Phylogenetic studies of Saprolegniomycetidae and related groups based on nuclear large subunit ribosomal DNA sequences

Alexandra Riethmüller, Michael Weiß, and Franz Oberwinkler

Abstract: To reveal phylogenetic relationships within the Peronosporomycetes (Oomycetes), we sequenced a part of the nuclear rDNA coding for the ribosomal large subunit of 46 Peronosporomycetes species and one representative of the Xanthophyta. The main emphasis of our study was put on the phylogenetic relationships within the Saprolegniomycetidae. We supplemented our data with a sequence of *Phytophthora megasperma* Drechsler from GenBank. Two sets of sequences were analysed using the neighbor-joining method, statistically supported by the bootstrap method, as well as the maximum parsimony method. Our results are well compatible with the tripartite subclassification of the Peronosporomycetes into Saprolegniomycetidae, Rhipidiomycetidae and Peronosporomycetidae, as well as with the placement of the orders Saprolegniales and Leptomitales in the Saprolegniomycetidae. *Pachymetra chaunorhiza* Croft & Dick, which has been placed in the Sclerosporales, was grouped within the Saprolegniales. Within the Peronosporomycetidae, the orders Peronosporales and Pythiales could not be separated. There are indications that *Phytophthora* de Bary and the Peronosporales form a common natural group. The genus *Achlya* Nees proved to be a heterogeneous group.

Key words: peronosporomycetes systematics, oomycetes systematics, stramenopiles, large subunit rDNA, 28S, molecular phylogeny.

Résumé : Afin de révéler les relations phylogénétiques au sein des Péronosporomycètes (Oomycètes), les auteurs ont séquencé une partie de l'ADN nucléïque codant pour la grande unité ribosomique chez 46 espèces de Péronosporomycètes et un représentant des Xanthophytes. Dans cette étude, on met l'accent sur les relations phylogénétiques au sein des Saprolegniomycetidae. Les auteurs ont combiné leurs données avec une séquence du *Phytophthora megasperma* Drechsler provenant de GenBank. Ils ont analysé deux ensembles de séquences en utilisant la méthode de neighbor joining, statistiquement supportée par la méthode de bootstrap, ainsi que par la méthode de maximum parsimony. Les résultats sont compatibles avec la subclassification tripartite des Péronosporomycètes en Saprolegniomycetidae, Rhipidiomycetidae et Peronosporomycetidae, ainsi qu'avec l'allocation des Saprolégniales et des Leptomitales aux Saprolegniomycetidae. On regroupe le *Pachymetra chaunorhiza* Croft & Dick, préalablement attribué aux Sclérosporales, dans les Saprolégniales. Au sein des Peronosporomycetidae, les ordres des Péronosporales et des Pythiales ne peuvent pas être séparés. Il y a des indications que les *Phytophthora* de Bary et les Péronosporales forment un groupe commun naturel. Le genre *Achlya* Nees s'avère être un groupe hétérogène.

Mots clés : systématique des péronosporomycètes, systématique des oomycètes, straménopiles, grande sous-unité de l'ADNr, 28S, phylogénie moléculaire.

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Introduction

Various molecular approaches have been tested to resolve species complexes and phylogenetic relationships in the Peronosporomycetes (Oomycetes). Preliminary phylogenetic studies of ribosomal DNA sequences were made by Walker and Doolittle (1982), who examined the 5S rRNA of *Saprolegnia ferax* (Gruithuysen) Thuret, *Pythium hydnosporum* (Mont.) Schroeter, and other organisms. Restriction fragment length polymorphisms (RFLPs) of nuclear DNA (e.g., internal transcribed spacer, ITS region) and mitochondrial DNA were used to identify individual species of *Phytophthora* (Förster et al. 1990; Förster and Coffey 1993; Tooley et al. 1996), *Pythium* Pringsheim (Klassen et al. 1987; Belkhiri and Dick 1988; Martin and Kistler 1990; Chen and Hoy 1993; Rafin et al. 1995) and *Saprolegnia* Nees (Molina et al. 1995). Kageyama (1997) sequenced the ITS region and was able to design species-specific primers for detecting *Pythium ultimum* Trow from diseased seedlings. Crawford et al. (1996) and Cooke and Duncan (1997) sequenced the ITS region of various species of the genus *Phytophthora* and

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found that molecular-based groupings generally agreed with groupings established using classical morphological criteria, primarily sporangial morphology. Lee and Taylor (1992) also used DNA sequencing of the ITS region to deduce phylogenetic relationships among species of *Phytophthora*. Daugherty et al. (1998) sequenced the ITS region of some species of the Saprolegniales to examine the evolution of spore release mechanisms.

Within the Peronosporomycetes, 28S rDNA was first sequenced in *Phytophthora megasperma* (van der Auwera et al. 1994). Briard et al. (1995) sequenced 177 base pairs (bp) of the 28S rDNA of 23 species of *Pythium* and *Phytophthora* to assess their phylogenetic relationships. They found a high level of diversity among members of the genus *Pythium*, and four subgroups could be distinguished that correlated with groups based on the sporangial form. In contrast, the genus *Phytophthora* proved to be very homogeneous.

A new taxonomic system for the Peronosporomycetes was proposed by Dick (1995). His classification into orders was based on morphological and ultrastructural characters, e. g. oosporogenesis, oospore wall, and protoplasmic structure of the oospore.

The work presented here concerns phylogenetic analysis of the 5′ terminal domain of the 28S rDNA of 46 species of Peronosporomycetes over a length of more than 650 bp, supplemented with a sequence from *Phytophthora megasperma* (van der Auwera et al. 1994). We construct hypotheses about phylogenetic relationships within the Peronosporomycetes, with a particular emphasis on the Saprolegniomycetidae, and we discuss our results particularly in comparison to Dick's (1995) system. This is the first time the phylogeny of the Peronosporomycetes has been discussed on the basis of 28S rDNA sequences to this extent.

Materials and methods

The organisms included in the study are listed in Table 1. We isolated genomic DNA from pure cultures using a modified version of the SDS method of Edwards et al. (1991) and Henrion et al. (1992). Hyphal material was ground in liquid nitrogen and incubated in 500 µL extraction buffer for 1 h at 65°C. The extract was centrifuged for 10 min at 13 793 × *g*, and the supernatant was transferred to a new Eppendorf tube and digested with 10 U RNAse for 1 h at 37°C. The DNA was precipitated with 50 µL 3 M sodium acetate and 1000 µL 100% ethanol, cooled for 30 min, and centrifuged for 15 min at 13 793 × *g*. The pellet was washed with 70% ethanol, dried at room temperature, and dissolved in 50 µL H_2O.

The 5′ terminal domain of the nuclear DNA coding for the large ribosomal subunit (LSU rDNA) was amplified using the polymerase chain reaction (PCR) (Mullis and Faloona 1987; White et al. 1990) with NL1 and NL4 as primers (O'Donnell 1993). PCR products were purified with the QIAquick™ kit (QIAGEN) according to the manufacturer's instructions. DNA was sequenced using the ABI PRISM™ dye terminator cycle sequencing kit (Perkin Elmer) and an automated DNA sequencer (ABI 373, Perkin Elmer).

The alignments were produced with the aid of the MEGALIGN module of the LASERGENE system (DNASTAR, Inc.) with minor manual corrections. They were analysed according to the neighbor-joining method (Saitou and Nei 1987), as well as the maximum parsimony method (e.g., Fitch 1971). For the neighbor-joining analysis, we used the computer programs DNAdist and NEIGHBOR from the PHYLIP package version 3.5c (Felsenstein 1993) with Kimura two-parameter distances (Kimura 1980) as modified by Felsenstein (1993) and a transition/transversion rate of 2.0. In addition, the bootstrap procedure of Felsenstein (1985) was employed with 1000 repetitions using the SeqBoot and CONSENSE programs of the PHYLIP package. For heuristic parsimony analysis (1000 replications with random addition, tree bisection–reconnection, MULPARS option without steepest descent, gaps treated as uncertainties), we used the PAUP* test version 4.0d64 written by D.L. Swofford (1998). The alignments are available from the corresponding author upon request.

Results

Two sets of sequences were analysed to obtain estimates of phylogenetic relationships within the Peronosporomycetes. The first set (Fig. 1) with sequences of five members of Peronosporomycetes and one representative of the Xanthophyta (*Heterococcus brevicellularis* Vischer) was drawn up to determine a basal group of the Peronosporomycetes. This alignment could be used over a length of 359 bp, after which the xanthophyte sequence could no longer be reliably aligned with the sequences of the Peronosporomycetes.

The second set (Fig. 2) with sequences of 47 species of Peronosporomycetes was assembled to show phylogenetic relationships within the Peronosporomycetes. This alignment could be used over a length of 669 bp, and the resulting topologies were rooted with the group of the Peronosporomycetidae, which appeared as a basal group of the Peronosporomycetes in the analysis of the first set of species.

Phylogenetic analysis of the first set of sequences

To determine a basal group of the Peronosporomycetes, we analysed one representative each of the Saprolegniales, Leptomitales, Rhipidiales, Pythiales, and Peronosporales and, additionally, one representative of the Xanthophyta. The results of the neighbor-joining analysis are shown in Fig. 1. Based on the studies of Leipe et al. (1994) and van der Auwera and de Wachter (1997), we used the representative of the Xanthophyta as an outgroup species for rooting.

Phylogenetic analysis of the second set of sequences

The data set included 47 sequences of species of Peronosporomycetes, containing species of six orders from all three subclasses of Peronosporomycetes. The topology of the neighbor-joining analysis is shown in Fig. 2. Rooting was done according to the results of the analysis of the first set.

In this alignment two insertions were noticed. The first insertion extends over a length of 58 bp beginning at alignment position 390 and is present in all examined species of the Pythiales and Peronosporales, as well as in the examined representatives of the genera *Sapromyces* Fritsch and *Apodachlya* Pringsheim. The second insertion of 7 bp begins at alignment position 531 and is present in all species showing the first insertion, and in *Leptomitus lacteus* (Roth) Agardh.

The topology of the neighbor-joining analysis is compatible with the classification of the Peronosporomycetes into the subclasses Saprolegniomycetidae, Rhipidiomycetidae, and Peronosporomycetidae (Dick 1995). The Saprolegniomycetidae group includes two groups, which correspond to the order Saprolegniales with representatives of 11 genera and a total of 34 sequences (clusters 1–9) and to the Leptomitales

Table 1. List of studied organisms.

Species	Source*	GenBank accession No.
Achlya americana Humphrey	AR 26	AF119574
Achlya caroliniana Coker	AR 13	AF119575
Achlya caroliniana Coker sensu Attaway	AR 97	AF119576
Achlya colorata Pringsheim	CBS 545.67	AF119677
Achlya dubia Coker	CBS 546.67	AF119578
Achlya klebsiana Pieters	CBS 101.49	AF119579
Achlya papillosa Humphrey	CBS 101.52	AF119580
Achlya racemosa Hildebrand	AR 48	AF119581
Achlya radiosa Maurizio	AR 2	AF119582
Achlya spinosa de Bary	AR 95	AF119583
Achlya treleaseana (Humphrey) Kauffmann	CBS 575.67	AF119584
Aphanomyces de Bary sp.	AR 11	AF119585
Aphanomyces laevis de Bary	AR 47	AF119586
Aphanomyces stellatus de Bary	AR 51	AF119587
Aplanes androgynus (Archer) Humphrey	AR 46	AF119588
Aplanopsis spinosa Dick	CBS 112.61	AF119589
Apodachlya brachynema (Hildebrand) Pringsheim	AR 93	AF119590
Brevilegnia bispora Couch	CBS 569.67	AF119591
Brevilegnia megasperma Harvey	AR 4	AF119592
Calyptralegnia achlyoides (Coker & Couch) Coker	AR 5	AF119593
Dictyuchus Leitgeb sp.	AR 29	AF119594
Dictyuchus monosporus Leitgeb	CBS 467.81	AF119595
Heterococcus brevicellularis Vischer	SAG 835-1	AF119596
Leptomitus lacteus (Roth) Agardh	AR 80	AF119597
Pachymetra chaunorhiza Croft & Dick	CBS 960.87**	AF119598
Peronospora bulbocapni Beck	AR 81	AF119599
Peronospora ficariae (Tul.) de Bary	AR 78	AF119600
Phytophthora fragariae Hickman	CBS 309.62	AF119601
Phytophthora infestans (Montagne) de Bary	CBS 560.95	AF119602
Phytophthora undulata (Petersen) Dick	AR 55	AF119603
Plasmopara aegopodii (Casp.) Trott.	AR 83	AF119604
Plasmopara pygmaea (Ung.) Schroet.	AR 86	AF119605
Plectospira myriandra Drechsler	CBS 523.87	AF119606
Pythium Pringsheim sp.	AR 100	AF119607
Pythium middletonii Sparrow	CBS 528.74	AF119608
Saprolegnia anisospora de Bary	CBS 537.67	AF119609
Saprolegnia diclina Humphrey	AR 12	AF119610
Saprolegnia eccentrica (Coker) Seymour	CBS 551.67	AF119611
Saprolegnia ferax (Gruithuysen) Thuret	AR 16	AF119612
Saprolegnia hypogyna (Pringsheim) de Bary	CBS 869.72	AF119613
Saprolegnia litoralis Coker	CBS 535.67	AF119614
Saprolegnia monilifera de Bary	CBS 558.67	AF119615
Saprolegnia torulosa de Bary	AR 77	AF119616
Saprolegnia unispora (Coker & Couch) Seymour	CBS 213.35	AF119617
Sapromyces elongatus (Cornu) Coker	AR 9	AF119618
Scoliolegnia asterophora (de Bary) Dick	AR 94	AF119619
Thraustotheca clavata (de Bary) Humphrey	AR 10	AF119620
Species sequenced by other authors		
Phytophthora megasperma Drechsler	van der Auwera et al. 1994	X75631

*Source acronyms are as follows: AR, A. Riethmüller; CBS, Centraalbureau voor Schimmelcultures, AG Baarn, the Netherlands; SAG, Sammlung von Algenkulturen, Universität Göttingen, Germany.

**CBS, isotype.

with two sequences (cluster 10). Within the Saprolegniales five further groups can be distinguished: the *Saprolegnia* clusters (clusters 1, 2, and 4) including *Scoliolegnia* Dick (3), the clusters containing some of the species of *Achlya* (clusters 5 and 6), the *Aphanomyces* de Bary cluster (cluster 7), the cluster with further species of *Achlya* (cluster 8) and the cluster with species of *Brevilegnia* Coker & Couch and *Dictyuchus* Leitgeb (cluster 9). The representative of the

Sclerosporales, *Pachymetra chaunorhiza*, appears in the *Aphanomyces* cluster (cluster 7) of the Saprolegniales. The Rhipidiomycetidae group is represented by one sequence (11). The Peronosporomycetidae group contains six representatives of the Pythiales and four representatives of the Peronosporales in two clusters (clusters 12 and 13). The orders Pythiales and Peronosporales according to Dick's (1995) classification do not appear separately in the two clusters (clusters 12 and 13).

Heuristic maximum parsimony analysis found 264 most parsimonious trees with equal number of changes. The resulting strict consensus topology shown in Fig. 3 is very similar to the topology of the neighbor-joining analysis (Fig. 2). Mainly, the same groups are formed; differences can be found among the representatives of the order Leptomitales, which do not appear as a monophyletic group, and within the Peronosporomycetidae.

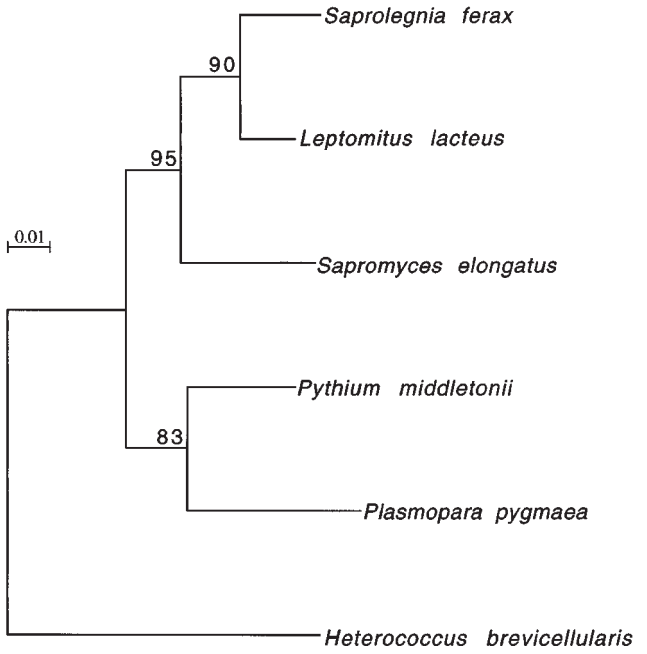
Discussion

Analyses of ribosomal small subunit rDNA by Förster et al. (1990) show the Peronosporomycetes to be a monophyletic group. Comparative ultrastructural studies position the Xanthophyta, the Peronosporomycetes and some colourless protists into a complex evolutionary assemblage, the Stramenopiles, tubulocristate protists with tripartite tubular hairs, or taxa derived from such organisms (Patterson 1989). Molecular phylogenetic studies carried out by Leipe et al. (1994), Silberman et al. (1996) and van der Auwera and de Wachter (1997) confirm that the Stramenopiles form a monophyletic group. For this reason, we used a representative of the Xanthophyta, *Heterococcus brevicellularis*, to root the topology shown in Fig. 1.

According to Dick (1995), three subclasses exist within the Peronosporomycetes: the Saprolegniomycetidae, Rhipidiomycetidae, and Peronosporomycetidae. This classification is reflected in the topologies of our neighbor-joining and maximum parsimony analyses (Figs. 1–3). The distribution of the detected insertions in the alignment (asterisks in Figs. 2 and 3) corresponds well with the adjacent position of Peronosporomycetidae, Rhipidiomycetidae, and Leptomitales in our analyses. In the topology in Fig. 2 the subclass Saprolegniomycetidae sensu Dick (1995) is only weakly supported with a bootstrap value of 68%, whereas the monophyly of the Peronosporomycetidae is optimally supported with a bootstrap value of 100%. The representative of the Rhipidiomycetidae, *Sapromyces elongatus* (Cornu) Coker, appears on a separate branch in all three topologies (Figs. 1–3). Our evaluations suggest, however, that the Rhipidiomycetidae are more closely related to the Saprolegniomycetidae than to the Peronosporomycetidae: the group consisting of Saprolegniomycetidae and Rhipidiomycetidae is optimally supported by a bootstrap value of 100% (Fig. 2), and this group appears in the maximum parsimony analysis as well (Fig. 3).

In the following, we will discuss the phylogenetic relationships resulting from our analyses within the individual groups and compare them with the results obtained by other authors. For results of the neighbor-joining analysis, we shall always refer to Fig. 2 and for the results of the maximum parsimony analysis to Fig. 3.

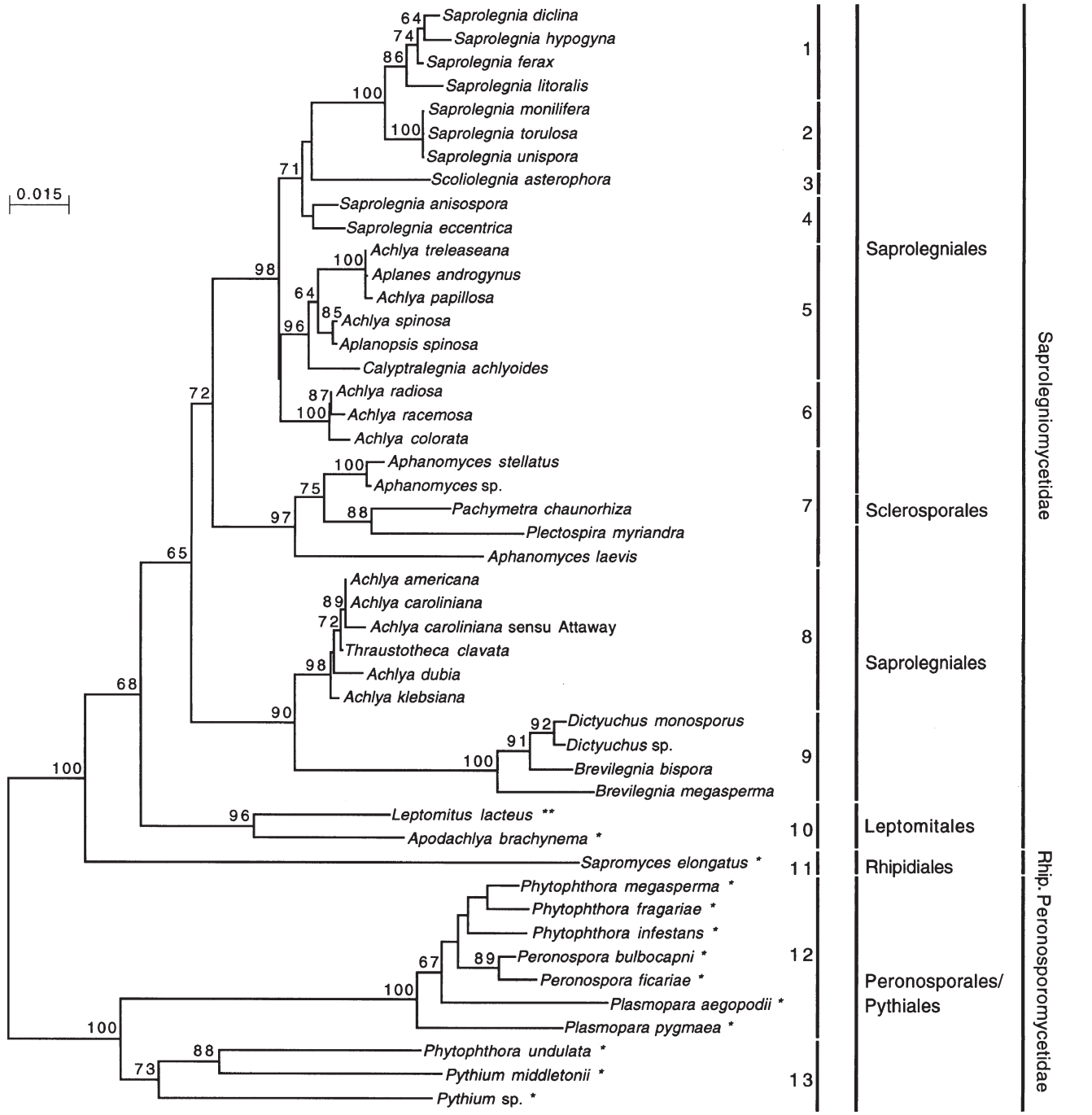
Fig. 1. Neighbor-joining analysis of partial 28S rDNA of five Peronosporomycetes and the xanthophyte *Heterococcus brevicellularis*. Genetic distances are Kimura two-parameter distances, numbers on branches are bootstrap values (1000 replicates, values smaller than 60% not shown). Branch lengths are scaled in terms of expected numbers of nucleotide substitutions per site. The topology was rooted with *Heterococcus brevicellularis*.



Saprolegniomycetidae

According to Dick (1995), who referred to analyses of rDNA restriction sites by Klassen et al. (1988), the Sclerosporales and Leptomitales should be removed from the Peronosporomycetidae and placed within the Saprolegniomycetidae. Our examinations confirm that the Sclerosporales and Leptomitales should be classified within the Saprolegniomycetidae (clusters 1–10). It is notable that *Pachymetra chaunorhiza* (cluster 7), which Dick et al. (1989) placed in the Sclerosporales, is included in the Saprolegniales. The Peronosporomycetes include both saprophytic as well as obligatory parasitic species. This diversity is reflected in our phylogenetic analyses, for example in cluster 7, which contains aquatic saprophytes (*Aphanomyces stellatus* de Bary, *Aphanomyces laevis* de Bary), the aquatic keratinophilic *Aphanomyces* sp., the species *Plectospira myriandra* Drechsler which is a parasite on rootlets of tomato seedlings and, as a representative of the order Sclerosporales, the obligatory parasite on grasses, *Pachymetra chaunorhiza*. Within the Leptomitales, only the sequence of *Apodachlya brachynema* (Hildebrand) Pringsheim contains the first insertion of 58 bp, thus supporting the topology of the maximum parsimony analysis, in which the representatives of the Leptomitales appear adjoining one another but do not form a monophyletic group (10a and 10b). Here *Apodachlya brachynema* appears to be more closely related to the representatives of the Rhipidiomycetidae and Peronosporomycetidae that all contain the first insertion, whereas *Leptomitus lacteus* appears to be more closely related to the Saprolegniales that lack the insertion. However, both species contain the

Fig. 2. Neighbor-joining analysis of partial 28S rDNA of 47 Peronosporomycetes. Genetic distances are Kimura two-parameter distances, numbers on branches are bootstrap values (1000 replicates, values smaller than 60% not shown). Branch lengths are scaled in terms of expected numbers of nucleotide substitutions per site. The topology was rooted with the group of the Peronosporomycetidae, as estimated in the phylogenetic analysis of a subset of sequences together with the sequence of a xanthophyte (see Fig. 1). *, species containing the insertion of 58 bp and the insertion of 7 bp; **, species containing only the insertion of 7 bp. Classification into orders and higher taxa according to Dick (1995). For details see text.

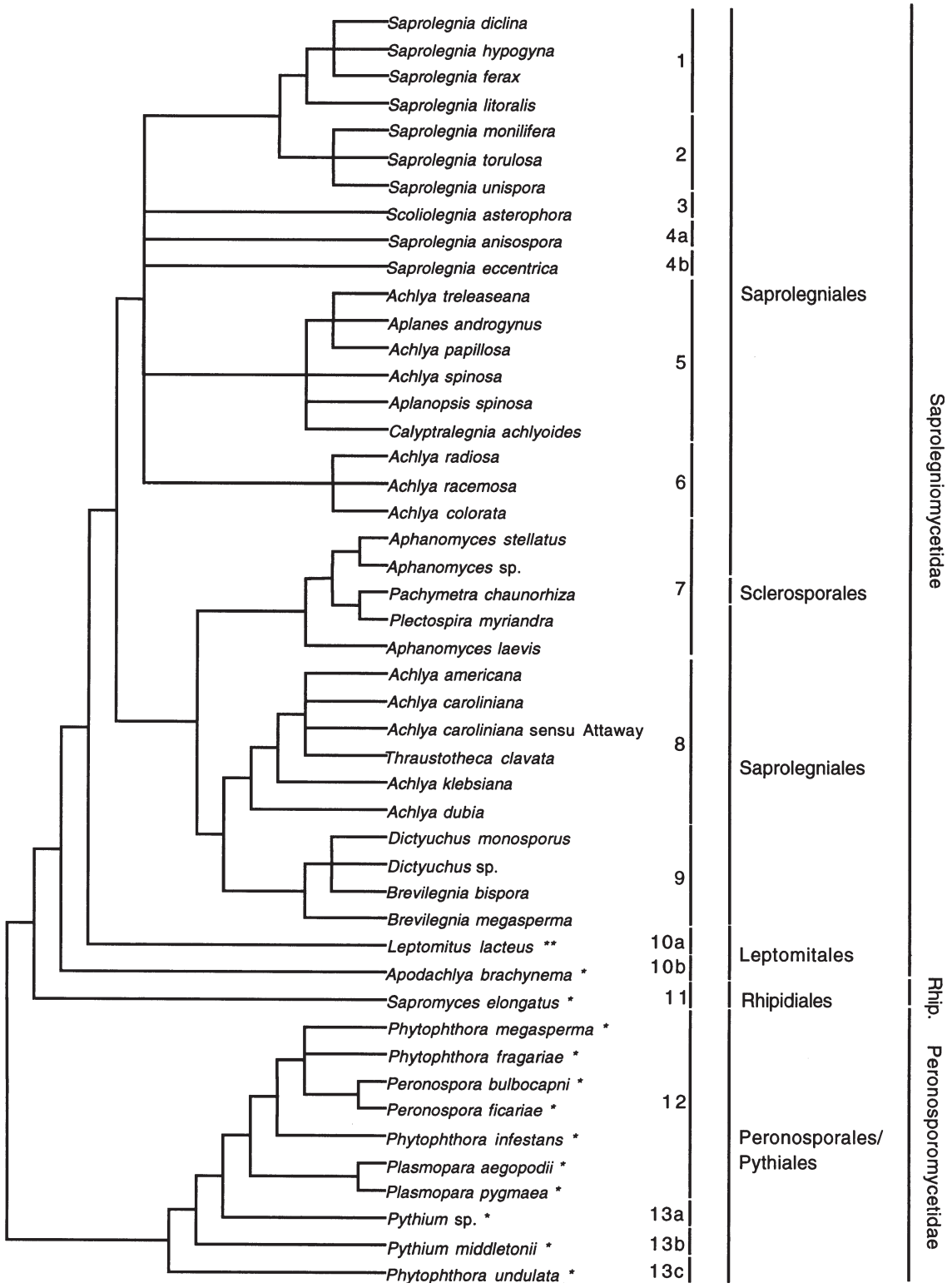


second insertion of 7 bp, thereby supporting their placement next to the Rhipidiomycetidae and Peronosporomycetidae, groups that also exhibit this character. In the topology of the neighbor-joining analysis the representatives of the Leptomitales appear as a monophyletic group well supported by a bootstrap value of 96%. The Saprolegniales, inclusive of the Sclerosporales (clusters 1–9), form one group, which is only moderately supported by a bootstrap value of 65%.

Saprolegniales

The overall group of the species of *Saprolegnia* (including *Scoliolegnia* sp.) is supported with a moderate bootstrap value of 71% in the neighbor-joining analysis and does not form a separate cluster in the maximum parsimony analysis. Three partial groups appear in the neighbor-joining analysis and in the maximum parsimony analysis (clusters 1, 2, and 4). In both analyses the species of clusters 1 and 2 together

Fig. 3. Heuristic maximum parsimony analysis of partial 28S rDNA of 47 Peronosporomycetes. Strict consensus of 264 most parsimonious trees. The topology was rooted with the group of the Peronosporomycetidae, as estimated in the phylogenetic analysis of a subset of sequences together with the sequence of a xanthophyte (see Fig. 1). *, species containing the insertion of 58 bp and the insertion of 7 bp; **, species containing only the insertion of 7 bp. Classification into orders and higher taxa according to Dick (1995). For details see text.



form a group within the genus *Saprolegnia* that is optimally supported by a bootstrap value of 100%. All of the species in clusters 1 and 2 form centric or subcentric oospores.

To clarify systematic groupings within the genus *Saprolegnia*, Beakes and Ford (1983) examined the esterase isoenzymes of various species and divided them into three

groups: (*i*) *S. australis* Elliott, *S. diclina* Humphrey, *S. ferax*, and *S. mixta* de Bary; (*ii*) *S. delica* Coker, *S. eccentrica* (Coker) Seymour, *S. furcata* Maurizio, *S. megasperma* Coker, *S. monoica* Pringsheim, *S. parasitica* Coker, and *S. toruloides* Kauffman; and (*iii*) *S. shikotsuensis* and *S. litoralis* Coker, in which *Achlya flagellata* Coker is located as well. This classification is partly reflected in our phylogenetic hypotheses. *Saprolegnia diclina*, *S. ferax*, and *S. litoralis* appear in cluster 1, while *S. eccentrica* appears in cluster 4.

The species *S. diclina*, *S. ferax*, *S. hypogyna* (Prings.) de Bary, and *S. litoralis* in cluster 1 (according to Dick (1973) *Saprolegnia* sensu stricto) all possess multiple to many oospores per oogonium, which, according to Dick (1969*a*) is typical for species of the genus *Saprolegnia*. This cluster is supported by a bootstrap value of 86%. Molina et al. (1995) examined the 18S rDNA of species of *Saprolegnia* using RFLP. Many of the RFLP patterns were identical, e.g., of *S. ferax*, *S. hypogyna*, and *S. delica* (now synonymized as *S. diclina*). However, according to our 28S rDNA sequences, these species can be differentiated. Molina et al. (1995) were also able to separate most of these species using additional RFLP analyses of the 5.8S rDNA and the ITS region.

According to Dick (1973) the species of *Saprolegnia* in clusters 2 and 4 belong to *Saprolegnia* sensu lato. Cluster 2 contains *S. monilifera* de Bary, *S. unispora* (Coker & Couch) Seymour, and *S. torulosa* de Bary. In the examined DNA region there are no differences in the sequences of the three taxa. According to Seymour (1970), *S. torulosa*, *S. monilifera*, and *Isoachlya toruloides* Kauffman & Coker form a complex of closely related species, and all three taxa of cluster 2 were once placed in the genus *Isoachlya* Kauffman. The type species of this genus, *I. toruloides*, should be sequenced to discover if this species groups with *S. unispora*, *S. torulosa*, and *S. monilifera*. Additionally, two other species of *Saprolegnia* that appear in different clusters in our analyses, *S. anisospora* de Bary and *S. eccentrica*, were temporarily transferred to the genus *Isoachlya*. Nowadays the species of the genus *Isoachlya* are again positioned in the genus *Saprolegnia* (Seymour 1970).

In the topology of the neighbor-joining analysis, the two species of *Saprolegnia* with eccentric oospores, *S. anisospora* and *S. eccentrica* (cluster 4), form a poorly supported cluster. In the maximum parsimony analysis (clusters 4a and 4b) they do not form a cluster. Therefore, it remains doubtful whether the group of eccentric species of *Saprolegnia* does form a natural group (cf., however, the well-supported group of eccentric species of *Achlya* in cluster 8 below).

Scoliolegnia asterophora (de Bary) Dick occupies an isolated position (cluster 3). Dick (1969*a*) transferred *Saprolegnia asterophora* to his new genus *Scoliolegnia*. He justified the erection of the new genus by a combination of vegetative, asexual, and sexual characters, including thin vegetative hyphae, proliferating zoosporangia, oogonia walls without pits and with more or less numerous papillae, as well as a variable internal structure of the mature oospores. In studies of the 18S rDNA by Molina et al. (1995), *Scoliolegnia asterophora* (as *Saprolegnia asterophora*) distinctly differed from the species of *Saprolegnia* studied. According to our results, it remains doubtful whether the genus *Scoliolegnia* can actually be separated from the genus *Saprolegnia*. In the phenetic analysis of 20 genera of Saprolegniaceae by Powell and Blackwell (1998), the genera *Scoliolegnia* and *Saprolegnia* also appear closely related; however, the authors did not consider sexual characteristics in their analysis.

In contrast to *Saprolegnia*, the examined species of *Achlya* do not form a monophyletic group either in the neighbor-joining analysis or in the maximum parsimony analysis. In both analyses three clusters are formed (clusters 5, 6, and 8), with cluster 8, which contains the eccentric species of *Achlya*, distinctly standing out from the others, being statistically well supported by a bootstrap value of 98%. Studies of GC content carried out by Storck and Alexopoulos (1970) and Green and Dick (1972) already revealed the heterogeneity of different species of *Achlya*. Our studies confirm the necessity to scrutinize the genus *Achlya*. They show that the achlyoid sporangia discharge character apparently does not unite the species in one natural group. According to our results, it would be conceivable to divide the genus *Achlya* into two major groups: (*i*) the monophyletic eccentric species (cluster 8) and (*ii*) the remaining species of *Achlya* (clusters 5 and 6). Together with the genera *Aplanes* de Bary, *Aplanopsis* Hoehnk, and *Calyptralegnia* Coker and the *Saprolegnia–Scoliolegnia* complex, the species of the second group form a natural group within the Saprolegniales, which is well supported by a bootstrap value of 98%. This hypothesis is confirmed by the maximum parsimony analysis.

Cluster 5 contains *Achlya papillosa* Humphrey, *Achlya treleaseana* (Humphrey) Kauffman, and *Achlya spinosa* de Bary, as well as *Aplanes androgynus* (Archer) Humphrey, *Aplanopsis spinosa* Dick, and *Calyptralegnia achlyoides* (Coker & Couch) Coker. This group is statistically well supported by a bootstrap value of 96%. Many oogonia of the species of *Achlya* appearing in cluster 5 have an apiculus, including our isolate of *Calyptralegnia achlyoides*. Apiculi can be found in *Aplanopsis spinosa* and *Aplanes androgynus* as well. Dick (1995) considered the formation of an apiculus on the oogonium as a nonterminal differentiation of the oogonium, because a vegetative hypha is often present. He connected this phenomenon with the location where oogonia are formed; oogonia can be found in an intercalary position in many groups including the Pythiales, Saprolegniales, and Leptomitales. Hence, Dick (1995) did not consider the intercalary oogonia formation a good phylogenetic character. However, regarding the existence of apiculi in all the species of this cluster without considering the intercalary oogonia formation, this common character appears quite interesting here. The results of our analyses do not confirm the phenetic analysis by Powell and Blackwell (1998) in which *Calyptralegnia* and *Aplanes* occur in the same group as *Brevilegnia*, *Thraustotheca* Humphrey, and *Dictyuchus*.

In our analyses, the taxa *Achlya treleaseana* and *Aplanes androgynus* cluster in direct proximity together with *Achlya papillosa*, which points to a close relationship between the three taxa. This is very well supported by a bootstrap value of 100%. Moreover, the sequences of the examined DNA region were identical in *Achlya treleaseana* and *Aplanes androgynus*. The isolates of *Aplanes androgynus* examined by us very rarely have a basal papilla on their oogonia, whereas the whole oogonium of *Achlya treleaseana* is typically covered with papillae. Interestingly, the oogonium of an isolate

of *Achlya treleaseana* in Dick (1973, Plate 3, Fig. 37) is also portrayed with only one basal papilla. Since some representatives of the genus *Aplanes* have already been transferred to the genera *Saprolegnia* or *Achlya*, Sparrow (1960) believed that it may be possible to dissolve the genus *Aplanes*.

In the DNA region examined there are slight differences (0.2% each) in the sequences of the species *Achlya treleaseana* and *Achlya papillosa*, as well as between *Achlya papillosa* and *Aplanes androgynus*. *Achlya papillosa* differs morphologically from the other two species, for example by its unpitted oogonial wall. *Achlya treleaseana*, *Achlya spinosa*, and *Aplanes androgynus* were placed in the "*Aplanes* group" by Dick (1973), which was defined, among other characters, by the existence of aplanoid cysts in the zoosporangium germinating by hyphae. Therefore, the morphological classification by Dick (1973) is confirmed by our results.

In the maximum parsimony analysis, *Achlya spinosa* and *Aplanopsis spinosa* appear together in cluster 5 but do not form a common cluster. However, based on the topology of the neighbor-joining analysis, the two species appear to be closely related, a fact that is statistically supported by a bootstrap value of 85%. Asexual sporulation is unknown in *Aplanopsis spinosa*. The oogonial wall of the only other member of the genus *Aplanopsis*, *A. terrestris* Hoehnk, has a mucilaginous outer layer (Dick 1995). *Aplanopsis spinosa* differs from the other species of the Saprolegniales in the structure of its sexual organs, although the hyphal stucture suggests relatedness to Saprolegniaceae (Dick 1960*a*). Although their general position is unclear, species of *Aplanopsis* were assigned to the family of Saprolegniaceae by Dick (1995) by merit of the formation of usually more than one oospore per oogonium. To determine whether *Achlya spinosa* and *Aplanopsis spinosa* possess further common characters, the ultrastructure of the cell wall and oogonia of *Achlya spinosa* should be examined more carefully.

Johnson (1956) saw a problem in discriminating the species *Achlya cornuta* Archer, *Achlya stellata* de Bary and *Achlya spinosa* and therefore described them as doubtful species. Dick (1960*b*) considered *Achlya cornuta* synonymous with *Achlya stellata*; he considered *Achlya stellata* and *Achlya spinosa* to be distinct species. He pointed out the similarities between *A. spinosa* and *A. treleaseana*, which are confirmed in our phylogenetic hypotheses by the appearance of the two species in the same cluster (cluster 5).

Within cluster 5, *Calyptralegnia achlyoides* has an isolated position, which suggests that it may be a member of a distinct genus.

A further *Achlya* cluster (cluster 6) contains the "*racemosa* group" introduced by Dick (1973), with the species *Achlya radiosa* Maurizio, *Achlya colorata* Pringsheim, and *Achlya racemosa* Hildebrand, which is statistically well supported with a bootstrap value of 100%. The shape, the position and the origin of the antheridium are important taxonomic criteria (Dick 1995). The "*racemosa* group" is distinguished by the apical attachment of the antheridium to the oogonium. In the examined DNA region, there are only slight differences of 0.9% in the sequences of *A. racemosa* and *A. colorata*, which points to a close relationship between these two species, as already shown by Johnson (1956). They differ merely in the existence of an ornamented oogonial wall in *A. colorata*. Pringsheim in Fischer (1892) considered the synonymy of the two species.

According to Johnson (1956), *Achlya racemosa*, *Achlya colorata*, and *Achlya papillosa* are members of the subgenus *Centroachlya* (with centric oospores among other characters), whereas *Achlya radiosa* and *Achlya treleaseana* are members of the subgenus *Subcentrica* (with subcentric oospores among other characters). This classification is not consistent with the distribution of these species in clusters 5 and 6; hence, a classification based on the structure of oospores is not supported here. Dick (1969*b*) remarked that within the Saprolegniaceae all gradations exist between centric and eccentric types of oospore structure.

The third *Achlya* cluster (cluster 8) is statistically well supported by a bootstrap value of 98%. All species of *Achlya* in this cluster belong to the subgenus *Achlya*, which, according to Johnson (1956), is distinguished from the other subgenera by eccentric oospores among other characters. These species are also assigned to a common group by Dick (1973).

There are no differences in the sequences of the examined DNA region of *Achlya americana* Humphrey and *Achlya caroliniana* Coker. However, the two are listed in Johnson (1956) as distinct species based on morphological characters. For example, the zoosporangia of *Achlya caroliniana* often have lateral discharge tubes, which do not occur in *Achlya americana*. Furthermore, usually in *Achlya caroliniana* no antheridia are formed on the oogonia. Interestingly enough, in the DNA region studied, there are slight differences (0.5%) in the two sequences of *Achlya caroliniana* and *Achlya caroliniana* sensu Attaway.

In our phylogenetic hypotheses, *Thraustotheca clavata* (de Bary) Humphrey clusters within the eccentric species of *Achlya* (cluster 8). The close relationship between *Thraustotheca clavata* and the eccentric species of the genus *Achlya*, characterised by small genetic distances, could perhaps make the assignment of this species to *Thraustotheca* questionable. According to Dick (1973), one species of *Thraustotheca*, *T. primoachlya* Coker & Couch, belongs to *Achlya* sensu lato, which is supported by the results obtained by Green and Dick (1972) for GC content.

Cluster 9, which directly adjoins the cluster of the eccentric species of *Achlya* (cluster 8), contains species of *Brevilegnia* and *Dictyuchus*. These two clusters form a branch of their own, which is statistically supported by a bootstrap value of 90%. Dick (1973) already suspected that some of the genera with eccentric oospores (*Thraustotheca*, *Geolegnia* Coker, *Brevilegnia*) are more closely related to the *Achlya* subgenus of *Achlya* than species of the subgenera within *Achlya* are to one another. Consideration of sporangia discharge types is interesting as well. In the eccentric *Achlya dubia* Coker, achlyoid, thraustothecoid, and dictyoid zoosporangia discharge are possible. Furthermore, for *Brevilegnia bispora* Couch both the dominant brevilegnoid as well as the achlyoid zoosporangia discharge occur. Since the achlyoid zoosporangia discharge occurs before the brevilegnoid discharge, Langsam (1986) suggested that this species should be transferred to *Achlya*. However, the appearance of *B. bispora* in the cluster of the species of *Brevilegnia* and

Dictyuchus (cluster 9), optimally supported by a bootstrap value of 100%, confirms the assignment of this species to the genus *Brevilegnia*.

In the topologies of the neighbor-joining and maximum parsimony analyses, the species of the genus *Aphanomyces* form a cluster of their own together with *Pachymetra chaunorhiza* and *Plectospira myriandra* (cluster 7), which is well supported by a bootstrap value of 97%. *Aphanomyces stellatus* and *Aphanomyces laevis* appear on different branches. Species of the genus *Aphanomyces* are divided into three subgenera, depending upon the appearance of their oogonia walls (Scott 1961). *Aphanomyces stellatus*, according to Scott (1961), is placed in the subgenus *Asperomyces*, *Aphanomyces laevis* in the subgenus *Aphanomyces*. It would be necessary to sequence further species of the genus *Aphanomyces* to ascertain whether the appearance of species of *Aphanomyces* from different subgenera on different branches suggested here is confirmed. *Plectospira myriandra* clusters among the species of *Aphanomyces*. According to Drechsler (1927), the two genera are very closely related. In the phenetic analysis by Powell and Blackwell (1998) *Aphanomyces* and *Plectospira* Drechsler also appear in the same group.

Sclerosporales

The representative of the Sclerosporales, *Pachymetra chaunorhiza*, appears together with *Plectospira myriandra* in the well-supported cluster of *Aphanomyces* species (cluster 7) of the Saprolegniales, which suggests a close relationship between the three genera. Dick et al. (1989) noted similarities between the genera *Pachymetra* Croft & Dick and *Aphanomyces*; still they placed *Pachymetra*, together with *Verrucalvus* Wong & Dick and the Sclerosporaceae, in the Sclerosporales. Based on a restriction fragment analysis of rDNA, Klassen et al. (1988) also felt that the genus *Pachymetra* should be placed near the orders Saprolegniales and Leptomitales and not, as in Dick et al. (1984), near the orders Pythiales and Peronosporales. This is confirmed in our studies. The type species of the genus *Sclerospora* Schroeter, *S. graminicola* (Sacc.) Schroet., should be sequenced in further studies to get further insight into the position of the Sclerosporales.

As was already presumed by Dick (1988), our studies suggest that obligate parasitism has evolved independently several times: it occurs in the Peronosporales as well as in the Sclerosporales, which in our analyses represent different lineages (clusters 7 and 12).

Rhipidiomycetidae and Rhipidiales

Dick (1995), referring to analyses of rDNA restriction sites (Klassen et al. 1988), considered the Rhipidiales to be distant from both the Saprolegniomycetidae and the Peronosporomycetidae and positioned them in a third subclass, the Rhipidiomycetidae. This is consistent with the results of our research, in which the representative of the order Rhipidiales, *Sapromyces elongatus* (11), represents the sister group of the Saprolegniomycetidae in the topologies in Figs. 1–3. The grouping of the Saprolegniomycetidae and Rhipidiomycetidae is optimally supported by a bootstrap value of 100% (Fig. 2); however, more members of the Rhipidiomycetidae must be sequenced to derive a more meaningful hypothesis about the phylogeny of this group.

Peronosporomycetidae

According to our analyses, the Peronosporales, represented by species of the genera *Peronospora* Corda and *Plasmopara* Schroeter (cluster 12), cannot be separated from the Pythiales as a monophyletic group (clusters 12 and 13). The species of the genus *Phytophthora*, which belong to the Pythiales according to Dick (1995), are distributed all over the cluster of the Peronosporomycetidae. Hence, the classification of the Peronosporomycetidae in the orders Peronosporales and Pythiales as suggested by Dick (1995) is not supported.

Pythiales and Peronosporales

In the topology of the neighbor-joining analysis (Fig. 2), *Phytophthora undulata* (Petersen) Dick (*Pythium undulatum* before Dick's (1989) transfer) appears in the *Pythium* cluster (cluster 13), which is supported by a bootstrap value of 73%. This could speak in favour of a reclassification of this species into the genus *Pythium*. Then the species of the genus *Pythium* would appear as a separate cluster (cluster 13), and the species of the genus *Phytophthora* would form a common cluster (cluster 12) together with the representatives of the order Peronosporales. This cluster (cluster 12) is optimally supported by a bootstrap value of 100% and, furthermore, shows a large genetic distance to other groups in the neighbor-joining analysis. According to Shaw (1970), species that normally produce zoospores may also germinate by germ tubes, which has been reported for representatives of the genus *Phytophthora*, e.g., *P. parasitica* Dast. and for species of the genus *Plasmopara*, e.g., *P. viticola* (B. et C.) Berl. et de T. Species of *Pythium* are only known to form zoospores. In subsequent studies, it would be interesting to sequence further species of *Phytophthora* and *Pythium*, to find out, if *Phytophthora* and the Peronosporales truly form a common natural group.

In the topology of the maximum parsimony analysis (Fig. 3), the two species of *Pythium* do not appear as a separate cluster together with *Phytophthora undulata*, but as representatives of basal groups of the Peronosporomycetidae (clusters 13*a*, 13*b*, and 13*c*). However, here as well, the other species of *Phytophthora* form a monophyletic group (cluster 12) together with the representatives of the Peronosporales and hence support the results of the neighbor-joining analysis in this point.

Pythium undulatum was transferred to the genus *Phytophthora* as *Phytophthora undulata* by Dick (1989), since sometimes the zoospores are already differentiated in the sporangium. This was also observed by Goldie-Smith (1952) and in our own isolate. Nevertheless, in most cases, the spores of our isolate matured within a vesicle, a property which is used to define the genus *Pythium*. Dick's transfer was also consistent with the examinations of GC content by Belkhiri & Dick (1988). Furthermore, the diameter of zoospore cysts in *Phytophthora undulata* reaches the upper limit for species of the genus *Pythium* (Dick 1989). Remarkably, sexual stages have not been found in *Phytophthora undulata* (Dick 1989), and the dimensions of the sporangia are unusual (Plaats-Niterink 1981). In the examined DNA region,

there are differences between the sequences of *Phytophthora undulata* and the other species of *Phytophthora* ranging from 17.2 to 18.4%, which are in the range of differences between species of *Pythium* and *Phytophthora*. By contrast, the differences within the other species of *Phytophthora* lie between 1.9 and 3.5%. This fact also suggests that *Phytophthora undulata* belongs to the genus *Pythium*.

The fact that *Phytophthora megasperma* and *Phytophthora fragariae* Hickman are close species in the neighbor-joining analysis agrees well with the classification of the genus into six groups by Stamps et al. (1990), where these species are both representatives of the same group.

Conclusions

The majority of the results of our examinations are compatible with the classification of the Peronosporomycetes into subclasses and orders according to Dick (1995). Within the Saprolegniales, we were able to confirm the heterogeneity of the genus *Achlya* already described by Green and Dick (1972). The generic concept requires revision; dividing this genus into two major groups could be considered, as well as a transfer of *Thraustotheca clavata* to the genus *Achlya*. The orders Peronosporales and Pythiales could not be separated. There are indications that *Phytophthora undulata* (formerly *Pythium undulatum*) belongs to the genus *Pythium* and that *Phytophthora* and the Peronosporales form a common natural group.

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