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ARTICLE

The inclusion of downy mildews in a multi-locus-dataset and its reanalysis reveals a high degree of paraphyly in *Phytophthora*

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Abstract: Pathogens belonging to the *Oomycota*, a group of heterokont, fungal-like organisms, are amongst the most notorious pathogens in agriculture. In particular, the obligate biotrophic downy mildews and the hemibiotrophic members of the genus *Phytophthora* are responsible for a huge variety of destructive diseases, including sudden oak death caused by *P. ramorum*, potato late blight caused by *P. infestans*, cucurbit downy mildew caused by *Pseudoperonospora cubensis,* and grape downy mildew caused by *Plasmopara viticola*. About 800 species of downy mildews and roughly 100 species of *Phytophthora* are currently accepted, and recent studies have revealed that these groups are closely related. However, the degree to which *Phytophthora* is paraphyletic and where exactly the downy mildews insert into this genus in relation to other clades could not be inferred with certainty to date. Here we present a molecular phylogeny encompassing all clades of *Phytophthora* as represented in a multi-locus dataset and two representatives of the monophyletic downy mildews from divergent genera. Our results demonstrate that *Phytophthora* is at least six times paraphyletic with respect to the downy mildews. The downy mildew representatives are consistently nested within clade 4 (contains *Phytophthora palmivora*), which is placed sister to clade 1 (contains *Phytophthora infestans*). This finding would either necessitate placing all downy mildews and *Phytopthora* species in a single genus, either under the oldest generic name *Peronospora* or by conservation the later name *Phytophthora,* or the description of at least six new genera within *Phytophthora*. The complications of both options are discussed, and it is concluded that the latter is preferable, as it warrants fewer name changes and is more practical.

Key words:
AU test
downy mildews
multigene phylogeny
Peronosporaceae
Phytophthora
taxonomy

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INTRODUCTION

Oomycetes are a group of organisms that superficially resemble fungi in their hyphal growth and absorptive way of nutrition. However, they are not closely related to *Mycota*, but belong to a group of heterokont organisms, *Straminipila* (Dick 2001), which also includes diatoms and sea-weeds. Oomycetes have adapted to parasitism of plants at least three times, once in the *Saprolegniales* in the genera *Aphanomyces* and *Pachymetra* (Riethmüller *et al.* 1999, Diéguez-Uribeondo *et al*. 2009), and separately in *Albuginales* and *Peronosporales* (Riethmüller *et al.* 2002, Hudspeth *et al.* 2003, Thines *et al*. 2008). While the evolution of obligate biotrophy seems to be an ancient occurrence for the white blister rusts (Thines & Kamoun 2010), the downy mildews have more recently arisen from *Phytophthora*-like ancestors (Riethmüller *et al*. 2002, Göker *et al*. 2003, 2007, Thines *et al.* 2008, 2009, Thines 2009). The close relationship of the downy mildews and *Phytophthora* revealed by these studies is in contrast to the widely used taxonomic classifications of Waterhouse (1973) and Dick (1984, 2001), in which *Phytophthora* and *Pythium* were grouped together in the family *Pythiaceae*. Although Cooke *et al*. (2000) inferred a position of *Peronospora sparsa* as a sister group of clade 4 (as defined in that study) based on ITS sequences alone, no substantial phylogenetic resolution was present on the phylogenetic backbone, thus failing to position this group within the genus *Phytophthora*. Other studies (including multi-locus studies) that included both downy mildew and *Phytophthora* species have so far not resolved the placement of downy mildews in relation to the different clades of *Phytophthora* (Riethmüller *et al*. 2002, Göker *et al*. 2007, Thines *et al.* 2009, Giresse *et al.* 2010). Additionally, Thines *et*

al. (2009) demonstrated that the support for the sister-group relationship of *Peronospora* and clade 4 inferred by Cooke *et al*. (2000) could have been the result of an alignment artefact. Conversely, a recent study by Blair *et al*. (2008) addressed the phylogenetic relationships of *Phytophthora* species with good resolution, but no downy mildew was included in that study, leaving their placement to speculation. Downy mildews have been shown to be a monophyletic assemblage by Göker *et al*. (2007). However, Göker & Stamatakis (2006) later (in spite of being published earlier than Göker *et al*. 2007) came to the conclusion that a placement of *Phytophthora* clade 1 within the downy mildews would also be possible, although no support could be obtained for this scenario. The question of which is the sister clade of the downy mildews, and how this clade is embedded among the different lineages of *Phytophthora* therefore continues to be controversial, but is fundamental for understanding the evolution of this group of important plant pathogens, especially with respect to the evolution of biotrophy. In addition, the taxonomic status of many *Phytophthora* species depends on the degree of paraphyly of the genus. At least with two clades, 9 and 10, *Phytophthora* is paraphyletic with respect to downy mildews (Cooke *et al*. 2000, Göker *et al*. 2007, Thines *et al*. 2009), but so far, the degree of paraphyly of *Phytophthora* could not be resolved. Therefore, it was the aim of this study to resolve the phylogenetic placement of the monophyletic downy mildews (represented by the two divergent downy mildew genera for which genome data are currently available) among *Phytophthora* and to test this placement statistically, to further clarify the relationships within this group of important plant pathogens.

MATERIALS AND METHODS

All sequences of *Phytophthora* and *Pythium* were obtained from the study of Blair *et al*. (2008) available in the National Center for Biotechnology Information (NCBI) nucleotide database, GenBank. The dataset includes sequences of seven different loci, and all species for which all seven loci were not available were discarded, except for two *Pythium* species for which only six of the seven loci could be obtained. This resulted in an overall dataset of 121 species sampled. The sequences of *Phytophthora infestans* were used to obtain homologous sequences from the genome of *Hyaloperonspora arabidopsidis* from the NCBI database using BLAST (Altschul *et al*. 1997) and from the genome of *Pseudoperonospora cubensis* (Tian *et al*. 2011) using the annotated EST sequence information. Because no sequence information for the 28S nuclear ribosomal DNA locus of *Pseudoperonospora cubensis* could be obtained from the EST library, which was enriched for protein-coding genes, sequence information was obtained from the NCBI database, using a sequence from the study of Riethmüller *et al*. (2002). GenBank accession numbers for all sequences included in the analyses are given in Table S1 (Supplementary Information, online only).

Each of the seven sets of sequences was edited (i.e. leading and trailing gaps were removed) using the DNASTAR computer package v. 8 (Lasergene, Madison, WI), and were aligned separately using MAFFT v. 6.240 (Katoh *et al*. 2005) using a webserver interface (http://www.genome.jp/tools/mafft/). The G-INS-i algorithm was chosen for all alignments. Subsequently, the aligned sequences were concatenated for phylogenetic analyses and no further editing was done on the alignment to ensure reproducibility and to prevent introduction of bias. After the removal of leading and trailing gaps 6282 nucleotide sites were included in the phylogenetic analyses. These comprised seven loci: 1119 bp of the beta-tubulin gene, 493 bp of the 60S ribosomal protein L10 gene, 873 bp of the translation elongation factor 1-alpha gene, 720 bp of the 28S nuclear ribosomal DNA gene, 646 bp of the glyceraldehyde-3-phosphate dehydrogenase gene, 1438 bp of the heat shock protein 90 gene, and 993 bp of the enolase gene. The alignment, together with the tree from the Bayesian Analysis shown in Fig. 1, has been deposited in TreeBASE (www.treebase.org) under the accession number S11829.

The general time reversible (GTR) model was selected for the concatenated alignment using Modeltest v. 3.7 (Posada & Crandall 1998) and PAUP v. 4.0b10 (Swofford 2002), with gamma-distributed substitution rates (shape parameter = 0.69) and proportion of invariable sites (pinv = 0.54). The values of these parameters were included in the Bayesian and Minimum Evolution analyses.

Minimum Evolution (ME) analysis was done using MEGA v. 4.0 (Tamura *et al*. 2007), with the gamma-distributed substitution rates as inferred by Modeltest and using the Maximum-Composite-Likelihood substitution model. For inferring tree robustness, 1000 bootstrap replicates (Felsenstein 1985) were computed.

For Maximum Likelihood (ML) inference, the RAxML webserver at http://phylobench.vital-it.ch/raxml-bb/ (Stamatakis *et al*. 2008) was used with standard settings and maximum likelihood search, including an estimation of invariable sites. The analysis was repeated five times with 100 bootstrap replicates each. The bootstrap support values obtained were averaged, because the rapid bootstrapping algorithm can lead to some deviation.

For Bayesian analysis, MrBayes (Huelsenbeck & Ronquist 2001) at the Phylemon2 webserver (http://phylemon.bioinfo.cipf.es/) and at a local server, for parallel runs, was used. Four incrementally heated simultaneous Markov Chain Monte Carlo chains were run for two million generations with every 1000th tree sampled, under the general time reversible (GTR) model with the gamma-distributed substitution rates and proportion of invariable sites as inferred by Modeltest. Maintaining that the standard deviation of split frequencies was constantly below 0.01 and the stationary phase of the likelihood values was reached after 10 % of sampled trees when quitting the analysis. The first 1000 trees sampled this way were discarded, and the remaining 1000 trees were used to compute a 50 % majority rule consensus tree and to estimate the posterior probabilities. To ensure general reproducibility, the analysis was repeated twice using the webserver, and twice on a local server using MrBayes v. 3.1.2.

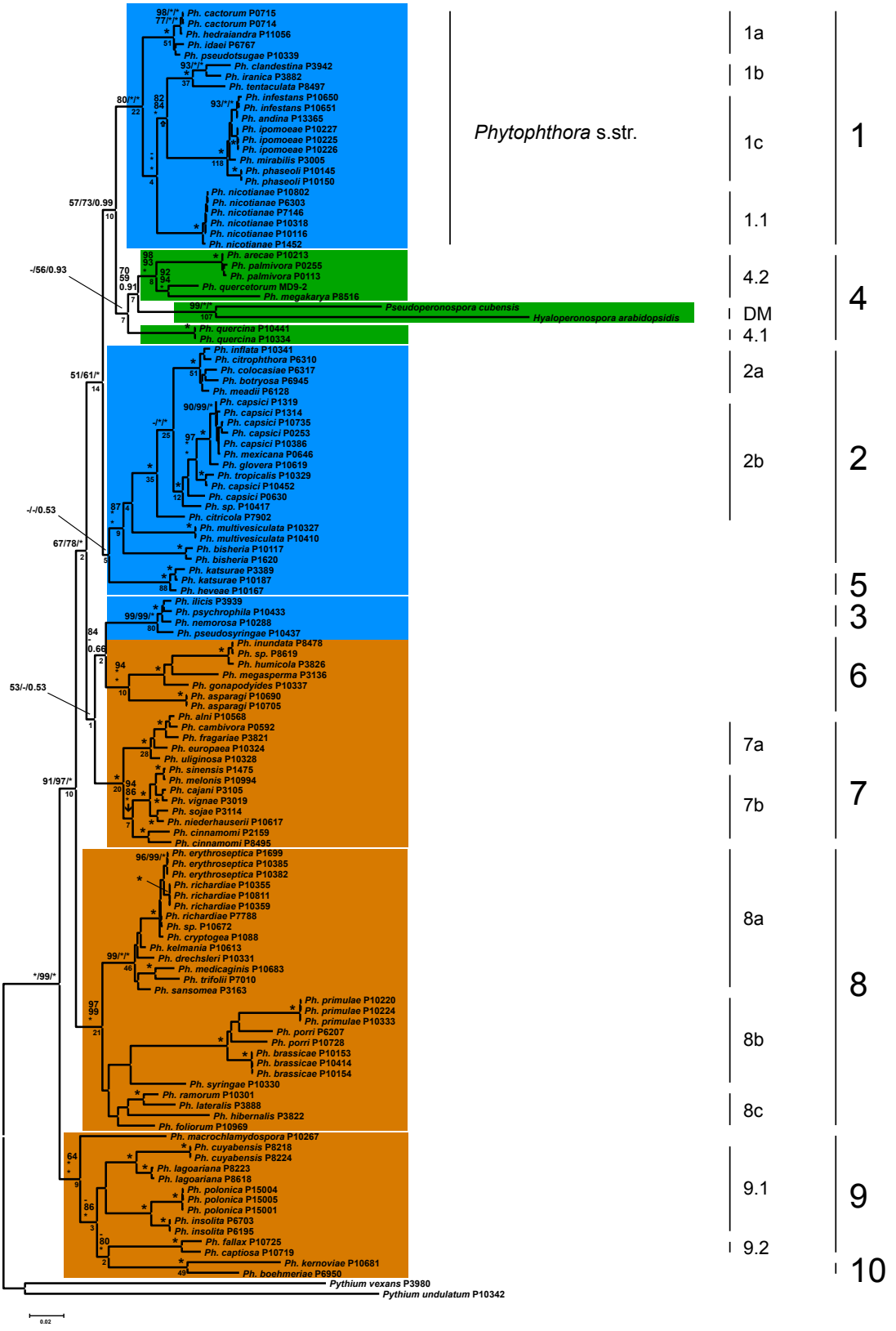

Fig. 1. Phylogenetic reconstruction for *Phytophthora* and the downy mildews (Bayesian Analysis), with support values in Minimum Evolution, Maximum Likelihood, and Bayesian Analysis, in the respective order, on the branches, and Bremer support below the branches. Small Asterisks denote maximum support in a single analysis, big asterisks denote maximum support in all three phylogenetic analyses. Clade designations are those of Blair *et al.* (2008), with some additional differentiation corresponding to the statistical testing of the tree topology as given in Table 1. Predominantly caducous and papillate clades are highlighted in blue, the clade containing downy mildews is highlighted in green and the clades with predominantly non-caducous, non-papillate or semi-papillate members are highlighted in brown. For *Phytophthora*, the highlighted areas are divided into blocks representing groups that lead to paraphyly of *Phytophthora* and could potentially serve as a basis for the description of new genera.

Table 1. Results of the site-wise log-likelihoods generated under possible associations of species in base edges. The first column gives the possible associations for which the site-wise log-likelihoods were produced. Columns show the support values for the approximately unbiased (AU) test, the observed log-likelihood differences of the edges (OBS), Bootstrap probability tests (NP, BP; and PP), Kishino-Hasegawa (KH) test, Shimodaira-Hasegawa (SH) test, weighted Kishino-Hasegawa (WKH) test, and the weighted Shimodaira-Hasegawa (WSH) test.

Possible associations	AU	OBS	NP	BP	PP	KH	SH	WKH	WSH
(4.2, DM)	0,983	-106,9	0,992	0,993	1,000	0,966	0,992	0,974	0,989
(1, 4, DM)	0,983	-106,9	0,992	0,993	1,000	0,966	0,992	0,974	0,989
(1, 2, 4, DM)	0,983	-106,9	0,992	0,993	1,000	0,966	0,992	0,974	0,989
(4, DM)	0,979	-39,4	0,985	0,985	1,000	0,901	0,988	0,94	0,996
(1c,1b)	0,882	-32,7	0,981	0,981	1,000	0,860	0,925	0,925	0,925
(3, 6)	0,713	-28,2	0,918	0,919	1,000	0,753	0,753	0,753	0,753
(1–8, 9.1, DM)	0,679	-14,1	0,648	0,646	1,000	0,721	0,909	0,666	0,916
(1–4, 6, DM)	0,670	-5,6	0,47	0,467	0,997	0,592	0,967	0,592	0,967
(2b, 2.2)	0,644	-5,1	0,407	0,399	0,973	0,593	0,911	0,593	0,927
(5, 7)	0,617	-14,7	0,741	0,742	1,000	0,653	0,807	0,653	0,831
(1, 2, 4, 5, 7, DM)	0,555	5,6	0,104	0,103	0,002	0,408	0,949	0,408	0,951
(1, 2, 4, 5, DM)	0,440	14,7	0,251	0,252	0,000	0,347	0,815	0,347	0,806
(1–6, DM)	0,383	14,7	0,259	0,258	0,000	0,347	0,678	0,347	0,676
(2.1, 2b)	0,356	5,1	0,593	0,601	0,027	0,407	0,585	0,407	0,569
(9.1,9.2)	0,321	14,1	0,352	0,354	0,000	0,279	0,678	0,334	0,668
(3,5–7)	0,302	5,8	0,093	0,091	0,000	0,232	0,911	0,232	0,821
(1–5, 7, DM)	0,287	28,2	0,082	0,081	0,000	0,247	0,636	0,247	0,645
(1–4, DM)	0,287	28,2	0,082	0,081	0,000	0,247	0,636	0,247	0,645
(1b,1.1)	0,118	32,7	0,019	0,019	0,000	0,140	0,596	0,075	0,330
(3, 6, DM)	0,022	106,9	0,007	0,006	0,000	0,034	0,093	0,015	0,065
(1, 4.1)	0,021	39,4	0,015	0,015	0,000	0,099	0,406	0,031	0,156
(1, 4)	0,017	106,9	0,008	0,007	0,000	0,034	0,093	0,015	0,051
(1, 2, 4, 5)	0,017	106,9	0,008	0,007	0,000	0,034	0,093	0,015	0,051
(1, 2, 4)	0,017	106,9	0,008	0,007	0,000	0,034	0,093	0,015	0,051

The following species were randomly chosen as representatives for the corresponding clades and subclades in the statistical analysis – 1c, *Phytophthora cactorum* ; 1b, *P. nicotianae*; 1c, *P. iranica*; 1.1, *P. infestans*; 2ab, *P. capsici*; 2.1, *P. bisheria*; 2.2, *P. multivesiculata*; 3, *P. nemorosa*; 4.1, *P. quercina*; 4.2, *P. palmivora*; 5, *P. katsurae*; 6, *P. humicola*; 7, *P. europaea*; 8, *P. ramorum*; 9.1, *P. polonica*; 9.2, *P. captiosa*; 10, *P. boehmeriae*; DM, *Pseudoperonospora cubensis*.

Inference of Bremer support was done using Maximum Parsimony with the Parsimony Ratchet implemented in PRAP2 (Müller 2003), using PAUP v. 4.0b10. The starting tree was obtained by stepwise addition and subsequently the tree-bisection-and-reconnection (TBR) algorithm was used. Two hundred replicates were run with 25 % randomly chosen characters weighted double and the shortest tree of each run was saved. Afterwards the decay index of each of the bisections was obtained in PRAP2.

The Approximately Unbiased (AU) test (Shimodaira 2002) was applied to the 100 bootstrap replicate trees of the first Maximum Likelihood analysis and to the last 100 sampled trees of the first Bayesian Analysis using the CONSEL computer package (Shimodaira & Hasegawa 2001). The respectively most probable trees were compared to the topologies of the resulting trees of the ML, ME and Bayesian analyses and no conflicting support was found to be present.

For conducting the AU testing of the position of the downy mildews within *Phytophthora* and additional statistical tests, representatives of each of the clades at a node important to infer the position of the downy mildews or the major monophyletic clades were chosen. For these 18 accessions, a Bayesian analysis was conducted as described above, but with estimation of the gamma-distribution and the proportion of invariable sites by MrBayes, for enabling the AU testing with CONSEL. The sampled accessions are given in Table 1. The resulting tree was compared to the original tree and no conflicting support was present, and only minor changes in topology (placement of clade 5) were observed, ensuring the validity of the results. One hundred trees (i.e. every 20 000th generation) of the Bayesian analysis were used to create a site-wise log-likelihood output in PAUP for bootstrap analysis and statistical testing in CONSEL. The TREEASS program of the CONSEL computer package assesses support for each possible association of species in base edges in the underlying trees and outputs *p*-values for the AU test, Bootstrap probability tests (NP, BP; and PP), Kishino-Hasegawa (KH) test, Shimodaira-Hasegawa (SH)

test, weighted Kishino-Hasegawa (WKH) test, and weighted Shimodaira-Hasegawa (WSH) test. Default settings of 10 scaling factors of 0.5–1.4, with 10 000 pseudoreplicates for each, were used. *Phytophthora boehmeria,* of the most basal clade of *Phytophthora*, was used as an outgroup for the analyses.

RESULTS

When used independently, the loci of the concatenated alignment always yielded topologies with no significantly supported inconsistencies (data not shown). The Maximum Likelihood (ML) analysis of the concatenated alignment resulted in a best tree with a log-likelihood of -62481.32, a Minimum Evolution (ME) tree with a sum of branch lengths of 1.04068070, and the best tree from Bayesian Analysis (BA) had a log-likelihood score of -62678.74. The best tree from the BA, with posterior probabilities and bootstrap support values from the other analyses, is given in Fig. 1. In addition, Bremer support values are given for all clades and subclades. Under the given tree, Bremer decay indices > 5 can be considered as significant support and values of 10 or higher as strong support. It should be noted that the Bremer support is not linearly correlated with bootstrap support. Species of *Phytophthora* were grouped into nine highly supported clades, with clade 9 also including clade 10 of Blair *et al.* (2008). Tree topology was similar to the one found in Blair *et al*. (2008) and no supported conflicts were observed, with the exception of the before-mentioned inclusion of clade 10 into clade 9. Downy mildews, represented by the two divergent genera, *Hyaloperonospora* and *Pseudoperonospora*, were grouped together with maximum support in ML and BA and strong support in ME inference, and were consistently found among the members of clade 4 of Blair *et al.* (2008) with varying support in the full dataset (Fig. 1). The sister-group relationship of downy mildews with a part of clade 4, comprised of *Phytophthora megakarya*, *P. quercetorum*, *P. palmivora*, and *P. areceae* received 70 % bootstrap support in ME, 59 % in ML and a posterior probability of 0.91, at a confidence interval at 95 % for the trees sampled. This group was found sister to *P. quercina*, although this grouping received significant support only in the BA. Clade 1 and the monophyletic group containing the downy mildews and the clade 4 species of *Phytophthora* were consistently grouped together in all analyses, with varying support of 57 % bootstrap support in ME, 73 % in ML, and a posterior probability of 0.99. The Bremer decay index was 7 for the grouping of DM with *P. megakarya*, *P. quercetorum*, *P. palmivora*, and *P. areceae* and also 7 for the sister-group placement of the above assemblage with *P. quercina*. The sister-group relationship of clade 1 with clade 4 (including downy mildews) was supported by a Bremer decay index of 10, thus providing an independent support for the monophyly of this grouping. The monophyly of clade 1 was well supported with moderate to maximum support in the phylogenetic analyses and a Bremer decay index of 24. The monophyly of clades 2 and 5 was also strongly supported; however, their sister-group relationship did not receive significant support in any of the analyses. Clades 1, 4 (plus downy mildews), 2, and 5 were grouped together with weak support in ME and ML analyses, but maximum support in the BA. This group was grouped together with clades 3, 6, and 7 with weak support in ME (67 %), moderate support in ML (78 %) and maximum support in the BA. Clades 3, 6, and 7 were all found to be monophyletic with strong to maximum support in all analyses. However, their grouping as a monophyletic assemblage received only weak support in ME and BA. Clade 8 was placed basal to the before-mentioned clades 1–7 and its monophyly received strong to maximum support in all analyses. A deep divergence was found between clades 1–8 on the one side and clades 9 and 10 on the other side, resulting in a strong to maximum support for the monophyly of the assemblage comprised of clades 1–8 in all phylogenetic analyses, and a Bremer decay index of 10. Clade 10 was found to be nested within clade 9 in ML and BA, and the monophyly of the group containing these clades was weakly supported in ME, but strongly supported in ML and BA, and also received a Bremer decay index of 9. In the reduced dataset (Fig. S1, Supplementary Information, online only) the downy mildews, represented by *Pseudoperonospora cubensis*, grouped together with *Phytophthora palmivora* of clade 4 with maximum support, and *P. quercina* was found to be the sister taxon of this group with strong statistical support. The group comprising the downy mildew and clade 4 representatives was found to be sister to clade 1 with maximum support. An alternative topology was observed for some weakly supported nodes, as the grouping of clades 3 and 6 as well as the grouping of clades 5 and 7 received significant support.

To test the robustness of the observed grouping of the clades, especially with respect to the placement of the downy mildews within *Phytophthora*, and to infer the probability of alternative groupings, several tests were performed, which are summarised in Table 1. The analyses were carried out without constraints, seeking for all possible groupings of the clades and subclades of *Phytophthora* and the downy mildews. The clustering of downy mildews with clade 4.2 had the highest AU values and also received the highest scores in all other analyses, and also the larger clusters of clades 1, 4, and DM, and 1, 2, 4, and DM scored equally high. The latter of these groupings is, in contrast to the tree presented in Fig. 1, as it excludes clade 5, which was grouped together in the full phylogenetic analysis with clade 2 without significant support. But in the phylogeny of the clade representatives, the grouping that scored high in the AU analysis could also be observed (Fig. S1). The nesting of the downy mildews within clade 4 received almost equally high support, with 0.979 in the AU analysis. Thus the topology of the tree presented in Fig. 1 with respect to the immediate relationships of the downy mildews received the highest support in the AU analysis and all other tests employed. Only four contradicting clusters were found to be possible. These include an alternative placement of the downy mildews with clades 3 and 6; the clustering of clades 1 and 4 with the exclusion of

downy mildews; the clustering of clades 1, 2, 4 and 5 with the exclusion of downy mildews; and the clustering of clades 1, 2, and 4 with the exclusion of downy mildews. But the high improbability of these groupings is reflected by very low AU scores, which were 0.022 for the first and 0.017 for the other groupings. Groupings of *Phytophthora* which received significant support are the clustering of clades 1b and 1c (AU 0.882); although these scored less than for the position of downy mildews as a sister group of clade 4.2 and their nested placement in clade 4. The grouping of clades 3 and 6, which were affiliated to other clades without significant support in the phylogenetic analyses, received moderate support (AU 0.713). Another grouping which was not observed in the phylogenetic analysis is the clustering of clades 5 and 7, which was also moderately supported (AU 0.617). Moderate support was also obtained for the grouping of clades 1–8, including downy mildews, together with 9.1 (AU 0.679), and clades 1–4, including downy mildews, together with clade 6 (AU 0.670).

DISCUSSION

The genus *Phytophthora* is one of the largest genera of the oomycetes and contains about 100 currently accepted species, of which about 60 species were included in the monograph of Erwin & Ribeiro (1996), and to which about 40 species have been added subsequently (Érsek & Ribeiro 2010). As many of the species are of ecological and economic interest, *Phytophthora* has received much attention in the past decades, and as a consequence, the genome sequencing of several of its members has been undertaken (Tyler *et al.* 2006, Haas *et al.* 2009). New species are being discovered in the previously species-poor basal clades (Brasier *et al.* 2005, Belbahri *et al.* 2006, Dick *et al.* 2006), and it seems likely that only a small fraction of the evolutionary diversity of this genus has been discovered. The genus *Phytophthora* has often been considered a member of *Pythiaceae* (Waterhouse 1973, Dick *et al.* 1984, Dick 2001), while the obligate biotrophic downy mildews were viewed as constituting the family *Peronosporaceae*. Dick *et al*. (1984) even placed the *Peronosporaceae* together with the *Albuginaceae* into the order *Peronosporales* and opposed this to the cultivable *Pythiales*, which also included *Phytophthora*. However, Gäumann (1952) already realised that *Phytophthora* and the downy mildews were likely to be closely related, and this hypothesis was later corroborated with the first molecular phylogenies including members of both *Phytophthora* and the downy mildews (Cooke *et al.* 2000, Riethmüller *et al*. 2002). The strict split between downy mildews and *Phytophthora* is rather synthetic, as there are species with intermediate character states that bridge the apparent gulf between the necrotrophic and hemibiotrophic members of *Phytophthora* and the obligate biotrophic downy mildews (Thines 2009). For example, the downy mildew genus *Viennotia* (Göker *et al.* 2003) possesses sporangiophores capable of additional growth after sporulation, *Poakatesthia* (Thines *et al*. 2007) forms intracellular mycelium apart from haustoria, and *Sclerophthora* has hyphal sporangiophores which do not form sporangia simultaneously (Payak & Renfro 1967). All of these features are usually attributed to *Phytophthora* species, although other characteristics place these genera among the downy mildews (Thines 2009). The chimeric appearance of *Sclerophthora* is so pronounced that it was even included in the monograph of *Phytophthora* by Erwin & Ribeiro (1996). It is also noteworthy that evolution of the downy mildews may have been initiated as parasites of grass relatives (Thines *et al.* 2007, Thines 2009). Support for this hypothesis is provided by *Phytophthora* species from *Cyperaceae* which have also been considered members of an independent genus, *Kawakamia*, and are not readily cultivable (Erwin & Ribeiro 1996). On the other hand, there are reports of axenic cultivation for *Sclerospora graminicola* (Tiwari & Arya 1969) and *Sclerophthora macrospora* (Tokura 1975), although these results have not been confirmed by independent experiments of other groups. Unfortunately, none of the above-mentioned parasites of grasses could be included in the present study because of difficulties of amplification using the primers available. Also, for downy mildews in general, the primers used by Blair *et al.* (2008) do not readily amplify the targeted genes, therefore we obtained these sequences directly from the genomes of *Hyaloperonospora arabidopsidis* (Baxter *et al*. 2010) and *Pseudoperonospora cubensis* (Tian *et al*. 2011). However, as the downy mildews most likely represent a monophyletic group (Göker *et al*. 2007), the inclusion of only these two exemplars from largely divergent downy mildew genera can be considered valid for inferring the placement of this group amongst the phylogenetic lineages currently placed in *Phytophthora*.

The topology of the tree shown here is mostly congruent with the topology presented by Blair *et al*. (2008). However, the inclusion of the downy mildews has in some cases resulted in lower support values, especially on the backbone and to a grouping of clades 2 and 5 without significant support. In Blair *et al*. (2008), clade 5 was inferred as being basal to clade 2 with weak to moderate support. In our investigations, however, the downy mildews were consistently grouped together with some members of clade 4, which is in line with the sister-group relationship for *Peronospora sparsa* with a group made up of *Phytophthora arecae*, *P. palmivora*, and *P. megakarya* as observed by Cooke *et al*. (2000) on the basis of ITS sequence data, although it cannot be ruled out that the finding in that study was influenced by alignment artefacts (Thines *et al*. 2009) and a bias of the Neighbour-joining analysis. In our study, which is based on the multi-locus dataset of Blair *et al*. (2008) to which sequences from downy mildew representatives have been added, the close relationship of the downy mildews with members of clade 4 is also supported by several phylogenetic methods and statistical tests, in which the sister-group relationship of clade 4.2 with the downy mildews and the grouping of downy mildews within clade 4 as a whole received strong support. As discussed in previous publications on the global phylogeny of *Phytophthora* (e.g. Blair *et al.* 2008, Cooke *et al*. 2000, Kroon

et al. 2004), there are no clear-cut synapomorphies identified for the different clades so far. However, four of the five groups with predominantly papillate or caducous sporangia (1, 2, 4, and 5), together with the downy mildews, form the crown group of *Phytophthora*, and it is thus likely that caducous and papillate sporangia represent a derived character state. This is in contrast to the conclusion of Kroon *et al*. (2004), who, based on a smaller set of loci, deduced that papillate sporangia could also be a plesiomorphic trait. Clade 3, which was considered papillate by Kroon *et al.* (2003), was found to sister to clade 6 in this study, although the support for this grouping, and also the further clustering of clades 3 and 6 with clade 7, was low. An alternative placement closer to the other predominantly papillate clades can therefore not be ruled out at present, although moderate support for a sister-group relationship of clades 3 and 6 was also observed in the AU analysis. In line with Blair *et al*. (2008), *P. quercina*, which was considered a member of clade 3 in Cooke *et al.* (2000), was placed in clade 4, and is referred to as clade 4.1 in this study, as this species was found to be basal to the group of the other members of clade 4 and the downy mildews. This placement received varying support in analysis of the full dataset and strong support in the reduced dataset. The predominantly non-papillate clades 6–10 were found predominantly in a basal position with respect to the crown group, providing evidence that the non-papillate stage might be ancestral, and the development of semi-papillate sporangia in clade 8b and clade 9 (*sensu* Blair *et al*. 2008) represents a homoplasy. Clade 9 (including clade 10) was found to be separated from the other *Phytophthora* clades with strong support and represented the most basal clade of *Phytophthora*. As was previously attested by Cooke *et al*. (2000), no obvious phylogenetic pattern with respect to temperature or climate adaptation can be observed from the phylogenetic analyses.

Cooke *et al*. (2000) doubted if the species in these clades could be retained in *Phytophthora* and stated that it is likely that further investigation would lead to their exclusion from *Phytophthora*. As revealed in this study, paraphyly of *Phytophthora* is pronounced, rendering *Phytophthora* a typical example of a paraphyletic genus, with the most derived linages sharing some synapomorphies with downy mildews, while the more basal clades are more similar to *Halophytophthora*, *Phytopythium* and *Pythium*. This is similar to the situation in *Peronosporales* as a whole, for which Hulvey *et al.* (2010) recently proposed a broad circumscription of *Peronosporaceae*, encompassing all downy mildew genera, *Halophytophthora*, and *Phytopythium*, to avoid the description of several new, poorly differentiated families. If a similar option were chosen for the genus *Phytophthora*, this would mean an inclusion of all downy mildew genera and *Phytophthora* into a single genus. The oldest available name for this assemblage on genus level would be *Peronospora* (Corda 1837), which was described much earlier than *Phytophthora* (de Bary 1876), thus, if *Phytophthora* were not conserved that would necessitate the inclusion of about 300 species of downy mildews, currently placed in other well-defined and widely accepted genera, e.g. *Basidiophora*, *Bremia*, *Plasmopara*, *Peronosclerospora*, *Pseudoperonospora*, and *Scleropsora* (Thines 2006, Voglmayr 2008), and about 100 species of *Phytophthora* (Waterhouse 1963, Erwin & Ribeiro 1996, Érsek & Ribeiro 2010) into this genus. This would not only be a nomenclatural nightmare but would also result in a highly heterogeneous group, encompassing species with divergent physiological, ecological, and morphological properties. For these reasons, but also because even more name-changes would be necessary, conservation of *Phytophthora* and an inclusion of all downy mildew genera (necessitating about 400–500 name changes for *Peronospora* alone), is not preferable. If this option were chosen, 700–800 names would have to be changed, including many well-known pathogens in the genera *Bremia* (e.g. *Bremia lactucae*), *Plasmopara* (e.g. *Plasmopara viticola* and *Pl. halstedii*), *Hyaloperonospora* (*Hyaloperonospora brassicae*, *H. arabidopsidis*, *H. parasitica*), and *Peronospora* (e.g. *Pe. tabacina*, *Pe. destructor*, *Pe. effusa*, *Pe. farinosa*, *Pe. lamii*).

An alternative solution would be to resolve the paraphyly of this group by introducing new generic names where none existed for the lineages not belonging to the monophyletic subtree that includes *Phytophthora infestans* (the type species of *Phytophthora*). Judging from the results of this study, *Phytophthora* is at least six times paraphyletic as revealed by the phylogenetic investigations, but possibly seven times paraphyletic with respect to the downy mildews judging from the results obtained from the statistical tests. This would necessitate the introduction of new generic names (or the adoption of currently unused generic names) for clades 4.1, 4.2, 8, and the group (9, 10). In addition to these clusters, additional generic names would have to be introduced for groups formed by members of clades 2, 3, 5, 6, and 7. In the phylogenetic analysis, while the groups (2, 5) and (3, 6, 7) were observed, their monophyly could not be ascertained; indeed, some support for alternative clusters (3, 6) and (5, 7), with clade 2 as an independent linage, was received in statistical tests. Several loci will need to be added in future phylogenetic studies to clarify the evolutionary relationships of these groups. Based on the current data, it can be assumed that *Phytophthora* is at least six, but possibly seven times paraphyletic with respect to downy mildews. Species of clade 1, which include the economically most important pathogen of the genus, *Phytophthora infestans*, as well as the well-known pathogens, *P. nicotianae* and *P. cactorum*, would retain their original names. This solution would need only a quarter of the name changes (less than 100) needed for the first option (inclusion of all downy mildew and *Phytophthora* species into *Peronospora*), and only about 15 % of the name changes that would be needed if *Phytophthora* were conserved and all downy mildews were transferred into this genus. In addition, it would leave the names of most of the most important pathogens of the *Peronosporaceae* unchanged, like *Bremia lactucae*, *Hyaloperonospora brassicae*, *Phytophthora infestans*, *Plasmopara halstedii*, *Plasmopara viticola*, *Pseudoperonospora cubensis* and

Pseudoperonospora humuli. Therefore, we feel that this solution is to be preferred. But to introduce the new names for the clades outlined above will necessitate a search for characters defining synapomorphies for these groups, which might not be easy, judging from the apparent discrepancies between the morphological classification of Waterhouse (1963), and recent phylogenetic studies (Cooke *et al*. 2000, Kroon *et al.* 2004, Blair *et al.* 2008). Probably, these genera might have to be defined with the aid on DNA sequence synapomorphies, rather than only morphology. But retaining the usage of the generic name *Phytophthora* for all the at least six monophyletic groups between *Halophytophthora* and at the same time retaining the 19 downy mildew genera, would not only be contrary to the widely accepted idea of ideally having monophyletic taxa only, but also hamper the awareness of the unique evolution of these organisms, stepwise towards obligate biotrophy (Thines & Kamoun 2010). For example, in terms of evolution, *Phytophthora infestans* is much closer to downy mildews than to *P. sojae* or even *P. ramorum*. But for the understanding of the evolution of obligate biotrophy, which is one of the most fascinating and fundamental evolutionary tipping points for any group of pathogens, it will be even more important to obtain genome sequences for members of the clades 4.1 and 4.2, which are apparently the closest relatives of the downy mildews, and of the neglected species of *Phytophthora* affecting *Cyperaceae*.

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