic relationships within Entomophthorales inferred from neighbor-joining analysis of 1581 nucleotides of nuclear SSU rDNA. Bootstrap percentages over 50% from 200 replicates are shown above each supported branch. The branches connecting Entomophthorales are shown with bolder lines. The scale bar indicates the evolutionary distance and corresponds to one change per 20 nucleotide positions.

TABLE 3

Kishino-Hasagawa Likelihood Test Results (Kishino and Hasagawa, 1989) for the Most Parsimonious Trees Generated from an Analysis without Any Constraints, for the Most Parsimonious Trees Generated from an Analysis where the Monophyly of the Zygomycota Were Constrained, and for the Most Parsimonious Trees Generated from an Analysis where the Monophyly of the Chytridiomycota Were Constrained

User tree	Log likelihood	Difference in log likelihood	SD	Significantly worse?
Most-parsimonious tree 1	14212.953			
Most-parsimonious tree 2	14214.093	−1.139	15.268	No
Most-parsimonious tree 3	14217.542	−4.588	16.264	No
Most-parsimonious tree 4	14219.359	−6.405	9.639	No
Most-parsimonious tree 5	14220.582	−7.628	18.382	No
Most-parsimonious tree 6	14224.025	−11.072	19.170	No
Most-parsimonious tree 7	14334.375	−121.421	21.973	Yes
Most-parsimonious tree 8	14340.450	−127.496	24.170	Yes
Zygomycota-constrained tree 1	15518.274	−1305.321	49.769	Yes
Zygomycota-constrained tree 2	15519.201	−1306.247	49.831	Yes
Zygomycota-constrained tree 3	15523.278	−1310.325	49.867	Yes
Chytridiomycota-constrained tree 1	15367.110	−1154.156	49.570	Yes
Chytridiomycota-constrained tree 2	15481.024	−1268.070	51.407	Yes
Chytridiomycota-constrained tree 3	15489.738	−1276.784	51.304	Yes

Note. Six of eight most parsimonious trees were not significantly worse than the most likely tree, which was also one of those six. Two of the most parsimonious trees, and all of the constrained trees were significantly worse than the most likely tree.

maximum likelihood and the neighbor-joining analyses these taxa formed a monophyletic group, but this clade was not well supported in any of these analyses. Relationships within the Entomophthorales, however, did not vary between the different trees from the three analyses.

In each of our analyses the Entomophthorales, exclusive of *Basidiobolus,* were monophyletic, supported by bootstrap percentages of 90 and 95% (Figs. 1 and 2). A branch uniting the Entomophthoraceae was supported by high bootstrap percentages of 99 and 100% (Figs. 1 and 2). Within this family, two strongly supported (100% bootstrap) clades were resolved, with the genera *Entomophthora, Entomophaga,* and *Eryniopsis* in one, and *Zoophthora, Pandora,* and *Strongwellsea* in the other (Figs. 1 and 2).

The two studied species of *Conidiobolus* appear to be paraphyletic, with *C. coronatus* on the most basal branch within the Entomophthorales; *C. thromboides* and *M. vermicola* are on the next branch to diverge, which forms a monophyletic group (100% bootstrap) with other members of the Entomophthorales (Figs. 1 and 2).

In all the analyses a sister group relationship between Mucorales and Entomophthorales was suggested. This was supported by bootstrap percentages of 74% (MP) and 99% (NJ).

We found a surprising amount of variation in the SSU rDNA sequences within the Entomophthorales. Taxa belonging to the family Entomophthoraceae contained up to 50 extra nucleotides in the region between helix 23 and helix E23-2, a variable region within the SSU (Van de Peer *et al.,* 1997). This region was alignable within the family, but could not be aligned for all members within the order, and was excluded from the analyses. Interestingly, the internal branches among Entomophthorales were quite long as compared with those within the Ascomycota and the Basidiomycota.

DISCUSSION

Entomophthorales and Mucorales have been suggested to be sister groups, although this was not well-supported by bootstrap values (Gehrig *et al.,* 1996; Nagahama *et al.,* 1995). In the present study this sister group relationship is supported with high bootstrap values in both NJ distance-based and MP cladistical analyses. However, long branches are present throughout these groups, which could result in long-branch attraction between taxa (Hillis *et al.,* 1994); to confirm this proposed relationship we need either sequences from other genes or more taxa.

Our analyses support monophyly of the Entomophthorales, and confirm that *Basidiobolus ranarum* is more related to chytrids from Chytridiales and Neocallimasticales than to the Entomophthorales (Nagahama *et al.,* 1995). Repetitive discharge of conidia has been proposed as a key character defining the order Entomophthorales. The molecular data provide evidence that repetitive conidial discharge may have developed in at least two independent lineages within the Zygomycota and Chytridiomycota. Members of *Basidiobolus* repetitively discharge conidia by

a "rocket mechanism," in which conidiophores disrupt just below the swollen portion of the conidiophore, resulting in discharge of the conidium plus the upper part of the conidiophore (Ingold, 1934). In contrast, members of the Entomophthorales repetitively discharge conidia by mechanisms described as a "rounding-up mechanism" as exemplified by *E. muscae* (Eilenberg *et al.,* 1986, 1995) or by papillar eversion exemplified by *Conidiobolus* spp. (Humber, 1981), both in which only the conidia are discharged.

Within Entomophthorales, members of the Ancylistaceae seem to be the first lineage to diverge, which is in agreement with the basal placement of this family within the Entomophthorales based on previous phylogenetic interpretations of their smaller nuclei and saprotrophic lifestyle (Humber, 1984a).

DNA-based phylogenies also support the segregation of the Entomophthoraceae as a monophyletic lineage of obligate insect pathogens. In addition, these results support using the character of large nuclei, which stain readily with orcein, to define the Entomophthoraceae (Humber, 1981).

There are at least two distinct lineages within the Entomophthoraceae: one with uninucleate conidia as represented by *Zoophthora, Pandora,* and *Strongwellsea,* and a second one with multinucleate conidia as represented by *Entomophthora, Entomophaga,* and *Eryniopsis.* Based on the available molecular data, the two lineages might deserve recognition at the subfamilial rank, but such taxonomic changes should not be made until more taxa from this family are studied.

The relationships of the genus *Eryniopsis* have been controversial in that their unitunicate and multinucleate primary conidia resemble the primary conidia of *Entomophaga,* but their secondary conidia resemble the secondary conidia of *Erynia sensu lato* and *Zoophthora.* In addition, like *E. sensu lato* and *Zoophthora, Eryniopsis* has branched conidiophores (Humber, 1984b). The morphology of the known species within *Eryniopsis* is diverse and the genus might be a polyphyletic assembly. Our results indicate that the studied species of *Eryniopsis, E. ptycopterae,* is more closely related to *Entomophaga* than to *E. sensu lato* and *Zoophthora,* as suggested by primary spore morphology (Keller and Eilenberg, 1993). To clarify the status of *Eryniopsis* as a genus more species should be examined.

The molecular data suggest the genus *Conidiobolus* to be paraphyletic; *C. coronatus* belongs to the subgenus *Delacroixia* (Bałazy, 1993), the only group within the Entomophthorales which produces microconidia. Molecular evidence supports separation of *Conidiobolus* based on the presence of microconidia.

It is worth noting that members of *Conidiobolus,* such as *C. coronatus,* occasionally occur as human pathogens, causing subcutaneous (Towersey *et al.,* 1988) or pulmonary (Walsh *et al.,* 1994) infections. Recognizing the apparently separate origins of the two studied *Conidiobolus* species, one of which includes clinically important strains, is essential information for risk assessments of *Conidiobolus.* Further studies including more species of the insect-pathogenic *Conidiobolus* and their relatives are needed for a more comprehensive phylogeny prior to the formal registration and approval of these fungi as commercial insect biocontrol agents.

Zygomycetes and chytrids, the most basal groups within Eumycota, are ancient and diverse, and our results support the nonmonophyly of the Zygomycota and of the Chytridiomycota. Studies on orders within these groups, such as the Entomophthorales with their obligate pathogenic relationships, may provide valuable information on evolution of various fungal lifestyles. Better resolution of these groups, and identifications of monophyletic lineages, will require sampling of a diversity of taxa and sequences from other genes. Future research, including groups such as the Entomophthorales, may provide information to help understand the origins and mechanisms of pathogenicity within the fungi.

ACKNOWLEDGMENTS

We thank R. Humber (USDA) for cultures of *Conidiobolus thromboides* and *Macrobiotophthora vermicola,* and R. Humber, K. O'Donnell, J. W. Taylor, and an anonymous reviewer for valuable advice. We thank C. Hansen for technical support. This work was supported by Danish National Research Council.

REFERENCES

Bałazy, S. 1993. Entomophthorales. In *Flora of Poland. Fungi* (*Mycota*), Vol. XXIV, pp. 60–86. Polish Academy of Sciences.

Beauvais, A., and Latgé, J-P. 1988. A simple medium for growing entomophthoralean protoplasts. *J. Invertebr. Pathol.* **51:** 175–178.

Berbee, M. L., and Taylor, J. W. 1993. Dating the evolutionary radiation of the true fungi. *Can. J. Bot.* **71:** 1114–127.

Bowman, B. H., Taylor, J. W., Brownlee, J. L., Lu, S-D., and White, T. J. 1992. Molecular evolution of the fungi: Relationship of the Basidiomycetes, Ascomycetes, and Chytridiomycetes. *Mol. Biol. Evol.* **9:** 258–296.

Bruns, T. D., Vilgalys, R., Barns, S. M., Gonzalez, D., Hibbett, D. S., Lane, D. J., Simon, L., Stickel, S., Szaro, T. M., Weisburg, W. G., and Sogin, M. L. 1992. Evolutionary relationships within the fungi: Analyses of nuclear small subunit RNA sequences. *Mol. Phylogenet. Evol.* **1:** 231–241.

Carruthers, R. I., and Hural, K. 1990. Fungi as natural occurring entomopathogens. In *New Directions: Biological Control: Alternatives for Suppressing Agricultural Pests and Diseases*, pp. 115–138. Liss, New York.

DePriest, P. 1993. Small subunit rDNA variation in a population of lichen fungi due to optional group I introns. *Gene* **134:** 67–74.

Eilenberg, J., Bresciani, J., and Latgé, J-P. 1986. Ultrastructural studies of primary spore formation and discharge in the genus *Entomophthora. J. Invertebr. Pathol.* **48:** 318–324.

Eilenberg, J., Bresciani, J., Olesen, U., and Olson, L. 1995. Ultrastructural studies of secondary spore formation and discharge in the genus *Entomophthora. J. Invertebr. Pathol.* **65:** 179–185.

Felsenstein, J. 1981. Evolutionary trees from DNA sequences: A maximum likelihood approach. *J. Mol. Evol.* **17:** 368–376.

Förster, H., Coffey, M. D., Elwood, H., and Sogin, M. L. 1990. Sequence analysis of the small subunit ribosomal RNAs of three zoosporic fungi and implications for fungal evolution. *Mycologia* **82:** 306–312.

Gargas, A., and DePriest, P. T. 1996. A nomenclature for fungal PCR primers with examples from intron-containing SSU rDNA. *Mycologia* **88:** 745–748.

Gargas, A., and DePriest, P. T. 1997. Origins of lichen associations within the Eumycota: Parsimony and maximum likelihood analyses of two SSU rDNA data sets. *Am. J. Bot. Suppl.* **84:** 9–10.

Gargas, A., DePriest, P. T., Grubbe, M., and Tehler, A. 1995. Multiple origins of lichen symbioses in fungi suggested by SSU rDNA phylogeny. *Science* **268:** 1492–1495.

Gargas, A., and Taylor, J. W. 1995. Positions of multiple insertions in SSU rDNA of lichen-forming fungi. *Mol. Biol. Evol.* **12:** 208–218.

Gehrig, H., Schüssler, A., and Kluge, M. 1996. *Geosiphon pyriforme*, a fungus forming endocytobiosis with *Nostoc* (Cyanobacteria), is an ancestral member of the Glomales: Evidence by SSU rDNA Analysis. *J. Mol. Evol.* **43:** 71–81.

Hajek, A. E. 1997. Ecology of terrestrial fungal entomopathogens. In *Advances in Microbial Ecology*, Vol. 15 (Jones, Ed.), pp. 193–249. Plenum Press, New York.

Hillis, D. M., Huelsenbeck, J. P., and Swofford, D. L. 1994. Hobgolbin of phylogenetics? *Nature* **369:** 363–364.

Humber, R. A. 1981. An alternative view of certain taxonomic criteria used in the Entomophthorales (Zygomycetes). *Mycotaxon* **13:** 191–240.

Humber, R. A. 1984a. Foundation for an evolutionary classification of the Entomophthorales (Zygomycetes). In *Fungus/Insect Relationships. Perspectives in Ecology and Evolution* (Q. Wheller and M. Blackwell, Eds.), pp 166–183. Columbia University Press, New York.

Humber, R. A. 1984b. *Eryniopsis*, a new genus of the Entomophthoraceae (Entomophthorales). *Mycotaxon* **21:** 257–264.

Humber, R. A. 1989. Synopsis of a revised classification for the Entomophthorales (Zygomycotina). *Mycotaxon* **36:** 441–460.

Ingold, C. T. 1934. The spore discharge mechanism of *Basidiobolus ranarum*. *N. Phytol.* **33:** 274–277.

Keller, S. 1987. Arthropod-pathogenic Entomophthorales of Switzerland I. *Conidiobolus, Entomophaga* and *Entomophthora. Sydowia* **40:** 122–167.

Keller, S. 1991. Arthropod-pathogenic Entomophthorales of Switzerland II. *Erynia, Eryniopsis, Neozygites, Zoophthora* and *Tarichium*. *Sydowia* **44:** 39–122.

Keller, S., and Eilenberg, J. 1993. Two new species of Entomophthoraceae (Zygomycetes, Entomophthorales) linking the genera *Entomophaga* and *Eryniopsis. Sydowia* **45:** 264–273.

Kishino, H., and Hasegawa, M. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *J. Mol. Evol.* **29:** 170–179.

Kumar, S., and Rzhetsky, A. 1996. Evolutionary relationships of eukaryotic kingdoms. *J. Mol. Evol.* **42:** 183–193.

Lee, S. B., and Taylor, J. W. 1990. Isolation of DNA from fungal mycelia and single spores. In *PCR Protocols: A Guide to Methods and Applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, Eds.), pp. 282–287. Academic Press, New York.

Mankin, A. S., Skryabin, K. G., and Rubtsov, P. M. 1986. Identification of ten additional nucleotides in the primary structure of yeast 18S rRNA. *Gene* **44:** 143–145.

Nagahama, T., Sato, H., Shimazu, M., and Sygiyama, J. 1995. Phylogenetic divergence of the entomophthoralean fungi: Evidence from nuclear 18S ribosomal RNA gene sequences. *Mycologia* **87:** 203–209.

Olsen, G. J., Matsuda, H., Hagstrom, R., and Overbeck, R. 1994. FastDNAml: A tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Comput. Appl. Biosci.* **10:** 41–48.

Paquin, B., Forget, L., Roewer, I., and Lang, B. F. 1995. Molecular phylogeny of *Allomyces macrogynus:* Congruency between nuclear ribosomal RNA- and mitochondrial protein-based trees. *J. Mol. Evol.* **41:** 657–663.

Simon, L., Bousquet, J., Lévesque, R. C., and Lalonde, M. 1993. Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. *Nature* **363:** 67–69.

Simon, L., Lalonde, M., and Bruns, T. D. 1992. Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. *Appl. Environ. Microbiol.* **58:** 291–295.

Sogin, M. L., Miotto, K., and Miller, L. 1986. Primary structure of the *Neurospora crassa* small subunit ribosomal RNA coding region. *Nucleic Acids Res.* **14:** 9540.

Swann, E. C., and Taylor, J. W. 1993. Higher taxa of basidiomycetes: an 18S rRNA gene perspective. *Mycologia* **85:** 923–936.

Swofford, D. L. 1993. *PAUP: Phylogenetic Analysis Using Parsimony*, Version 3.1.1. Illinois Natural History Survey, Champaign, IL.

Taylor, J. W. 1993. A contemporary view of the holomorph: Nucleic acid sequences and computer databases are changing fungal classification. In *The Fungal Holomorph: Mitotic. Meiotic and Pleomorphic Speciation in Fungal Systematics* (D. R. Reynolds and J. W. Taylor, Eds.), pp. 3–15. C. A. B. International, Wallingford.

Towersey, L., Wanke, B., Estrella, R. R., Londero, A. T., Mendonca, A. M. N., and Neves, R. G. 1988. *Conidiobolus coronatus* infection treated with ketoconazole. *Arch. Dermatol.* **124:** 1392–1396.

Van de Peer, Y., and De Wachter, R. 1994. TREECON for Windows: A software package for the construction and drawing of evolutionary trees

for the Microsoft Windows environment. *Comput. Appl. Biosci.* **10:** 569–570.

Van de Peer, Y., Jansen, J., De Rijk, P., and De Wachter, R. 1997. Database on the structure of small ribosomal subunit RNA. *Nucleic Acids Res.* **25:** 111–116.

Wainright, P. O., Hinkle, G., Sogin, M. L., and Stickel, S. K. 1993. The monophyletic origins of the metazoa: An unexpected evolutionary link with fungi. *Science* **260:** 340–342.

Walsh, T. J., Renshaw, G., Andrews, J., Kwon-Chung, J., Cunnion, R. C., Pass, H. I., Taubenberger, J., Wilson, W., and Pizzo, P. A. 1994. Invasive zygomycosis due to *Conidiobolus incongruus*. *Clin. Infec. Dis.* **19:** 423–430.

Waterhouse, G. M. 1973. Entomophthorales. In *The Fungi*, Vol. IVB (G. C. Ainsworth, F. K. Sparrow, and A. S. Sussman, Eds.), pp. 219–229. Academic Press, New York.

White, T. J., Bruns, T. D., Lee, S. B., and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, Eds.), pp. 315–321. Academic Press, New York.