A *cox2* phylogenetic hypothesis for the downy mildews and white rusts

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Mitochondrially encoded *cox2* sequences were used to infer evolutionary relationships of downy mildew and white rust taxa in a data set of 36 peronosporomycete isolates. The data set of 599 aligned nucleotides was analysed using neighbor joining, maximum parsimony, and maximum likelihood. These phylogenetic analyses did not infer separate monophyletic orders for the *Pythiales* versus downy mildews (*Peronosporales*), but do indicate that separation of monocotyledonus and dicotyledonous-infecting downy mildews into separate subclasses is not justified. Analyses of three species of *Albugo*, however, infer that *Peronosporales* are a polyphyletic group, unless this order is expanded to include species of the *Pythiales* and *Rhipidiales*. Whereas all examined downy mildew *cox*2 amino acid sequences bore the signature indel LEF/Y characteristic of the subclass Peronosporomycetidae the three *Albugo* species did not. Instead, the LEF/Y signature indel was replaced by a highly variable indel unique to each *Albugo* species. Collectively, these results indicate that the white rusts are only distantly related to downy mildews and constitute a distinct order basal to other orders within the Peronosporomycetidae.

Key words: *Albugo*, cytochrome *c* oxidase, *Peronosclerospora*, *Peronospora*, *Peronosporales*, *Sclerosporales*

Introduction

The fungal-like protists of the class Peronosporomycetes (= Oomycetes) within the newly erected Kingdom Straminipila (Dick, 2001) include a large number of diverse plant and animal parasites. A significant number of these have historically impacted the economics of agriculture, forestry, and aquaculture. Worldwide costs in agricultural crop disease control and yield reductions alone are in the billions of dollars per annum. Yet the evolutionary relationships of these organisms still remain problematic.

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Recent molecular studies have been used to analyse relationships within the Peronosporomycetes in attempts to resolve conflicting morphologically and/or biochemically based hypotheses. The resulting molecular phylogenetic hypotheses have been inferred from a variety of DNA sequence data including SSU-rDNA (Dick *et al*., 1999), LSU-rDNA (Riethmueller *et al*., 1999; Leclerc *et al*., 2000; Petersen and Rosendahl, 2000), and the mitochondrial *cox2* locus (Hudspeth *et al*., 2000; Cook *et al*., 2001). All the molecular studies strongly support the division of the Peronosporomycetes into at least two of the major subclasses initially proposed by Dick *et al*. (1984).

The Peronosporomycetidae is the most successful of the subclasses in terms of recognized species and economic impact. As currently constituted (Dick, 2001) this group includes in the order *Peronosporales* those obligate biotrophic genera responsible for both the downy mildews of dicotyledonous hosts and the white rusts. The typically facultative saprobes as well as the more specialized hemibiotrophic parasites are also included in this subclass, but within the less specialized, and presumably more primitive, order *Pythiales*. The *Sclerosporales*, consisting of the obligate biotrophic graminicolous downy mildews, however, have been assigned to the other major subclass, the Saprolegniomycetidae (Dick *et al*., 1989). Molecular analyses using various rDNA data sets (Riethmueller *et al*., 1999; Cooke *et al*., 2000; Rehmany *et al*., 2000; Petersen and Rosendahl, 2000) have all convincingly placed representative dicotyledonicolous downy mildews within a *Phytophthora* clade as polyphyletic members of the *Pythiales*, rather than retaining them in a distinct order (the *Peronosporales*). However, one of the more recently characterized graminicolous downy mildews, *Pachymetra*, has been placed in the Saprolegniomycetidae (Riethmueller *et al*., 1999) in support of the Dick *et al*. (1989) hypothesis.

Three of the rDNA studies (Cooke *et al*., 2000; Rehmany *et al*., 2000; Petersen and Rosendahl, 2000) also addressed the relatedness of the white rusts to other members of the Peronosporomycetidae by the inclusion of one or more isolates of *Albugo candida*. These analyses, however, could infer only that *Albugo* is distantly related to the downy mildew-*Phytophthora* clades, but still within the Peronosporomycetidae.

The aim of this study was to more precisely infer evolutionary relationships of the white rusts and both downy mildew groups in the broader context of the peronosporomycetous taxa present in the *cox2* data set. Specifically, we re-evaluated the separation of the graminicolous (sclerosporalean) and dicotyledonicolous (peronosporalean) downy mildews into different subclasses by examining representative downy mildews; and, we

more precisely inferred the evolutionary relationships of the white rusts within the Peronosporomycetidae by the inclusion of diverse species of *Albugo*.

Materials and methods

Isolates and media

The isolates and their sources included in this study are listed in Table 1. Axenic cultures were maintained on potato dextrose agar (Difco Laboratories, Troy, MI) and propagated for DNA preparation in aerated liquid peptone yeast glucose (PYG) medium (Griffin *et al*., 1974) at ambient temperatures. Spores of *Peronospora tabacina* were harvested from infected tobacco leaves by rinsing with sterile water and concentrating by centrifugation at 10,000 × g, 5 minutes. Pelleted spores were stored at -80ºC. Host tissues infected with *Pe. manshurica*, *Pe. parasitica*, *Peronosclerospora sorghi*, *Albugo candida*, *A. occidentalis*, and *A. tragopogonis* were mechanically trimmed to enrich for hyphae, sporangiophores or conidiophores, and sporangia or conidia. Tissues were then lyophilised, and stored at -80ºC.

Table 1. Peronosporomycete *cox2* sequences novel to this study.

Isolates	Host	Origin	Source
Albugo candida Kuntze	*Capsella*	DeKalb Co, IL	D. Stenger
Albugo occidentalis G.W. Wilson	Spinach	Texas	Black
Albugo tragopogonis Gray	*Helianthus*	South Africa	vanWyk
Peronosclerospora sorghi C.G. Shaw*	Sorghum	Texas A&M	Michelmore
Peronosclerospora sorghi C.G. Shaw	Sorghum	Mead, NE	Jensen
Peronospora manshurica Syd.	*Glycine*	South Africa	vanWyk
Peronospora parasitica Fr.	*Capsella*	DeKalb Co, IL	D. Stenger
Peronospora tabacina Adam	*Nicotiana*	Fayette Co, KY	Nesmith
Peronospora tabacina Adam	*Nicotiana*	Jackson Co, KY	Nesmith
Peronospora tabacina Adam **	*Nicotiana*	Univ. Ky	Nesmith
Pythium acanthicum Drechsler			ATCC 34036

*SDM pathotype III (obtained as DNA sample)
**KY79

Isolation of DNA and molecular techniques

Total DNA from axenic cultures was prepared from late log-phase mycelia as detailed in Hudspeth *et al*. (1983). All other DNAs were prepared essentially according to the protocol of White *et al*. (1990).

The *cox2* peronosporomycete specific primers and the conditions for PCR amplification from total DNA were as described (Hudspeth *et al*., 2000). Amplification products were assessed by electrophoresis on 1% agarose gels and prepared for sequencing using ExoSap (Stratagene, La Jolla, CA) according to the manufacturer's recommendations.

DNA sequence data were obtained using a Beckman Coulter CEQ 2000XL eight capillary automated DNA sequencer.

Sequence data management and phylogenetic analysis

Sequencing data files were organized and maintained using the PCGENE program group (Intelligenetics Inc., Mountain View, CA). Clustal X was used for preliminary multiple alignments of both nucleotide and amino acid sequences (Higgins *et al*., 1992). Final alignments were manually adjusted to ensure that codon alignments were maintained. For phylogenetic analysis each homologous sequence position was treated as a discrete character with four possible unordered states (G, A, T, or C). Gaps were treated as missing data. PAUPSTAR (test version 4.0.0d63) was used for maximum parsimony (MP), maximum likelihood (ML), and neighbor joining (NJ) analyses. Gaps (indels present as 3, 7, or 9 codons) were treated as missing data in the analyses. Recoding of the gaps as one of five character states and their addition to the nucleotide data set as an additional character (data not shown) did not change the topology of any of the inferred trees. Heuristic parsimony searches (with tree-bisection-reconnection branch swapping) were performed using 1000 replicates of random stepwise addition of taxa. Characters were treated as unordered and equally weighted data. Reported values for consistency indices exclude uninformative characters. The ML analysis was performed using the Kimura 2 parameter model of nucleotide substitution. Stepwise addition of taxa was used to build a starting tree followed by heuristic searches using TBR branch swapping. Bootstrap resampling was used to estimate the relative reliability of inferred groups for all analyses. *Hyphochytrium catenoides* was used as the outgroup in all analyses (Hudspeth *et al*., 2000).

Accession numbers

The nucleotide sequence data presented in this work have been deposited in GenBank as accession numbers AY286219-AY286229

Results

Identification of COII indels

The deduced cytochrome *c* oxidase subunit 2 (COII) amino acid sequences from the 11 peronosporomycete isolates examined in this work were aligned with those from the 24 taxa from Cook *et al*. (2001), plus *Phytophthora infestans* (Paquin *et al*., 1997; GenBank accession NC 002387). This allowed us to locate and identify the peronosporomycete signature COII indels (Hudspeth *et al*., 2000; Cook *et al*., 2001) and to provide the rationale for maintaining codon positions in subsequent nucleotide alignments. Both the graminicolous downy mildews, represented by the two *Peronosclerospora sorghi* isolates, and the dicotyledonicolous downy mildews (five *Peronospora* isolates) uniformly encoded the signature LEF COII tripeptide indel. This molecular character is retained as LEF/Y by all previously examined members of the Peronosporomycetidae. *Albugo* isolates, however, encoded a new set of longer and variable indels (FSFENED for *A. candida*, LNYFDNN for *A. tragopogonis*, and LNYFENTPD for *A. occidentalis*). These are notably unique in both size and sequence throughout the known deduced COII amino acid sequences of the Peronosporomycetes. No additional COII indels were identified by these alignments.

Analysis of nucleotide data

The data set for all 36 peronosporomycete isolates consisted of 599 total characters exclusive of PCR primers and indels. Alignment of the nucleotide positions was independently verified and manually adjusted when necessary to maintain codon positions. Of 380 variable nucleotide positions 286 were phylogenetically informative by parsimony criteria. The 50% majority rule consensus of only three MP trees found (CI = 0.379, tree length = 1522) is shown in Figure 1. The three MP trees differed only in the placement of *Halocrusticida okinawaensis* in a separate clade basal to the *Haliphthoros* clade, or the placement of *Atkinsiella dubia* basal to the subclass separation of the Saprolegniomycetidae and the Peronosporomycetidae.

Both graminicolous and dicotyledonicolous downy mildews along with the white rusts are included in a single modestly supported (67% bootstrap value) MP clade inclusive of Peronosporomycetidae taxa. There is no support for the inclusion of the downy mildews within a single monophyletic clade within the subclass, but there is strong support (89% bootstrap value in MP) for the separation of the white rusts from the downy mildews and other members

of the subclass. The *Albugo* spp. are placed as a basal clade in the Peronosporomycetidae, such that *Sapromyces elongatus* (sole representative of the order *Rhipidiales*) was inferred to share a more recent common ancestor with the downy mildews and taxa of the *Pythiales* than *Albugo*.

Topologies similar to the MP trees are also inferred from both NJ and ML analyses. In these two analyses strong bootstrap support (Fig. 1) is similarly provided for the subclass division which includes both groups of downy mildews in the Peronosporomycetidae. No support, however, is given for a monophyletic dicotyledonicolous downy mildew clade, since the clade containing the downy mildews also included other taxa of the *Peronosporales* and *Pythiales*. Finally, NJ and ML analyses also demonstrated strong support for a monophyletic white rust clade basal to all other members of the Peronosporomycetidae.

The relatively modest MP bootstrap value (67%) supporting separation of the Peronosporomycetes into the two major subclasses (Saprolegnomycetidae and Peronosporomycetidae) prompted an additional set of analyses in which *A. dubia*, the basal taxon of the Saprolegniomycetidae, was excluded. In the resulting MP analysis (data not shown) six parsimonious trees were found and bootstrap support for subclass separation rose to 83%. Separation of the Saprolegnomycetidae and Peronosporomycetidae was similarly supported by NJ (90% bootstrap) and ML (78% bootstrap) analyses (data not shown).

Discussion

Sequence data derived from the *cox*2 locus has permitted a re-evaluation of evolutionary relationships within the Peronosporomycetes. The inclusion of taxa representative of downy mildews infecting grasses or dicotyledonous hosts, and several species of white rusts has facilitated a refinement of the taxonomic status of these economically important obligate plant pathogens. Specifically, we addressed whether separation of the downy mildews into two subclasses corresponding to plant host taxa is justified, and whether the white rusts and downy mildews comprise a monophyletic grouping.

The downy mildews

Shaw (1978) initially proposed separation of the downy mildews into grass- and dicotyledon-infecting lineages in his analysis of *Peronosclerospora*. While his proposed graminicolous lineage was restricted to hosts of the *Poaceae*, the dicotyledon-infecting group also included taxa which infected

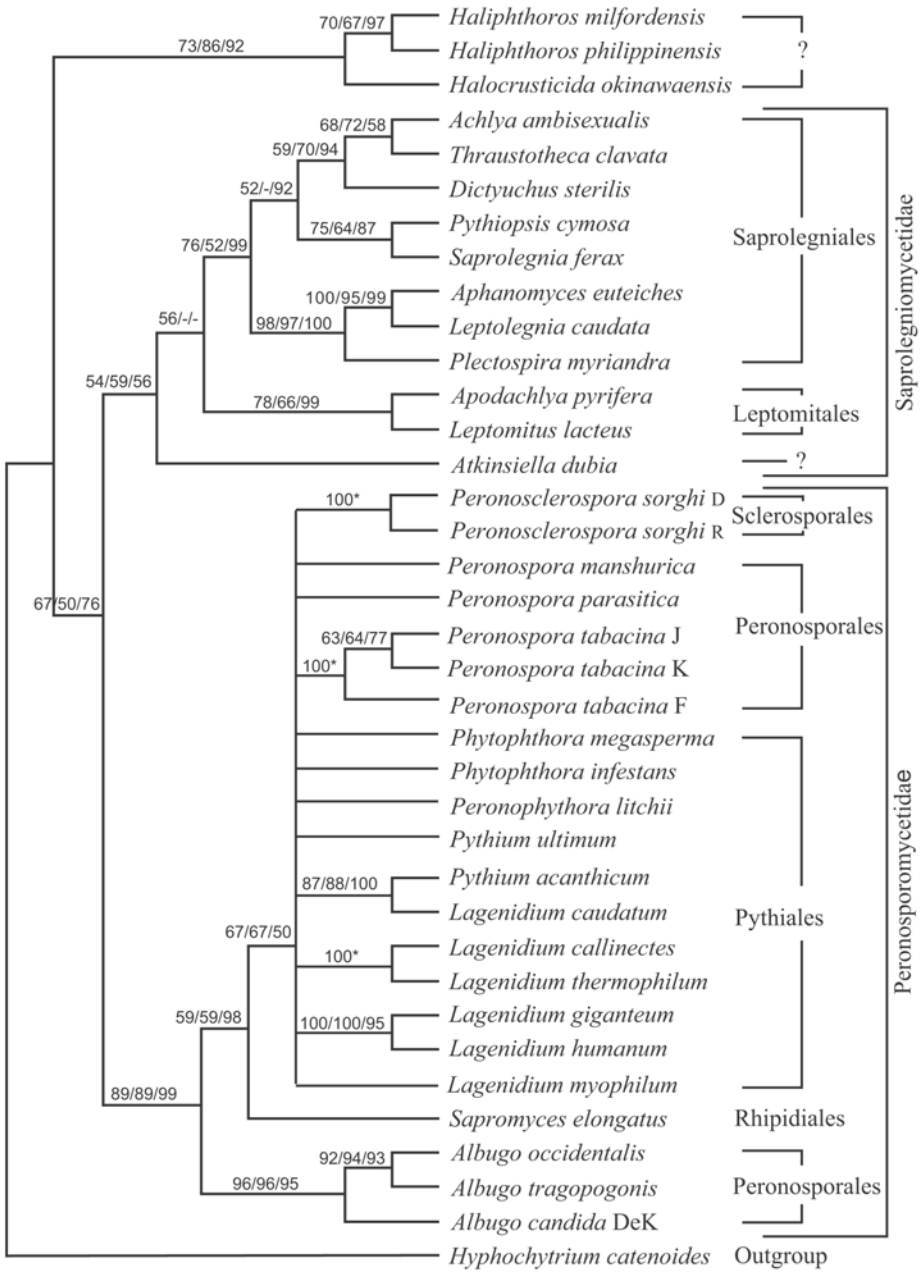

Fig. 1. The 50% majority rule consensus tree of three trees inferred by maximum parsimony using the *cox2* nucleotide data set. Bootstrap values over 50% are indicated at the nodes for maximum likelihood (500 replicates), MP (1000 replicates), and NJ (1000 replicates), respectively. The asterisk indicates equivalent values for all three analyses. Brackets indicate order and subclass designations. GenBank accession numbers for taxa not listed in Table 1 are given in Cook *et al*. (2001).

grasses. Both downy mildew lineages of Shaw (1978), along with the white rusts, were proposed to have segregated from a *Pythium-Phytophthora* line of development. Dick *et al*. (1984) formalized this separation by erecting the order *Sclerosporales* for the grass-infecting lineage, while retaining the other lineage, along with the white rusts, in the *Peronosporales*. Dick *et al*. (1984) also assigned the remaining Peronosporomycetidae taxa in the newly erected order *Pythiales*. Following the descriptions of *Verrucalvus* and *Pachymetra* (Dick *et al*., 1984; 1989) which possess some saprolegnean characters the *Sclerosporales* were transferred to the Saprolegniomycetidae. As currently constituted (Dick, 2001) the *Sclerosporales* consists of two families - the *Sclerosporaceae* (comprised of *Sclerospora*, *Peronosclerospora*, and *Sclerophthora*), and the *Verrucalvaceae* (comprised of *Verrucalvus* and *Pachymetra*).

Several recent molecular phylogenies (Riethmueller *et al*., 1999; Cooke *et al*., 2000; Petersen and Rosendahl, 2000) used either LSU-rDNA or ITS sequences to infer phylogenies involving representative downy mildews. These analyses clearly confirmed placement of the dicotyledonicolous genera *Peronospora* and *Plasmopara* in the Peronosporomycetidae. Riethmueller *et al*. (1999), however, also included a graminicolous taxon (*Pachymetra*), and in tentative support of the Dick *et al*. (1989) proposal, inferred a phylogeny with *Pachymetra* placed within the Saprolegniomycetidae.

The analyses presented here do not support the Dick *et al*. (1989) separation of grass- and dicotyledon-infecting downy mildews into the separate subclasses. Rather, based on both nucleotide and indel data, our analysis limit the inclusion of the examined taxa to the Peronosporomycetidae. Specifically, the *cox*2 phylogeny (Fig. 1) place a representative species of the *Sclerosporaceae* (*Peronosclerospora sorghi*) in the same subclass as three *Peronospora* spp. Likewise, conservation of the LEF indel independently supports retention of *Peronosclerospora* within the Peronosporomycetidae.

The taxonomic placement of the other family of grass-infecting downy mildews (*Verrucalvaceae*) was not addressed in this study, as *cox2* sequences for this taxon were not available for comparison. However, LSU-rDNA sequence data of Riethmueller *et al*. (1999) indicate *Pachymetra* to be allied with *Aphanomyces* in the Saprolegniomycetidae. Together, our analyses and that of Riethmueller *et al*. (1999) suggest that the *Verrucalvaceae* may be phylogenetically distinct from the *Sclerosporaceae*. Given the variable taxonomic placement of *Peronosclerospora* and *Pachymetra* based on phylogenetic analyses of different genes, we propose that the classical viewpoint of *Verrucalvaceae* as downy mildews may not be justified. Indeed, as parasites of roots rather than aerial tissues, both *Pachymetra* and its sister

taxon *Verrucalvus* are unique among the downy mildews. Furthermore, as it was saprolegnean characters of the *Verrucalvaceae* that were the impetus for transfer of the *Sclerosporales* to the Saprolegniomycetidae, the analyses of Riethmueller *et al*. (1999) and Dick *et al*. (1984; 1989) are likely correct to infer *Pachymetra* as a member of the Saprolegniomycetidae. An analysis of *cox2* sequences for *Pachymetra* and *Verrucalvus* (should samples become available) would be instrumental in confirming the sub-class placement of the *Verrucalvaceae*.

The white rusts

Our *cox2* analyses of *Albugo* infer placement of the *Albuginaceae* as the basal taxon in a clade that includes all other members of the Peronosporomycetidae. This placement is strongly supported by bootstrap values for both MP (89%) and ML (99%). Both analyses exclude the *Albuginaceae* from the *Pythiales* and the *Peronosporales*, and, surprisingly, infer a position basal to the *Rhipidiales*. This placement is independently supported by the presence in *Albugo* of unique indels in lieu of the LEF/Y signature indel conserved among the *Pythiales*, *Peronosporales*, *Sclerosporales*, and *Rhipidiales*.

Biotrophism and oospore morphology were key characteristics that have been used to place and retain the *Albuginaceae* in the *Peronosporales*. However, as noted by Shaw (1978) the asexual structures of *Albugo*, in which chains of sporangia are formed subepidermally prior to release by eruption through the host epidermal tissue, are decidedly unique and clearly differentiate the *Albuginaceae* from the downy mildews and other taxa of the *Peronosporales*. Our analyses support Shaw's contention that the unique asexual state of *Albugo* is indicative of an early separation of the *Albuginaceae* from the *Pythium-Phytophthora* lineage.

A basal position of *Albugo* within the Peronsporomycetidae also has been inferred from LSU-rDNA data (Riethmueller *et al*., 1999; Petersen and Rosendahl, 2000). However, it is the diversity of the *cox2* database presented here that allows exclusion from the *Pythiales* and *Peronosporales* and more precisely places *Albugo* basal to the *Rhipidiales*. As such, *Albugo* deserve consideration of elevation to the ordinal level. Clearly, the *Peronosporales*, as currently constituted to include both downy mildews and white rusts, is a polyphyletic assemblage in need of revision.

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Note: Similar conclusions were recently reported using LSU rDNA sequence data (Riethmuller et al (2002). Mycologia 94: 834-849).

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