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How do obligate parasites evolve? A multi-gene phylogenetic analysis of downy mildews

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Abstract

Plant parasitism has independently evolved as a nutrition strategy in both true fungi and Oomycetes (stramenopiles). A large number of species within phytopathogenic Oomycetes, the so-called downy mildews, are defined as obligate biotrophs since they have not, to date, been cultured on any artificial medium. Other genera like *Phytophthora* and *Pythium* can in general be cultured on standard or non-standard agar media. Within all three groups there are many important plant pathogens responsible for severe economic losses as well as damage to natural ecosystems. Although they are important model systems to elucidate the evolution of obligate parasites, the phylogenetic relationships between these genera have not been clearly resolved. Based on the most comprehensive sampling of downy mildew genera to date and a representative sample of *Phytophthora* subgroups, we inferred the phylogenetic relationships from a multi-gene dataset containing both coding and non-coding nuclear and mitochondrial loci. Phylogenetic analyses were conducted under several optimality criteria and the results were largely consistent between all the methods applied. Strong support is achieved for monophyly of a clade comprising both the genus *Phytophthora* and the obligate biotrophic species. The facultatively parasitic genus *Phytophthora* is shown to be at least partly paraphyletic. Monophyly of a cluster nested within *Phytophthora* containing all obligate parasites is strongly supported. Within the obligate biotrophic downy mildews, four morphologically or ecologically well-defined subgroups receive statistical support: (1) A cluster containing all species with brownish-violet conidiosporangia, i.e., the genera *Peronospora* and *Pseudoperonospora*; (2) a clade comprising the genera with vesicular to pyriform haustoria (*Basidiophora*, *Benua*, *Bremia*, *Paraperonospora*, *Plasmopara*, *Plasmoverna*, *Protobremia*); (3) a group containing species included in *Hyaloperonospora* and *Perofascia* which almost exclusively infect Brassicaceae; (4) a clade including the grass parasites *Viennotia oplismeni* and *Graminivora graminicola*. Phylogenetic relationships between these four clades are not clearly resolved, and neither is the position of *Sclerospora graminicola* within the downy mildews. Character analysis indicates an evolutionary scenario of gradually increasing adaptation to plant parasitism in Peronosporales and that at least the most important of these adaptive steps occurred only once, including major host shifts within downy mildews.

Keywords: β-Tubulin; Cytochrome oxidase; Downy mildews; LSU rDNA; NADH dehydrogenase; Obligate parasites; Oomycetes; Phylogenetic analysis; *Phytophthora*

1. Introduction

Students of plant pathogenic fungi are well aware that some fungal parasites can easily be cultivated on standard media whereas others cannot. Well-known examples for obligate parasitism in fungi include powdery mildews (Braun et al., 2002), rust fungi (Savile, 1976), and the dikaryotic stage of smut fungi (Bauer et al., 2001). If at all possible, elaborated techniques and highly specific conditions are necessary to induce growth of such fungi in culture (e.g., Hottson and Cutter, 1951). Downy mildews (Stramenopiles, Oomycetes, Peronosporales) and white rusts (Stramenopiles, Oomycetes, *Albugo* spp.) have also been

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reported not to grow in culture, except for biphasic host-tissue cultures (see references in Hall, 1996). As these taxa do not belong to true fungi and as their closest relatives are saprotrophs or facultative parasites, which are considered to be more primitive traits, obligate biotrophism must have independently evolved in Oomycetes.

Most morphological and ecological studies conclude that downy mildew species are highly host-specific (e.g., Gäumann, 1918, 1923; Gustavsson, 1959a,b), however many of these investigations have not been corroborated by sound experimental data (for a comprehensive review, see Hall, 1996). Recent molecular investigations in *Peronospora* (Voglmayr, 2003) and *Hyaloperonospora* (Choi et al., 2003; Göker et al., 2004) also indicate that downy mildews usually have rather narrow host ranges, although rare exceptions exist, e.g., in *Pseudoperonospora* (Choi et al., 2005). *Pythium* (Stramenopiles, Oomycetes, Pythiales) and *Phytophthora* (Stramenopiles, Oomycetes, Peronosporales) species, on the contrary, are closely related to downy mildews, but hardly host-specific (Erwin and Ribeiro, 1996). Many of them cause soil-born plant diseases, some of which may affect whole habitats (McDougall et al., 2003). Both genera can be cultivated on standard (*Pythium*) or adapted (*Phytophthora*) media (Erwin and Ribeiro, 1996). Hence, obligate biotrophism seems linked to increased host-specificity in these Oomycetes (Gäumann, 1964, pp. 69–70).

These two features may also be positively correlated with fungal speciation. Dick (2001) lists 137 binomials in *Pythium* and 95 in *Phytophthora*. However, number of epithets described in *Peronospora* (inclusive of *Hyaloperonospora* and *Perofascia* at that time) and *Plasmopara*, the two largest genera of downy mildews, are given as 623 and 174, respectively. Irrespective of these differences, *Pythium*, *Phytophthora*, and downy mildews all contain dangerous plant pathogens like, e.g., *Pythium* spp. causing root rot (Martin and Loper, 1999), *Phytophthora infestans*, the causal agent of late blight of potato (Erwin and Ribeiro, 1996), and *Plasmopara viticola*, the downy mildew of grapevine (Burruano, 2000).

Downy mildews and their relatives thus present an interesting model system for the evolution of modes of parasitism, host-specificity, and parasite diversity. However, reliable reconstructions of character evolution require robust phylogenies. Since taxonomically useful morphological or ecological characters are few in Oomycetes, they present a lot of difficulties for a natural classification. A couple of recent publications have applied modern methods of phylogenetic inference based on DNA sequence information to elucidate the evolutionary history of these organisms.

In an important pioneering contribution, Cooke et al. (2000) used nuclear *rDNA* internal transcribed spacer (*ITS*) sequences to investigate evolutionary relationships within *Phytophthora* and of *Phytophthora* to other Oomycetes. They concluded that *Phytophthora* is paraphyletic with respect to downy mildews, but the latter were represented by a single *Peronospora* sequence only. Cooke et al. (2002) came again to the same conclusion in their later study including a couple of *Peronospora* species, but no other downy mildew genera. Paraphyly of *Phytophthora* was corroborated by Göker et al. (2003) based on nuclear large ribosomal subunit (*LSU*) *rDNA* sequences, but only few *Phytophthora* sequences were considered. Voglmayr (2003) included obligate parasitic Peronosporales from four genera as well as a selection of *Phytophthora* species and performed Bayesian analyses which also indicated paraphyly of *Phytophthora*. However, this result could not be confirmed by maximum parsimony bootstrapping (Voglmayr, 2003), and parsimony consensus topology in the latter study was in disagreement with the trees presented in Cooke et al. (2000). Furthermore, the *ITS* tree and bootstrap analysis presented by Constantinescu and Fatehi (2002) were not in complete accordance with the results of Voglmayr (2003).

Based on *LSU rDNA*, the study of Göker et al. (2003) provided some evidence that obligate biotrophism evolved twice within the *Phytophthora*-downy mildew lineage, rendering the downy mildews polyphyletic. However, statistical support for this conclusion could only be obtained by Bayesian inference of phylogeny, but not by maximum likelihood bootstrapping. A number of recent publications showed, based on simulation (e.g., Suzuki et al., 2002; Douady et al., 2003; Erixon et al., 2003) or empirical (Simmons et al., 2004; Taylor and Piel, 2004) studies, that Bayesian analysis may severely overestimate branch support. Although the debate has not been settled so far (e.g., Huelsenbeck and Rannala, 2004), we conclude that phylogenetic relationships of downy mildew genera and *Phytophthora* could not be sufficiently clarified to date.

Literature remained quite controversial about whether an increase in the number of taxa (e.g., Wheeler, 1992; Zwickl and Hillis, 2002; Hillis et al., 2003; Bergsten, 2005) or the number of characters (e.g., Rosenberg and Kumar, 2001; Whelan et al., 2001; Rokas and Carroll, 2005) is more likely to increase resolution and topological accuracy in phylogenetic studies. To resolve origin and phylogenetic interrelationships of downy mildews, the present study used a twofold strategy of increased sampling. Initially, we considered more taxa to receive a more representative set of species within Peronosporales. In addition to *Halophytophthora*, which is comprised of marine species formerly assigned to *Phytophthora* (Ho and Jong, 1990), members of nearly all *Phytophthora* subgroups, as recognised by Cooke et al. (2000), were included. However, we could not obtain enough sequences for *Phytophthora arecae* (ITS clade 4 in Cooke et al., 2000). However, *Phytophthora arecae* appears to be closely related to *Phytophthora litchii* (Riethmüller et al., 2002; Voglmayr, 2003), which may hence be regarded as a substitute for clade 4. Furthermore, we were able to include all genera of Peronosporales as recognised by Dick (2001), including the more recent additions, i.e., *Sclerospora* (Riethmüller et al., 2002), *Hyaloperonospora* and *Perofascia* (Constantinescu and Fatehi, 2002), *Viennotia* (Göker et al., 2003), *Protobremia* (Voglmayr et al., 2004), *Plasmoverna* (Constantinescu et al., 2005), and *Graminivora* (Thines et al., 2006). *Pythium undulatum* and *Pythium monosper-*

mum were selected as outgroup taxa based on the results of Riethmüller et al. (2002). We also considered the somewhat aberrant *Pythium vexans* (Belkhiri and Dick, 1988; Cooke et al., 2000; Voglmayr, 2003; Lévesque and De Cock, 2004).

Second, we increased the number of molecular characters. Although *LSU rDNA* proved to be useful in inferring phylogenetic relationships of downy mildews and relatives (Riethmüller et al., 1999; Riethmüller et al., 2002; Göker et al., 2003; Voglmayr et al., 2004; Thines et al., 2006), this gene alone does by far not guarantee enough resolution. Based on the work of Hudspeth et al. (2003), we thus included the mitochondrial gene for Cytochrome c oxidase (*COX*) subunit 2 in the sampling. Additionally, we followed Kroon et al. (2004) and sequenced the mitochondrial gene for *NADH dehydrogenase* subunit 1. Furthermore, we developed primers to sequence the nuclear gene for *β-tubulin* based on published GenBank sequences.

Following the well-known "total evidence" approach (Kluge, 1989), we decided not to perform separate analyses of the different loci sequenced in the course of this study. Instead, we analysed the concatenated dataset under a variety of phylogenetic methods. Achieving the same results with different methods increases the probability that these results are not due to an artefact of the method like long-branch attraction (Felsenstein, 1978; Bergsten, 2005) or compositional heterogeneity (Steel et al., 2000; Phillips et al., 2004; Jermiin et al., 2004).

2. Materials and methods

The organisms included in this study, along with the Genbank accession numbers of the respective sequences, are listed in Table 1. *ITS* clade assignment of the *Phytophthora* species is according to Cooke et al. (2000). The classification system of downy mildews used is mainly as described in Riethmüller et al. (2002), but also includes some recent changes (Constantinescu and Fatehi, 2002; Göker et al., 2003; Voglmayr et al., 2004; Constantinescu et al., 2005; Thines et al., 2006). Morphological and ecological characters that appear on the simplified version of the phylogenetic trees summarising the main results were collected from literature (De Bary, 1876; Erwin and Ribeiro, 1996; Fraymouth, 1956; Constantinescu and Fatehi, 2002; Göker et al., 2003; Voglmayr et al., 2004) or directly observed microscopically as previously described (Göker et al., 2003). We selected the depicted characters (Fig. 3) which appeared uniquely derived and unreversed and/or were of main interest with respect to the evolution of obligate biotrophism. These characters as well as apparently more homoplasious characters are discussed in detail below.

DNA extraction, PCR, and sequencing of the fragments containing the D1-D2-D3 and D7-D8 regions of the nuclear large subunit *rDNA*, respectively (Larson, 1991; Hopple and Vilgalys, 1999), were done as previously described (Riethmüller et al., 2002; Göker et al., 2003). Regarding their length compared to the other genes sequenced, we will in the following refer to the two fragments of *LSU rDNA* as two different loci. For the amplification of *COX* 2, the forward (5′-GGCAAATGGGTTTT CAAGATCC) and reverse (5′-CCATGATTAATACCAC AAATTTCACTAC) primers of (Hudspeth et al., 2000) were used. *NADH* 1 amplification was done with the primers NADHF1 and NADHF2 of Kroon et al. (2004).

β-Tubulin fragments were obtained with a set of primers developed in our lab based on the sequences of *Achlya klebsiana*, *Pythium ultimum*, and *Phytophthora cinnamomi* published in Genbank Accession Nos. J05597, AF115397/AF218256, and U22050, respectively. These primers could be used in nested and semi-nested PCR approaches in different combinations: bTub136-OW (5′-CGCATCAAY GTRTACTACAAYG) and bTub292-OW (5′-GGTAAY AAYTGGGCCAARCG) as forward primers as well as bTub1005R-OW (5′-CGAAGTAYGACGARTTYTTG), bTub1024R-O (5′-CGAAGTACGAGTTCTTGTTC), bTub1048R-OW (5′-ATRTCACACACRCTGGCT), and bTub1064R-O (5′-TCACACACGCTGGCCTTG) as reverse primers.

Sequences of all fragments were aligned with MAFFT 5.532 (Katoh et al., 2002) using the FFT-NSi option, respectively --nj --maxiters = 1000. To obtain reproducible results, no further manual "corrections" or "adjustments" of the alignment were done in case of the two *LSU rDNA* fragments. Nucleotide alignment of the coding fragments was corrected according to the underlying amino acids with Se-Al v.2.0a11 (Rambaut, 1996) which was straightforward since alignment ambiguities were restricted to deletions of single amino acids. The aligned fragments were concatenated and positions with a large amount of leading or trailing gaps due to incomplete sequencing were excluded. The whole alignment and the trees computed as described below were deposited in Treebase (http://www.treebase.org/).

To obtain an appropriate model of site substitution for use in maximum likelihood searches, the data were analysed with Modeltest 3.6 (Posada and Crandall, 1998) in conjunction with PAUP* (Swofford, 2002). We chose the corrected Akaike information criterion (AICc) to distinguish between the different models as recommended by Posada and Buckley (2004). The best model of nucleotide site substitution was used to search for best trees and for bootstrapping under the maximum likelihood criterion (ML; Felsenstein, 1981). Five hundred bootstrap replicates were computed with the fast likelihood software PHYML 2.4.4 (Guindon and Gascuel, 2003).

In contrast to PHYML 2.4.4, TREEFINDER (Jobb et al., 2004; Jobb, 2005) is able to assign partition-specific substitution rates as additional parameters of the maximum likelihood model. We conducted searches for best likelihood trees and 200 bootstrap replicates in TREEFINDER with equal partition rates as well as optimisation of rates computed for five partitions (D1/D2/D3, D7/D8, *COX* 2, *β-tubulin*, *NADH* 1) or four partitions (non-coding sites as well as first, second, and third positions in triplets corresponding to amino acids), respectively. TREEFINDER as well as the χ^2 test, as imple-

Table 1
Collection data and GenBank accession numbers of the taxa and loci studied

Species	Collection No.	DNA isolation No.	Host	Collection data	*LSU*: D1/D2/D3	*LSU*: D7/D8	*Cox2*	*β-Tubulin*	*NADH*
* *Basidiophora entospora* Roze & Cornu	HV 123	HV 123	*Conyza canadensis* (L.) Cronquist	Austria, Niederösterreich, Krems, Langenlois; 22.04.1999; leg. HV (WU)	AY035513 *	AY273990 *	DQ365699	~	DQ361169
Benua kellermanii (Sacc.) Constant.	HV 2071	MG 42-9	*Iva xanthiifolia* Nutt.	Moldova, Lăpuşna, Chişinău; 16.6.1993; leg. GN (UPS, ex BUCM127.045)	DQ361226	~	DQ365700	~	DQ361170
Bremia graminicola Naumov	RK 995	AR 327	*Arthraxon hispidus* (Thunb.) Makino	China, Yunnan, Kunming; 2.8.2001; leg. RK (HOH 738)	DQ195167 ***	DQ195168 ***	DQ365702	~	~
* *Br. lactucae* Regel	HV 759	HV 759	*Cirsium oleraceum* (L.) Scop.	Austria, Oberösterreich, Schärding, St. Willibald; 11.11.2000; leg. HV (WU)	AY035507 *	AY273984 *	DQ365701	~	DQ361171
Halophytophthora batemanensis (Gerr.-Corn. & J.A. Simpson) H.H. Ho & S.C. Jong	~	MG 25-3, 33-5	~	CBS 679.84	DQ361227	DQ361246	DQ365703	DQ361105	~
Hyaloperonospora brassicae (Gäum.) Göker et al.	MG 1866	MG 14-3, 14-4	*Sinapis alba* L.	Germany, Baden-Württemberg, Tübingen; 26.10.2000; leg. MG (TUB)	AY035503 *	AY273974 *	DQ365704	DQ361106	DQ361172
H. erophilae (Gäum.) Göker et al.	MG 1961	MG 19-4	*Erophila verna* (L.) Chev.	Germany, Baden Württemberg, Criesbach; 28.04.2001; leg. MG (TUB)	AY271998 *	AY273972 *	DQ365705	~	DQ361173
	MG 1884	MG 17-6	*Erophila verna*	Germany, Baden-Württemberg, Niedernhall; 16.04.01; leg. MG (TUB)	~	~	~	DQ361107	~
H. lunariae (Gäum.) Constant.	MG 1946	MG 18-10, 34-6	*Lunaria rediviva* L.	Germany, Bayern, Munich; 11.05.2001; leg. MG (TUB)	AY271997 *	AY273970 *	~	DQ361108	DQ361174
	HV 364	MG 37-7	*Lunaria rediviva*	Austria, Niederösterreich, Lilienfeld; 07.05.2000; leg. HV (WU)	~	~	DQ365706	~	~
H. niessleana (Berlese) Constant.	MG 1843	MG 4-1	*Alliaria petiolata* (M. Bieb.) Cavara & Grande	Germany, Baden-Württemberg, Tübingen; 15.04.2000; leg. MG (TUB)	AY035498 *	AY273971 *	~	~	~
	MG 1865	MG 3-1	*Alliaria petiolata*	Germany, Baden-Württemberg, Füßbach; 08.04.00; leg. MG (TUB)	~	~	~	DQ361109	DQ361175
	MG 738	MG 2-12	*Alliaria petiolata*	Germany, Baden-Württemberg, Honau; 12.06.99; leg. MG (TUB)	~	~	DQ365707	~	~
H. parasitica (Persoon: Fries) Constant. s.l. 1	MG 1821	MG 5-8	*Cardamine hirsuta* L.	Germany, Nordrhein-Westfalen, Wuppertal; 23.04.2000; leg. MG (TUB)	AY035505 *	AY273975 *	DQ365708	~	DQ361176
	MG 1885	MG 17-8	*Cardamine pratensis* L.	Germany, Baden-Württemberg, Niedernhall; 08.04.01; leg. MG (TUB)	~	~	~	DQ361110	~
H. parasitica (Persoon: Fries) Constant. s.l. 2	MG 1939	MG 18-6, 34-5	*Cardamine impatiens* L.	Germany, Baden-Württemberg, Bebenhausen; 06.06.2001; leg. MG (TUB)	AY272000 *	AY273976 *	DQ365709	~	DQ361177
	MG 1840	MG 13-9	*Cardamine impatiens*	Austria, Tirol, Schattwald; 05.10.00; leg. MG (TUB)	~	~	~	DQ361111	~
* *H. parasitica* (Persoon: Fries) Constant. s.str.	MG 1964	MG 19-1	*Capsella bursa-pastoris* (L.) Medik.	Germany, Baden-Württemberg, Criesbach; 24.04.2001; leg. MG (TUB)	AY271996 *	AY273969 *	DQ365710	~	DQ361178
	MG 1878	MG 17-10	*Capsella bursa-pastoris*	Germany, Baden-Württemberg, Dußlingen/Kreßbach; 26.04.01; leg. MG (TUB)	~	~	~	DQ361112	~
H. thlaspeos-perfoliati (Gäum.) Göker et al.	MG 1882	MG 17-4	*Thlaspi perfoliatum* L.	Germany, Baden-Württemberg, Niedernhall; 08.04.2001; leg. MG (TUB)	AY271999 *	AY273973 *	~	DQ361113	~

	MG 1879	MG 17-9	*Thlaspi perfoliatum*	Germany, Baden-Württemberg, Öschingen; 26.04.01; leg. MG (TUB)	~	~	DQ365711	~	DQ361179
* *Paraperonospora leptosperma* (De Bary) Constant.	HV 383	HV 383	*Tripleurospermum perforatum* (Mérat) M. Lainz	Austria, Oberösterreich, Schärding, St. Willibald; 10.06.2000; leg. HV (WU)	AY035515 *	AY273989 *	DQ365712	~	DQ361180
* *Perofascia lepidii* (McAlpine) Constant.	HJ 2068/01	MG 22-9	*Lepidium ruderale* L.	Germany, Sachsen-Anhalt, Röden; 30.07.2001; leg. HJ (TUB)	DQ361228	~	DQ365713	~	~
	HJ 3189/01	MG 27-11	*Lepidium ruderale*	Germany, Sachsen-Anhalt, Wendelsheim; 22.09.2001; leg HJ (TUB)	~	DQ361247	~	DQ361114	DQ361181
Peronospora aestivalis Syd. in Gäum.	MG 1941	MG 18-4	*Medicago sativa* L.	Germany, Bavaria, Munich; 31.07.2001 leg. MG (TUB)	AY035482 *	AY273948 *	DQ365714	DQ361115	~
P. alpicola Gäum.	MG 1945	MG 18-9, 34-7	*Ranunculus aconitifolius* L.	Germany, Baden-Württemberg, Titisee; 18.05.2001; leg. MG (TUB)	AY271990 *	AY273953 *	DQ365715	DQ361116	DQ361182
P. alta Fuckel	MG 1831	MG 8-8	*Plantago major* L.	Germany, Baden-Württemberg, Tübingen; 29.06.2000; leg. MG (TUB)	~	~	~	DQ361117	DQ361183
	MG 1854	MG 8-9	*Plantago major*	Austria, Tirol, Schattwald; 05.10.2000; leg. MG (TUB)	AY035493 *	AY273962 *	DQ365716	~	~
P. aparines (De Bary) Gäum.	MG 1822	MG 4-5	*Galium aparine* L.	Germany, Baden-Württemberg, Tübingen; 16.04.2000; leg. MG (TUB)	AY035484 *	AY273955 *	DQ365717	~	DQ361184
P. aquatica Gäum.	MG 1968	MG 19-5, 19-6	*Veronica anagallis-aquatica* L.	Germany, Bayern, Birkenried near Günzburg; 18.07.2001; leg. MG (TUB)	AY271991 *	AY273956 *	DQ365718	DQ361118	DQ361185
P. arvensis Gäum.	MG 1871	MG 15-9, 15-10	*Veronica hederifolia* L.	Germany, Baden-Württemberg, Tübingen; 25.03.2000; leg. MG (TUB)	AY035491 *	AY273957 *	DQ365719	DQ361119	~
	MG 1856	MG 3-6	*Veronica hederifolia*	Germany, Baden-Württemberg, Criesbach; 08.04.00; leg. MG (TUB)	~	~	~	~	DQ361186
P. boni-henrici Gäum.	AR 167	MG 7-4	*Chenopodium bonus-henricus* L.	Germany, Bayern, Oberjoch; 02.07.1997; leg. MP (TUB)	AY035475 *	AY273952 *	DQ365720	~	DQ361187
P. calotheca De Bary	MG 1828	MG 6-2, 6-6	*Galium odoratum* (L.) Scop.	Germany, Baden-Württemberg, Tübingen; 16.05.2000; leg. MG (TUB)	AY035483 *	AY273960 *	DQ365721	DQ361120	DQ361188
P. conglomerata Fuckel	MG 1947	MG 18-11	*Geranium pyrenaicum* L.	Germany, Baden-Württemberg, Heidelberg; 21.05.2001; leg. MG (TUB)	AY271993 *	AY273961 *	DQ365723	DQ361122	DQ361189
P. hiemalis Gäum.	MG 1544	MG 4-4	*Ranunculus acris* L.	Germany, Baden-Württemberg, Tübingen; 15.04.2000; leg. MG (TUB)	AY271992 *	AY273958 *	DQ365724	DQ361123	DQ361190
P. lamii A. Braun	MG 1867	MG 14-1, 14-2	*Lamium purpureum* L.	Germany, Baden-Württemberg, Tübingen; 26.10.2000; leg. MG (TUB)	AY035494 *	AY273968 *	DQ365725	DQ361124	DQ361191
P. potentillae-sterilis Gäum.	MG 1833	MG 14-5, 14-6	*Potentilla sterilis* (L.) Garcke	Germany, Baden-Württemberg, Tübingen; 02.11.2000; leg. MG (TUB)	AY035486 *	AY273967 *	DQ365726	DQ361125	DQ361192
P. pulveracea Fuckel	MG 1763	MG 9-5, 9-6	*Helleborus niger* L.	Austria, Styria, Mariazell; 12.07.2000; leg. WM (TUB)	AY035470 *	AY273959 *	DQ365727	DQ361126	DQ361193
* *P. rumicis* Corda	HV 300	HV 300	*Rumex acetosa* L.	Austria, Oberösterreich, Schärding, Kopfing; 25.04.2000; leg. HV (WU)	AY035476 *	AY273951 *	DQ365728	DQ361127	DQ361194
P. sanguisorbae Gäum.	MG 1839	MG 12-6	*Sanguisorba minor* Scop.	Austria, Tirol, Schattwald; ?.08.2000; leg. MG (TUB)	AY035487 *	AY273954 *	~	~	~
	MG 1799	MG 12-1, 12-2	*Sanguisorba officinalis* L.	Germany, Baden-Württemberg, Hirschhorn; 23.09.00; leg. MG (TUB)	~	~	DQ365729	DQ361128	DQ361195
P. sordida Berk. et Broome	MG 1943	MG 18-7, 18-8, 45-6	*Scrophularia nodosa* L.	Germany, Baden-Württemberg, Heidelberg; 21.05.2001; leg. MG (TUB)	AY271995 *	AY273964 *	DQ365730	DQ361129	DQ361196

(*continued on next page*)

Table 1 (*continued*)

Species	Collection No.	DNA isolation No.	Host	Collection data	*LSU*: D1/D2/D3	*LSU*: D7/D8	*Cox2*	*β-Tubulin*	*NADH*
P. trifolii-alpestris Gäum.	MG 1771	MG 10-9, 10-10	*Trifolium alpestre* L.	France, Le Bout du Monde; 26.07.2000; leg. MG (TUB)	AY271989 *	AY273946 *	DQ365731	DQ361130	~
	MG 1798	MG 13-1	*Trifolium alpestre*	Germany, Baden-Württemberg, Tübingen; 02.06.00; leg. FO (TUB)	~	~	~	~	DQ361197
P. trifolii-repentis Sydow	AR 226	MG 16-9	*Trifolium repens* L.	Austria, Tirol, Tannheim; 30.09.2000; leg. AR (TUB)	AY271988 *	AY273945 *	DQ365732	DQ361131	DQ361198
P. cf. *trifoliorum* De Bary	MG 1797	MG 10-7, 10-8	*Trifolium* cf. *medium* L.	France, Mont Blanc; 28.07.2000; leg MG (TUB)	AY035478 *	AY273947 *	DQ365722	DQ361121	~
P. trivialis Gäum.	MG 1803	MG 6-4, 6-8	*Cerastium fontanum* Baumg.	Germany, Baden-Württemberg, Niedernhall; 30.04.2000; leg. MG (TUB)	AY035471 *	AY273950 *	DQ365733	DQ361132	~
P. variabilis Gäum.	MG 1651	MG 8-6, 8-7	*Chenopodium album* L.	Germany, Baden-Württemberg, Tübingen; 16.06.2000; leg. MG (TUB)	AY035477 *	AY273949 *	DQ365734	DQ361133	DQ361199
P. verna Gäum.	MG 1969	MG 17-7	*Veronica arvensis* L.	Germany, Baden Württemberg, Niedernhall; 08.04.2001; leg. MG (TUB).	AY271994 *	AY273963 *	DQ365735	DQ361134	~
Phytophthora boehmeriae Sawada	~	MG 42-6	~	CBS 291.29	DQ361229	DQ361248	DQ365736	DQ361135	DQ361200
Ph. cactorum (Lebert & Cohn) J. Schröt.	~	MG 25-7, 34-2	~	CBS 279.37	DQ361230	DQ361249	DQ365737	DQ361136	DQ361201
Ph. cambivora (Petri) Buisman	~	AR 245, MG 33-1, 41-2	~	IMI 340630	DQ361231	DQ361250	DQ365738	DQ361137	DQ361202
Ph. capsici Leonian	~	AR 244	~	IMI 352321	DQ361232	DQ361251	DQ365739	DQ361138	DQ361203
Ph. drechsleri Tucker	~	MG 25-4, 34-1, 41-4	~	CBS 359.52	DQ361233	DQ361252	DQ365740	DQ361139	DQ361204
Ph. fragariae Hickman	~	MG 40-4	~	CBS 309.62	DQ361234	DQ361253	DQ365741	DQ361140	~
Ph. heveae A.W. Thomps.	~	MG 25-8, 42-3	~	CBS 269.29	DQ361235	DQ361254	DQ365742	DQ361141	DQ361205
* *Ph. infestans* (Mont.) De Bary	~	AR 69	~	CBS 560.95	AF119602 *	AY273991 *	DQ365743	DQ361142	~
Ph. insolita Ann & W.H. Ko	~	MG 25-2, 33-8, 41-3	~	CBS 691.79	DQ361236	DQ361255	DQ365744	DQ361143	DQ361206
Ph. lateralis Tucker & Milbrath	~	MG 33-7	~	CBS 168.42	DQ361237	DQ361256	DQ365745	DQ361144	DQ361207
Ph. litchii (= *Peronophythora litchii* W.H. Ko et al.)	~	AR 178, MG 33-3	~	CBS 100.81	AY035531 *	AY273993 *	DQ365746	DQ361145	DQ361208
Ph. megasperma Drechsler	~	MG 42-1	~	CBS 402.27	DQ361238	DQ361257	DQ365747	DQ361146	DQ361209
Ph. multivesiculata Ilieva et al.	~	AR 239, MG 33-6	~	CBS 545.96	DQ361239	DQ361258	DQ365748	DQ361147	DQ361210
Ph. nemorosa E.M. Hansen & Reeser	~	MG 42-7	~	CBS 1148.70	DQ361240	DQ361259	DQ365749	DQ361148	DQ361211
Ph. nicotianae Breda de Haan	~	AR 238	~	CBS 305.29	DQ361241	DQ361260	DQ365750	DQ361149	DQ361212
Ph. quercina T. Jung	~	MG 25-6, 34-3, 40-6	~	CBS 768.95	DQ361242	DQ361261	DQ365751	DQ361150	DQ361213
Ph. richardiae Buisman	~	MG 42-2	~	CBS 240.30	DQ361243	DQ361262	DQ365752	DQ361151	DQ361214
Ph. sojae Kaufm. & Gerd.	~	MG 25-5, 34-4	~	CBS 312.62	DQ361244	DQ361263	DQ365753	DQ361152	DQ361215
Plasmopara baudysii Scalický	HV 571	HV 571	*Berula erecta* (Huds.) Coville	Austria, Niederösterreich, Gramatneusiedl; 02.08.2000; leg. HV (WU)	AY035517 *	AY273985 *	~	DQ361153	DQ361216
Pl. densa (Rab.) J. Schröt.	MG 1823	MG 6-1	*Rhinanthus alectorolophus* (Scop.) Poll.	Germany, Baden-Württemberg, Tübingen; 18.05.2000; leg. MG (TUB)	AY035525 *	~	~	~	~

	HV 2209	MG 39-8, 45-9	*Rhinanthus minor* L.	Austria, Niederösterreich, Mödling, Gießhübl; 15.05.2005; leg. HV (WU)	~	AY273983 *	~	DQ361154	DQ361217
	MG 686	MG 1-6	*Rhinanthus alectorolophus*	Germany, Baden-Württemberg, Neckartailfingen; XX.04.1999; leg. RB (TUB)	~	~	DQ365754	~	~
Pl. megasperma (Berl.) Berl.	HV B.M. 4.4	HV B.M. 4.4, MG 39-4	*Viola rafinesquii* Greene	USA, Tennessee, Knoxville; 04.04.2000; leg. HV (WU)	AY035516 *	AY273981 *	DQ365755	~	DQ361218
* *Pl. nivea* (Unger) J. Schröt.	MG 1829	MG 7-2	*Aegopodium podagraria* L.	Germany, Baden-Württemberg, Tübingen-Bebenhausen; 23.04.2000; leg. AR (TUB)	AF119604 *	AY273982 *	DQ365756	~	~
	HV 1012	MG 45-5	*Aegopodium podagraria*	Austria, Oberösterreich, Schärding, Raab; 15.09.2001; leg. HV (WU)	~	~	~	DQ361155	~
Pl. obducens (J. Schröt.) J. Schröt.	HV 306	HV 306	*Impatiens noli-tangere* L.	Austria, Oberösterreich, Schärding, Kopfing; 25.04.2000; leg. HV (WU)	AY035522 *	AY273980 *	DQ365757	DQ361156	~
Pl. pimpinellae O. Savul.	HV 634	HV 634	*Pimpinella major* (L.) Huds.	Austria, Tirol, Lienz, Obertilliach; 27.08.2000; leg. HV (WU)	AY035519 *	AY273988 *	DQ365758	~	~
	HV 635	MG 45-2	*Pimpinella major*	Austria, Tirol, Lienz, Obertilliach; 27.08.2000; leg. HV (WU)	~	~	~	DQ361157	~
Pl. pusilla (De Bary) J. Schröt.	MG 1861	MG 8-10	*Geranium pratense* L.	Germany, Baden-Württemberg, Tübingen; 27.06.2000; leg. MG (TUB)	AY035521 *	AY273979 *	DQ365759	DQ361158	~
Pl. viticola (Berk. & M. A. Curtis) Berl. & De Toni	MG 1751	MG 11-4, 11-5	*Vitis vinifera* L.	Germany, Baden-Württemberg, Tübingen; ?.08.2000; leg. MW (TUB)	AY035524 *	AY273978 *	DQ365760	DQ361159	DQ361219
* *Plasmoverna pygmaea* (Ung.) Constant. et al.	AR 86	AR 86	*Anemone ranunculoides* L.	Germany, Baden-Württemberg, Tübingen-Bebenhausen; 24.04.1998; leg. AR (TUB)	~	AY273986 *	~	DQ361160	DQ361220
	MG 1846	MG 4-6	*Anemone ranunculoides*	Germany, Baden-Württemberg, Tübingen; 16.04.00; leg. MG (TUB)	AF119605 *	~	DQ365761	~	~
Protobremia sphaerosperma (Savul.) Voglmayr et al.	HV 1050	MG 36-4	*Tragopogon orientalis* L.	Austria, Niederösterreich, Mödling, Gießhübl; 30.05.2002; leg. HV (WU)	AY250150 **	~	DQ365762	~	DQ361221
	HV 2118	MG 45-1	*Tragopogon orientalis*	Austria, Wien, 14th District, Halterbachtal; 02.05.2004; leg. HV (WU)	~	DQ361264	~	DQ361161	~
Pseudoperonospora humuli (Miyabe & Takah.) G. W. Wilson	HV 129	HV 129	*Humulus lupulus* L.	Austria, Niederösterreich, Krems, Langenlois; 22.04.1999; leg. HV (WU)	AY035496 *	AY273965 *	DQ365763	DQ361162	DQ361222
Ps. urticae (Libert ex Berk.) E. S. Salmon & Ware	HV 713	HV 713	*Urtica dioica* L.	Austria, Oberösterreich, Schärding, St. Willibald; 01.10.2000; leg. HV (WU)	AY035497 *	AY273966 *	DQ365764	DQ361163	~
* *Pythium monospermum* Pringsh.	~	AR 213, MG 40-10	~	Culture collection Reading, UK, strain no. 4114a	AY035535 *	AY273995 *	DQ365765	DQ361164	DQ361223
Py. undulatum H. E. Petersen	AR 207	AR 207, MG 33-2, 40-11	~	Culture collection Reading, UK, strain no. APCC 4701b	AF119603 *	AY273994 *	DQ365766	DQ361165	DQ361224
Py. vexans De Bary	~	MG 25-1, 42-4	~	CBS 339.29	DQ361245	DQ361265	DQ365767	DQ361166	~
* *Sclerospora graminicola* (Sacc.) J. Schröt.	HV 532	HV 532	*Setaria viridis* (L.) P. Beauv.	Austria, Niederösterreich, Wr. Neustadt/Land, Theresienfeld; 27.07.2000; leg. HV (WU)	AY035514 *	AY273987 *	DQ365768	DQ361167	DQ361225
* *Viennotia oplismeni* (Vienn.-Bourg.) Göker et al.	HV 11	HV 11, MG 45-11	*Oplismenus hirtellus* (L.) Beauv.	Africa, Guinea, Kindia; leg. JK (GZU)	AY035527 *	AY273977 *	DQ365769	DQ361168	~

Acronyms of collectors: AR, Alexandra Riethmüller; FO, Franz Oberwinkler; GN, G. Negrean; HJ, Herrmann Jage; HV, Hermann Voglmayr; JK, J. Kenneth; MG, Markus Göker; MP, Meike Piepenbring; MW, Michael Weiß; RB, Robert Bauer; RK, Roland Kirschner; WM, Wolfgang Maier. Vouchers: BUCM, Institute of Botany, Bucuresti; GZU, University of Graz; HOH, University of Hohenheim; TUB, University of Tübingen; UPS, University of Uppsala; WU, University of Vienna. Type species are marked with an asterisk. One asterisk after the accession number indicates that the sequence was obtained by Göker et al. (2003); two asterisks, by Voglmayr et al. (2004); three asterisks, by Thines et al. (in press). All other sequences were obtained in the course of the present study.

mented in PAUP* (BASEFREQS command), was used to test for base composition heterogeneity between the sequences which may strongly disturb phylogenetic analyses as it violates the assumptions of most current algorithms (e.g., Phillips et al., 2004).

PAUP* was used to conduct heuristic searches under the maximum parsimony criterion (MP; e.g., Fitch, 1971). Equal costs were assigned to all changes and sites; gaps were treated as missing data. Thousand rounds of random sequence addition and subsequent TBR branch swapping (MULTREES option in effect, STEEPEST option not in effect) were applied, collapsing branches if it was possible for them to have zero length (PSET COLLAPSE = MINBRLEN). After excluding uninformative characters, parsimony bootstrap analysis with 1000 replicates (Felsenstein, 1985) was performed by 10 rounds of random sequence addition and subsequent TBR branch swapping during each bootstrap replicate. The retention index (Farris, 1989) was also computed with PAUP* based on the most parsimonious tree found.

Based on maximum likelihood estimates of pair-wise distances computed under the best substitution model and the best parameter estimates found as described above, a BIONJ (Gascuel, 1997) tree was computed with PAUP*. As Holland et al. (2002) remarked, the best-fit maximum likelihood model needs not result in pair-wise distance estimates optimal for phylogenetic inference with neighbour-joining or other distance methods. Since most distance methods are guaranteed to infer the correct tree from completely additive distances, one could instead use the substitution model resulting in the distance matrix with the least departure from additivity to infer the tree (Swofford et al., 1996, p. 458; Holland et al., 2002) and use the best ML model only to estimate branch lengths. Holland et al. (2002) computed a "Delta Value" from each distance matrix which is minimal (0) in the optimal case. Here, we used PAUP∗ in conjunction with DeltaStats, a Python script written by B. Holland (pers. comm.) to compute the Delta Values for all substitution models considered by Modeltest 3.6. As a third distance approach, log-determinant (LogDet; Swofford et al., 1996, pp. 459–461) distances were computed with PAUP* after excluding constant sites (Steel et al., 2000). In each case, BIONJ bootstrapping was done with 1000 replicates.

RRTree (Robinson et al., 1998; Robinson-Rechavi and Huchon, 2000) was used to compare substitution rates between downy mildews and *Phytophthora*. RRTree was run with K2P distances and both with and without topological weighting based on the PhyML tree; the *Pythium* species were used as the outgroup.

3. Results

3.1. Sequencing, DNA alignment, and substitution model

LSU rDNA sequences covering D1, D2, and D3 were obtained for all 72 species under study. The D7/D8 part was lacking for one taxon only, as did the *COX* 2 region. The *β-tubulin* locus could not be sequenced for eight taxa, whereas *NADH* 1 sequencing did not work in 15 species. These sequencing difficulties are most likely due to suboptimal preservation of herbarium material. However, at least four of the five loci examined could be sequenced for all species except *Graminivora graminicola* and *Benua kellermanii* for which only three loci were obtained. Further details of sequencing results are found in Table 1.

After exclusion of sites with a large amount of leading or trailing gaps from the individual alignments, 3921 nucleotide sites remained in the concatenated dataset, 1672 of which were variable, and 1314 of which were parsimony-informative; see Supplementary Data for further information. A compositional heterogeneity check conducted with TREEFINDER revealed that deviations from mean nucleotide composition were lower than 10% and should not be a matter of concern, a result which was confirmed by PAUP*'s built-in χ^2 test.

The AICc criterion as implemented in Modeltest suggested GTR + I + G as an appropriate model with very low selection uncertainty as judged by its Akaike weight of 1.000 (Posada and Buckley, 2004; see Swofford et al., 1996 or Felsenstein, 2004 for an introduction to DNA substitution models). DeltaStats, on the other hand, suggested a model of much lower complexity, SYM, as best suited for distance analysis since it resulted in the lowest Delta value (0.3315; Delta value under GTR + I + G was 0.3456).

3.2. Maximum likelihood analyses

The likelihood tree inferred with PHYML 2.4.4 under a single GTR + I + G substitution model together with support values from 500 bootstrap replicates is shown in Fig. 1. The picture also indicates bootstrap values from the three analyses conducted with TREEFINDER. These analyses are based on GTR + G instead of GTR + I + G as recommended in the TREEFINDER manual (Jobb, 2005) for gamma value estimates below 1. Furthermore, TREEFINDER consistently estimated a negligible proportion of invariable sites in preliminarily analyses in which GTR + I + G was used (data not shown).

Likelihood of the best tree found was highest ($-\ln L = 48298.15$) if partitioning into non-coding parts as well as first, second, and third triplet positions was applied. Splitting the dataset according to the different loci was not optimal, as it resulted in a lower likelihood value ($-\ln L = 48999.19$), although more parameters had to be estimated (five instead of four partitions). Best likelihood values obtained without partitioning were even lower ($-\ln L = 49606.67$ obtained with PHYML, $-\ln L = 49780.64$ obtained with TREEFINDER). Irrespective of these differences between models and partitioning applied, the four likelihood approaches resulted in nearly identical topologies and, as evident from Fig. 1, nearly identical branch support values. The following description of ML results is therefore based on the analysis conducted with PHYML.

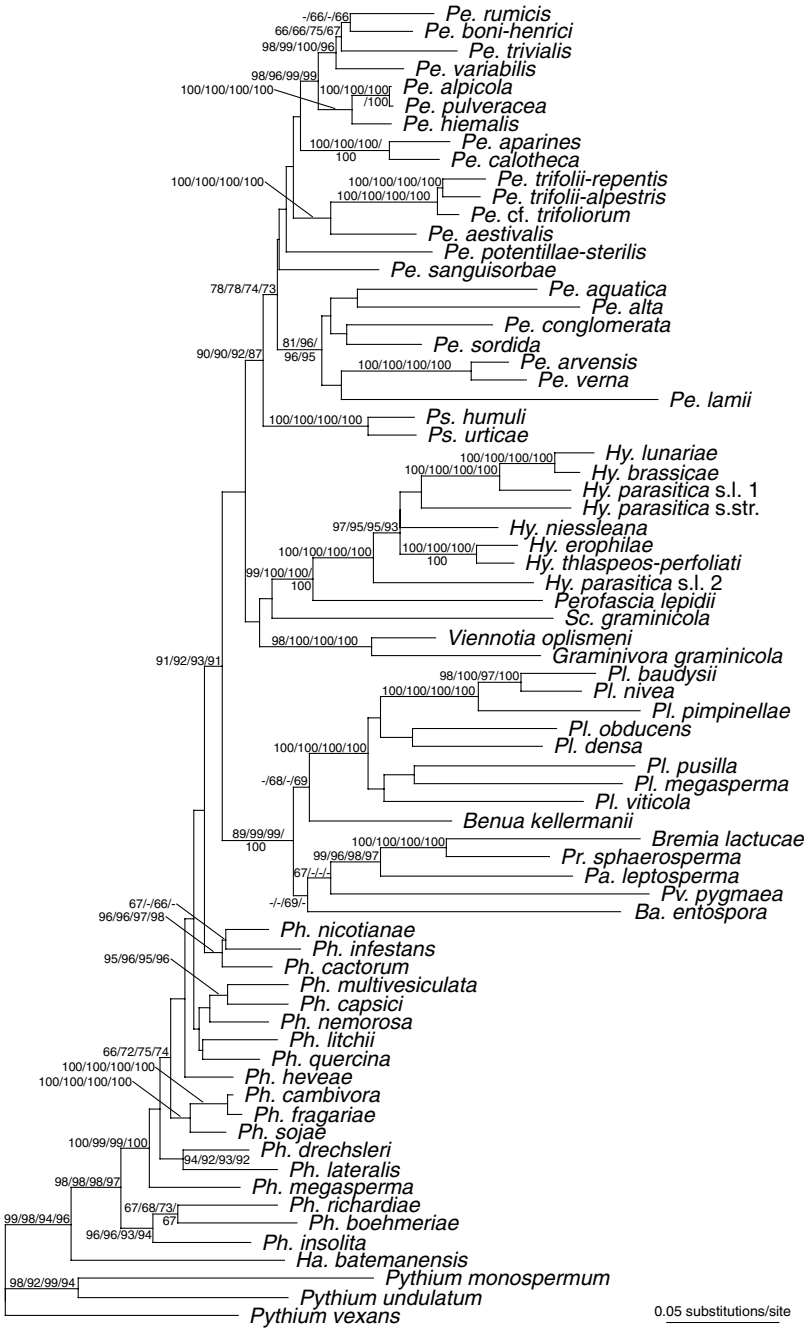

Fig. 1. Maximum likelihood phylogram computed with PHYML under a GTR + I + G model of site substitution. The tree is rooted so as to indicate that we remain agnostic with respect to taxonomic status of *Pythium*, because it cannot be inferred from the present taxon sampling. The *Pythium* species included could be either regarded as paraphyletic and divided into two or three subgroups, or as monophyletic. Numbers above branches denote bootstrap support values higher than 65% from 500 (PHYML) or 200 (TREEFINDER) replicates. Dashes indicate support lower than 65%. Bootstrap values from left to right: PHYML, GTR + I + G, one single partition; TREEFINDER, GTR + G, one single partition; TREEFINDER, GTR + G, four partitions (non-coding loci as well as first to third codon positions); TREEFINDER, GTR + G, five partitions, according to the five loci. Abbreviations: *Ba.*, *Basidiophora*; *Ha.*, *Halophytophthora*; *Hy.*, *Hyaloperonospora*; *Pa.*, *Paraperonospora*; *Pe.*, *Peronospora*; *Ph.*, *Phytophthora*; *Pl.*, *Plasmopara*; *Pv.*, *Plasmoverna*; *Ps.*, *Pseudoperonospora*; *Sc.*, *Sclerospora*.

Fig. 1 shows the ML tree inferred with PHYML together with branch support values above 65%, which were obtained by bootstrapping the differently partitioned dataset. Strong (99%) support is achieved for a bipartition of the taxa into the *Pythium* species on the one hand and all remaining taxa on the other hand. Furthermore, a bootstrap value of 98% clearly supports a bipartition of the taxa into *Pythium undulatum* and *Pythium monospermum* in one group and all remaining species in the other. We thus rooted the tree in a way to indicate that we remain agnostic with respect to the taxonomic status of *Pythium*, because it cannot be inferred from our sampling. In the present

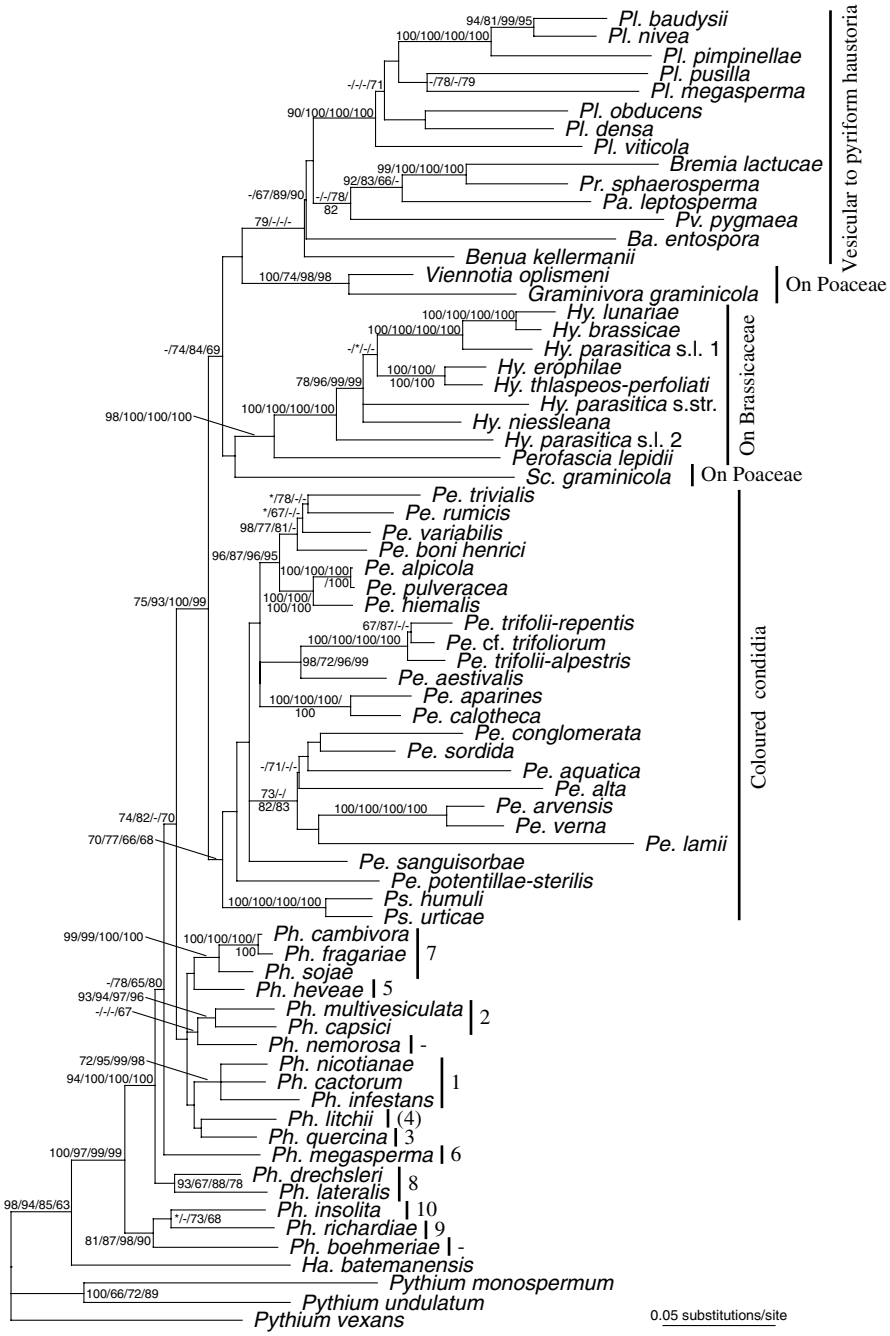

Fig. 2. BIONJ topology obtained with SYM as substitution model. Rooting is as in Fig. 1. Branch lengths were estimated with PAUP* under ML and a GTR + I + G model of site substitution. Numbers above branches denote bootstrap support values from 1000 replicates conducted with PAUP*. Dashes indicate support lower than 65%; stars indicate support for a conflicting grouping higher than 65%. Boostrap values from left to right: heuristic search under unweighted maximum parsimony; BIONJ based on pair-wise distances estimated under ML and GTR + I + G; BIONJ based on pair-wise distances estimated under ML and SYM; BIONJ based on LogDet distances. Numbers to the right of *Phytophthora* taxon labels correspond to the *ITS* clades as described by Cooke et al. (2000). *Phytophthora litchii* represents *ITS* clade 4 according on the results of Riethmüller et al. (2002) and Voglmayr (2003). Major autapomorphic features of downy mildew subclades are also indicated. Abbreviations are as in Fig. 1.

context, the genus could be regarded as either paraphyletic or monophyletic.

Irrespective of whether *Pythium* has to be treated as monophyletic or paraphyletic, strong support (99%) is achieved for a bipartition of the taxa into two groups: one containing *Pythium* and another containing *Halophytophthora*, *Phytophthora*, and downy mildew species. Within the latter, a clade containing all species of *Phytophthora* and downy mildews is highly supported (98%). Within that clade, strong support is achieved for a basal subdivision into a clade containing *Phytophthora ITS* clades 9 and 10 (see Fig. 2 for the assignment of *Phytophthora* species to *ITS* clades according to Cooke et al., 2000) as well as *Phytophthora boehmeriae* (96% support for monophyly), and a

large clade containing all remaining species (100% support for monophyly). *Phytophthora* as presently circumscribed thus forms a paraphyletic assemblage. Within the smaller of the two clades, the sister group relationship between *Phytophthora boehmeriae* and *Phytophthora richardiae* is only weakly (67%) supported. Within the larger clade, backbone resolution is low, indicating that all remaining *Phytophthora* groups could well form a monophylum. Only 66 and 56% bootstrap support is achieved for the paraphyletic arrangement of these clades with respect to obligate biotrophic parasites shown in the likelihood tree, i.e., for *ITS* clades 1–5 and 7 being more closely related to downy mildews than to *Phytophthora ITS* clades 6 and 8. Monophyly of the individual *Phytophthora* clades (in case more than one member could be considered), however, is mostly strongly supported, i.e., with 94% (*ITS* clade 8), 100% (*ITS* clade 7), 95% (*ITS* clade 2), or 96% (*ITS* clade 1) support.

Obligate biotrophic species (downy mildews) are supported as monophyletic with a bootstrap value of 91%. Within the downy mildews, a clade comprising *Plasmopara*, *Plasmoverna*, *Bremia*, *Protobremia*, *Paraperonospora*, *Basidiophora*, and *Benua* is revealed as monophyletic with 89% support. Within this clade, monophyly of *Plasmopara* is supported with a bootstrap value of 100% and monophyly of a clade containing *Paraperonospora*, *Protobremia*, and *Bremia* with a bootstrap value of 99%, whereas further intergeneric relationships are unsupported. Within *Plasmopara*, parasites of Apiaceae form a monophylum with 100% bootstrap support.

A sister-group relationship between *Graminivora graminicola* and *Viennotia oplismeni*, both occurring on grasses (Poaceae), is highly supported (98% bootstrap value). However, phylogenetic relationships of these taxa to *Sclerospora graminicola*, which also infects members of the grass family, *Perofascia*, and *Hyaloperonospora* are not sufficiently resolved. The latter two genera are the closest relatives with high support (99%). *Hyaloperonospora* monophyly is also highly supported (100%). Within *Hyaloperonospora*, several subgroups receive high support, e.g., a clade containing parasites of *Erophila verna* and *Microthlaspi perfoliatum*.

The *Peronospora* and *Pseudoperonospora* species included in our sample fall inside of one group with 90% support. *Pseudoperonospora* is strongly (100%) and *Peronospora* moderately (78%) supported as monophyletic. Backbone resolution within *Peronospora* is low, but some subgroups are well supported, e.g., the parasites of Fabaceae (100%), the parasites of Caryophyllales (98%) or the group containing species mainly occurring on Lamiales (81%).

Irrespective of whether topological weighting according to the PHYML tree was applied or not, RRTree reported highly significant ($p < 0.0001$) differences in substitution rates between *Phytophthora* and downy mildews.

3.3. Maximum parsimony and distance analyses

Equally weighted parsimony analysis resulted in a single most parsimonious tree of length 9693 and a retention index of 0.447. Parsimony bootstrap values higher than 65% are shown in Fig. 2. In general, they were lower than their maximum likelihood counterparts; e.g., support for downy mildew monophyly was 75% in parsimony analysis compared to 91% obtained with PHYML and support for *Phytophthora* paraphyly (i.e., *Phytophthora ITS* clades 9 and 10 as sister group of the remaining *Phytophthora* species and downy mildews) was 94% instead of 100%. *Peronospora* monophyly was unsupported under maximum parsimony. On the other hand, 74% instead of 66% bootstrap support was revealed for a sister-group relationship between *Phytophthora ITS* clades 6 and 8 and all other species within the latter cluster. Albeit lower in general, parsimony bootstrap all in all agreed well with branch support under maximum likelihood (Figs. 1 and 2) and will not be discussed in more detail.

Topology of the BIONJ tree inferred under SYM is shown in Fig. 2 with branch lengths estimated under maximum likelihood with GTR + I + G as substitution model. In addition to MP bootstrap support as described above, BIONJ bootstrap values computed with SYM, GTR + I + G, or LogDet distances are indicated on the branches. Tree topology and branch support values from different distance analyses agreed well with each other all in all and the results of character-based analyses as described above. An important exception is in the support for the clade consisting of *Plasmopara*, *Plasmoverna*, *Bremia*, *Protobremia*, *Paraperonospora*, *Basidiophora*, and *Benua,* which is considerable with ML (89–100%) and MP (79%), but disappears in the BIONJ analyses. On the contrary, distance methods result in moderate (67%) to strong (90%) support for a clade comprising the same species except *Benua kellermanii* (Fig. 2). Support for the clade containing *Bremia*, *Protobremia*, and *Paraperonospora* is moderate (83%) under GTR + I + G, but lacking in the remaining BIONJ bootstrap runs. LogDet and SYM BIONJ analyses result in moderate (78–82%) support for a sister-group relationship between the latter three genera and *Plasmoverna*. As seen in MP analysis, moderate (70–82%) support was achieved for a sister-group relationship between *Phytophthora ITS* clades 1–5 and 7 and downy mildews in two of the three runs of BIONJ bootstrap. Similar to parsimony analysis, too, is the lack of support for *Peronospora* monophyly. Support for other branches was comparable to bootstrap values under ML, although sometimes higher; e.g., downy mildew monophyly was supported by a BIONJ bootstrap value of 99% under SYM and of 100% if LogDet distances were applied.

Further information on the phylogenetic results is included in the Supplementary Data file.

4. Discussion

4.1. Consistency of phylogenetic results

Generally speaking, the results obtained with different methods of phylogenetic inference agreed well. The main

exception is in the support for monophyly of the clade containing downy mildew species with globose to pyriform haustoria (Figs. 1 and 2). Strong support for the whole clade is obtained with MP and ML analyses, whereas it is unsupported with distance methods. These strongly favour a clade comprising the same genera except *Benua kellermanii* which is not revealed with character-based methods. We hypothesise that these discrepancies are due to poor character sampling in *Benua*, as only three of the five loci could be sequenced for this species. Estimation of missing character states by ML and MP based on the current tree during tree search has no real parallel in pair-wise distance methods. In contrast to character-based methods, distance approaches rely on pair-wise comparisons only, leading to a certain loss of information (Penny, 1982). Individual Delta values as inferred with DeltaStats (Holland et al., 2002) support our interpretation, since *Benua* had a Delta value of 0.4039 compared to the average Delta of 0.3315 obtained with the SYM distance matrix.

For these reasons, our discussion of *Phytophthora* and downy mildew phylogeny below will mainly be based on MP and ML bootstrap values. However, we want to emphasise that, with the exception of the placement of *Benua*, results obtained with distance methods agreed well with those of character-based approaches. This is also evident from the high correlation values between bootstrap support values obtained under the respective optimality criteria (see Supplementary Data). Bootstrap support inferred with BIONJ with SYM as the substitution model is more similar to results from likelihood bootstrapping, than is BIONJ bootstrap support with GTR + I + G, even though SYM is a much less complex model and likelihood analyses were based on GTR(+I) + G. These results are in accordance with the opinion of Holland et al. (2002) that the best ML model needs not be optimal for tree search with distance methods.

It has been reported in literature that bootstrap values obtained under maximum parsimony frequently are lower than values inferred with parametric methods (Buckley and Cunningham, 2002), an observation our results agree well with. It could also be demonstrated that bootstrapping may underestimate support if branches are very unequal in length (Hillis and Bull, 1993), but that it accurately estimates branch support if branch lengths are relatively equal (Taylor and Piel, 2004). Regarding the considerable differences in branch lengths in our trees (Figs. 1 and 2), it thus seemed reliable to denote branches as well-supported if their MP bootstrap value was equal to or higher than 70% and the majority of ML bootstrap values was equal to or higher than 90%. Fig. 3 summarises the phylogenetic results with this assumption.

4.2. The phylogenetic relationships of Pythium and Halophytophthora

The phylogenetic relationships of *Pythium* and *Halophytophthora* revealed in the present study are in disagreement with the results of Cooke et al. (2000), who reported strong support for a sister-group relationship between *Pythium vexans* and *Halophytophthora batemanensis* based on a 5.8S and *ITS*2 alignment. Analysing a similar alignment, Voglmayr (2003) observed support for the same grouping. Due to the probable limitations of *ITS rDNA* multiple sequence alignments in inferring higher-level Oomycete relationships and since the present study is based on many more characters, we believe our rooting approach to be well founded. It is also the taxonomically most conservative treatment, as it does not imply non-monophyly of *Pythium*. *Halophytophthora* shares many attributes with *Phytophthora* and was only recently segregated from the latter genus (Ho and Jong, 1990; Erwin and Ribeiro, 1996).

4.3. The phylogenetic relationship of Phytophthora and downy mildews and its evolutionary implications

Our analyses, which are based on a larger dataset containing more conserved genes and a more representative sample of obligate parasites, confirm with high statistical support the results of Cooke et al. (2000, 2002) and Voglmayr (2003) that the genus *Phytophthora* is paraphyletic. Obligate parasites (downy mildews) are more closely related to *Phytophthora ITS* clades 1 to 8 than are those to*ITS* clades 9 and 10 (Figs. 2 and 3). We do not regard this result as being in disagreement with morphological or ecological features. Main characteristics of *Phytophthora* are most easily interpreted as plesiomorphic if compared with downy mildews. First of all, facultative parasitism should represent the ancestral condition, because it is intermediate between a saprotrophic lifestyle and obligate parasitism; *Halophytophthora* mainly consists of saprotrophs. Interestingly, *Phytophthora* species are known to depend on thiamine in culture, which is not required by *Pythium* (Erwin and Ribeiro, 1996). Since obligate biotrophism is usually due to loss of biochemical pathways, thiamine dependency may be interpreted as a synapomorphy of *Phytophthora* and downy mildews (Fig. 3).

Furthermore, some *Phytophthora* species share attributes with obligate parasites which are not found in other *Phytophthora* species. For instance, *Phytophthora ITS* clades 1–5 mainly comprise species which do not have a soil-borne habit, but have conidiosporangia dispersed through the air like downy mildews (Cooke et al., 2000; Fig. 3). In addition, presence of papillate sporangia is a common feature in *Phytophthora ITS* clades 1–5 and downy mildews. In MP and ML analyses, the *Phytophthora ITS* clades 1–5 appear more closely related to obligate parasites than to *Phytophthora ITS* clades 6–7. Unfortunately, very little is known about the distribution of haustoria in *Phytophthora*. De Bary (1876) and Erwin and Ribeiro (1996) depict haustoria of *Phytophthora infestans*, but data on haustoria in other species are (to our knowledge) not available, possibly since most morphological examinations have been carried out with culture material. Topology of ML trees (Fig. 1) could indicate a transformation series

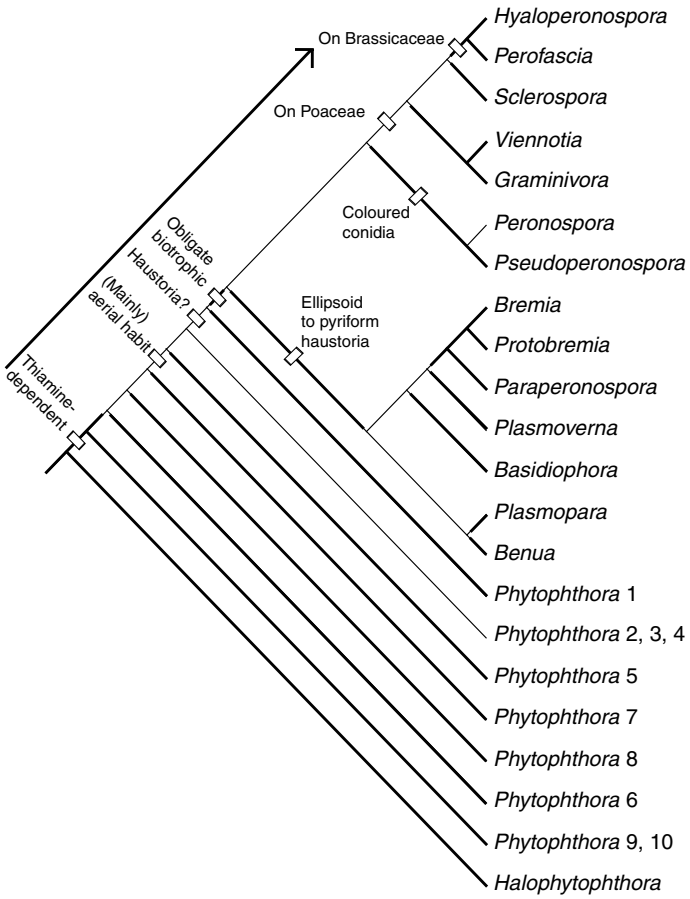

Fig. 3. Evolutionary scenario for downy mildews and their closest relatives, depicting our main findings as a summarised version of the ML tree presented in Fig. 1. Branches with significant bootstrap support in MP and ML analyses are drawn in bold. Synapomorphies and potential synapomorphies as discussed in the text are indicated on the corresponding branches. The arrow connects major adaptive steps related to plant parasitism. Reconstruction of some steps is ambiguous due to lack of resolution (aerial habit, haustoria in *Phytophthora*, parasitism of Poaceae) and/or insufficient knowledge of character state distribution (haustoria in *Phytophthora*). Rooting with *Pythium* instead of *Halophytophthora* would not affect character reconstructions except for lack of thiamin dependency, which is reported for *Pythium* (Erwin and Ribeiro, 1996), but to our knowledge not entirely evident from the literature on *Halophytophthora*.

leading from soil-borne *Phytophthora* species lacking haustoria to air-borne *Phytophthora* species capable of forming haustoria, the latter of which are sister to the downy mildews (Fig. 3). However, backbone resolution with respect to *Phytophthora ITS* clades 1–7 is low, as in the multi-gene analysis conducted by Kroon et al. (2004). Furthermore, these authors also pointed to many characters being rather homoplasious in *Phytophthora*, as did Cooke et al. (2000).

For the first time, substantial molecular phylogenetic evidence is provided that downy mildews are monophyletic. This is in contradiction to the results of Bayesian analyses conducted by Göker et al. (2003), which used a subset of the current dataset. However, Bayesian inference of phylogeny is now known to overestimate support, in particular when there are very short branches (Alfaro et al., 2003). Indeed, branches supported by high bootstrap values in Göker et al. (2003) were confirmed by the present study. Tree topologies from ML and Bayesian analyses conducted by Göker et al. (2003) indicated a sister-group relationship between *Phytophthora infestans* and the clade comprised of downy mildews with vesicular to pyriform haustoria. In the current analysis, bootstrap support for a group consisting of *Phytophthora ITS* clade 1 and obligate parasites with vesicular to pyriform haustoria was 20% with MP, 7–10% with ML, 1% with BIONJ and GTR + I + G as substitution model, and below 1% in the other BIONJ analyses. Thus, some support for the arrangement reported by Göker et al. (2003) is clearly present in the data, even though support for monophyly of downy mildews is much higher in the current analyses.

Regarding the branch lengths observed in obligate parasites which are much higher than those in *Phytophthora* and outgroup species (Figs. 1 and 2), one could argue that monophyly of downy mildews is due to a long-branch attraction artefact (Felsenstein, 1978). However, the clade was revealed under both non-parametric as well as parametric methods of phylogenetic inference relying on the most complex substitution models currently available (Bergsten, 2005). Furthermore, presence of paired long branches alone does not at all indicate an artefact, but may simply be caused by substitution rates being similarly high due to common ancestry (Siddall and Whiting, 1999). With

respect to downy mildews, one could conclude that the shift to obligate parasitism did not only trigger an increase in both host-specificity and speciation (Gäumann, 1964, pp. 69–70), but also in rates of sequence evolution. As evident from the results obtained with RRTree, substitution rates are significantly higher in obligate parasites than in *Phytophthora*.

Thus, obligate biotrophism seems to have arisen only twice independently in Oomycetes, i.e., in white rusts classified in the genus *Albugo* (Hudspeth et al., 2003; Riethmüller et al., 2002; Thines and Spring, 2005; Voglmayr and Riethmüller, 2006) and in downy mildews. Within Peronosporales, two discrete steps leading to full downy mildew habit occurred only once: shift to plant parasitism (apparently accompanied by loss of the ability to produce thiamine) at the most basal node of the *Phytophthora*-downy mildew clade and shift to obligate parasitism at the most basal node of downy mildews.

4.4. Phylogenetic relationships within downy mildews

Compared to earlier studies, our multi-gene approach clearly resulted in greater resolution of the phylogenetic relationships of downy mildews. Monophyly of three main groups could be demonstrated, which are easy to interpret in terms of morphological or peculiar ecological synapomorphies (Fig. 3). These are coloured conidiosporangia (*Peronospora* and *Pseudoperonospora*), vesicular to ellipsoid haustoria (*Basidiophora*, *Bremia*, *Benua*, *Paraperonospora*, *Plasmopara*, *Plasmoverna*, and *Protobremia*), or parasitism of Brassicaceae (*Hyaloperonospora* and *Perofascia*). Sister-group relationship between two graminicolous genera (*Graminivora* and *Viennotia*) could be confirmed, and the sister group of the third genus (*Sclerospora*) could not be identified.

The genera *Peronospora* and *Pseudoperonospora* are characterised by brownish-violet conidiosporangia. This trait is probably synapomorphic, since conidiosporangia in *Phytophthora* are hyaline (Erwin and Ribeiro, 1996; Fig. 3). In addition to support from morphology, our multi-locus molecular analysis reveals high bootstrap values for a sister-group relationship between both genera. Skalický (1966) proposed to merge *Peronospora* and *Pseudoperonospora* based on the similarity in conidiosporangiophore shapes. Waterhouse and Brothers (1981) as well as Constantinescu (2000) did not follow Skalický's suggestion. Instead, they pointed to conidiosporangia being poroid in *Pseudoperonospora* but possessing a continuous wall in *Peronospora*. In our analyses, *Pseudoperonospora* appears as monophyletic with high bootstrap values (Figs. 1 and 2). Although higher than in previous studies (Voglmayr, 2003), statistical support for monophyly of *Peronospora* is only moderate under maximum likelihood (Fig. 1). On the other hand, non-poroid conidiosporangia are likely to represent an autapomorphy of *Peronospora*, since non-poroid conidiosporangia are often observed when germination with hyphae is present. This may point to increased adaptation to dry conditions. *Peronospora sparsa* was interpreted as intermediate by Voglmayr (2003). Maintaining generic rank of both *Peronospora* and *Pseudoperonospora* thus seems appropriate.

Regarding the hyphal haustoria recorded for *Phytophthora* (De Bary, 1876; Erwin and Ribeiro, 1996), vesicular to pyriform haustoria have to be interpreted as apomorphic (Göker et al., 2003; Voglmayr et al., 2004; Fig. 3). Indeed, strong support under ML and MP is achieved for monophyly of a clade consisting of the genera *Plasmopara*, *Plasmoverna*, *Bremia*, *Protobremia*, *Paraperonospora*, *Basidiophora*, and *Benua*, the haustoria of which are vesicular to pyriform (Fraymouth, 1956; Göker et al., 2003; Voglmayr et al., 2004). Relationships within that clade have so far remained largely unresolved (Riethmüller et al., 2002; Göker et al., 2003), although it could be shown that *Plasmopara sphaerosperma* should be transferred to a genus of its own and is most closely related to *Bremia* (Voglmayr et al., 2004).

Here, increased sampling results in strong MP and ML bootstrap support for *Paraperonospora* as a sister group of *Bremia* and *Protobremia*. The three genera are unified by their conidiosporangiophores branching regularly in *Bremia* or irregularly dichotomous in *Protobremia* and *Paraperonospora* (Constantinescu, 1989; Voglmayr et al., 2004). Conidiosporangiophores of *Plasmopara* and *Plasmoverna* are monopodial (Voglmayr et al., 2004; Constantinescu et al., 2005); those of *Benua* and *Basidiophora* are unbranched except the most terminal parts (Constantinescu, 1998). However, it is unclear at present which of these different states of the character "conidiosporangiophore shape" is plesiomorphic. Furthermore, the character as a whole is, at least to some degree, homoplasious as evident from the similarity between the conidiosporangiophores of *Viennotia* and *Plasmopara* and between *Graminivora* and *Bremia* (Göker et al., 2003; Thines et al., 2006). Irregularly dichotomous conidiosporangiophores also were described in *Perofascia* (Constantinescu and Fatehi, 2002).

Obligate parasites of grasses have only recently been recognised as members of Peronosporales, in the case of *Sclerospora* (Riethmüller et al., 2002), or as genera of their own, in the case of *Viennotia* and *Graminivora* (Göker et al., 2003; Thines et al., 2006). These taxonomic conclusions are strongly corroborated by the present work. Additionally, a sister-group relationship between *Viennotia* and *Graminivora* is well supported. Haustoria observed in graminicolous downy mildews are hyphal and similar to each other, as they are coiled in *Sclerospora* and even more heavily coiled in *Viennotia* and *Graminivora* (Göker et al., 2003; Voglmayr et al., 2004; Thines et al., 2006).

The phylogenetic placement of the grass-inhabiting genera within downy mildews still remains enigmatic. Likelihood analyses point to a single origin of parasites of Poaceae and a single jump back to brassicaceous dicots (Figs. 1 and 3). This is in accordance with the single most parsimonious tree (not shown), but neither ML nor MP achieve statistical support for this topology, nor do dis-

tance methods, which result in a somewhat different topology (Fig. 2). *Peronosclerospora* was demonstrated by Hudspeth et al. (2003) to not belong to Saprolegniales in which it had been previously placed as a sister taxon of *Sclerospora* (e.g., Dick, 2002). Even though *Peronosclerospora* could not be considered here, *Sclerospora* and *Peronosclerospora* most probably are sister taxa within Peronosporales.

4.5. Host shifts and host range in downy mildews

Descriptions of plant-fungus relationships are often referred to as "studies in co-evolution" and frequently discuss host taxa as characters of the parasite. However, such a treatment is not as straightforward as it might seem at first glance. As Siddall (1997) remarked, host taxa do not represent characters dependent on the parasite. Furthermore, plant species need not be homogeneous within taxa of higher rank with respect to major determinants of susceptibility to parasitic fungi. On the other hand, monocot taxa have very rarely been colonised by downy mildews (Palti and Kenneth, 1981; Dick, 2002). The shift to grass parasitism may represent the widest host jump observable in downy mildews and may point to a peculiar amount of ecological adaptations distinguishing graminicolous from other downy mildews. However, one should keep in mind that this conclusion implies an extrapolation from extrinsic characters (occurrence on hosts) to intrinsic, i.e., genetically inherited characters, which have not been directly observed (Schuh, 2000).

Parasitism of Brassicaceae is just as peculiar in downy mildews. As recognised by Constantinescu and Fatehi (2002), *Peronospora* species occurring on Brassicaceae should be treated in two genera of their own. This taxonomic arrangement was confirmed by molecular data (Choi et al., 2003; Voglmayr, 2003; Göker et al., 2003). Here, we additionally show that *Hyaloperonospora* and *Perofascia* are more closely related to each other than to any other genus of downy mildews. As conidiosporangiophores in both genera are quite different (Constantinescu and Fatehi, 2002), host relationships is the sole synapomorphic character in *Hyaloperonospora* and *Perofascia* known so far. Few *Hyaloperonospora* species parasitize hosts from other families. Two of these species appeared to be deeply nested within parasites of Brassicaceae, and thus are likely to represent later reversals in host range (Göker et al., 2004).

The approach of Constantinescu and Fatehi (2002) to merge almost all species of *Hyaloperonospora* into *H. parasitica* could not be confirmed by more exhaustive molecular studies (Voglmayr, 2003; Göker et al., 2003; Choi et al., 2003; Göker et al., 2004). These were in accordance with narrow species concepts as advocated by Gäumann (1918) and Gustavsson (1959a,b). As a consequence, the number of *Hyaloperonospora* species infecting Brassicaceae may well exceed 100 (Constantinescu, 1991; Dick, 2002). In our view, listing species numbers is only a first step in documenting parasite biodiversity. At the very least, the parasitological rule formulated by Eichler (1942), later on called "Eichler's rule" by Stammer (1957; see also Brooks and McLennan, 1993, p. 14) should be considered. It simply states that, within a selection of comparable groups of parasites, taxonomic groups of hosts which are more diverse than comparable host clades harbour more parasites. Constantinescu (1991) lists 121 valid binomials of *Hyaloperonospora* on Brassicaceae (still included in *Peronospora* at that time); the second largest number of epithets mentioned is 91 on Fabaceae. However, species numbers are estimated by Heywood (1993) as 11,300 in Fabaceae and only about 3000 in Brassicaceae. Colonisation of Brassicaceae having occurred only once but having led to such considerable diversification (in absolute as well as relative terms) points to a unique role of the family in downy mildew evolution.

Challenges for further studies in downy mildew phylogeny are to clarify interrelationships of the main downy mildew clades as described above, as well as to determine the closest *Phytophthora* relatives of obligate parasites. A reasonable approach to achieve these goals is to further increase character sampling by sequencing of additional loci. Furthermore, integrated molecular-morphological analyses would be of use which could incorporate new characters such as those obtained by scanning electron microscopy (e.g., Constantinescu et al., 2005) or biochemistry (Spring et al., 2005). Cladistic interpretation of these characters in addition to greater resolution of phylogenies is likely to further improve our understanding of the evolution of this important group of plant pathogens. As summarised in Fig. 3, phylogenetic analyses so far point to an evolutionary scenario of gradually increasing adaptation to plant parasitism in Peronosporales. Strikingly, the most important of these adaptive steps, listed in Fig. 3, occurred only once: at the basal node in Peronosporales, at the origin of obligate parasites, and within downy mildews.

4.6. Taxonomic consequences of the present study

Traditional Oomycete taxonomy (e.g., Waterhouse, 1973; Dick, 2001, 2002) was revealed in previous studies to be only partly satisfactory. Is has been demonstrated that *Phytophthora*, including *Phytophthora litchii*, should be transferred from Pythiaceae to Peronosporaceae (Riethmüller et al., 2002). Molecular studies (Hudspeth et al., 2003; Riethmüller et al., 2002; Thines and Spring, 2005) also showed that Albuginaceae should not be considered as a member of Peronosporales (as done in, e.g., Dick, 2001, 2002). Hence, a single family, Peronosporaceae, remained in Peronosporales and could have been regarded as synonymous to the order. However, there was no molecular support for downy mildew monophyly at that time.

Based on the phylogenetic results of the present study, we suggest that "Peronosporaceae" henceforth be restricted to downy mildews, i.e., the obligate parasitic genera within Peronosporales. So far, these are represented by *Basidiophora*, *Benua*, *Bremia*, *Graminivora*, *Hyaloperonospora*, *Paraperonospora*, *Perofascia*, *Peronosclerospora*, *Peronos-*

pora, *Plasmopara*, *Plasmoverna*, *Protobremia*, *Sclerospora*, and *Viennotia*. *Phytophthora* should be assigned to Peronosporales, but not to Peronosporaceae. No formal taxonomical changes have to be proposed as "Peronosporaceae" has previously been published. Further studies are necessary to achieve a more satisfactory taxonomy for *Phytophthora*.

There is sound molecular and morphological evidence that maintenance of the genus *Peronophythora* should be dismissed and *P. litchii* be transferred to *Phytophthora* (Riethmüller et al., 2002; Voglmayr, 2003), which is confirmed by the present study. As the transfer of *Peronophythora litchii* to *Phytophthora* by Chi et al. (1982) is invalid due to absence of basionym indication and literature reference (Art. 33.3 of the ICBN; see Greuter et al., 2000), the formal nomenclatural combination needs to be made:

Phytophthora litchii (C.C. Chen ex W.H. Ko, H.S. Chang, H.J. Su, C.C. Chen & L.S. Leu) Voglmayr, Göker, Riethm. & Oberw., comb. nov.

Basionym: *Peronophythora litchii* C.C. Chen ex W.H. Ko, H.S. Chang, H.J. Su, C.C. Chen & L.S. Leu, Mycologia 70: 381 (1978).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2006.07.005.

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