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ORIGINAL ARTICLE

# Multigene analyses with a broad sampling in *Phytophthora* and related genera provide evidence for the monophyly of downy mildews

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## Abstract

Downy mildews are the most species-rich group of oomycetes, with more than 700 known species. The relationships within the main downy mildew lineages (i.e. the downy mildews with pyriform haustoria, the downy mildews with coloured conidia, and the brassicolous downy mildews) are increasingly well resolved, and 20 well-characterised monophyletic genera have been described. However, their relationships to each other, the various lineages of graminicolous downy mildews, and to the species subsumed in *Phytophthora* are still unresolved. Recent phylogenomic studies have suggested a polyphyly of the downy mildews, but with a limited taxon sampling within *Phytophthora*. As taxon sampling is crucial for inferring relationships between large groups, we have conducted a multigene analysis with a set of 72 *Phytophthora* species and included all known downy mildew lineages. In addition, we performed approximately unbiased (AU) testing as an additional approach to evaluate major nodes. Our analyses resolve the downy mildews as a monophyletic assemblage in all phylogenetic algorithms used. We thus conclude that the evolution of the obligate biotrophy characteristic of downy mildews was a singular event and that all downy mildew pathogens can be traced to a single ancestor.

**Keywords** Evolution · *Kawakamia* · Morphology · Obligate biotrophy · Oomycetes · *Peronophythora* · *Peronosporaceae* · *Phloeophthora* · Polyphyly · One new taxon

## Introduction

Downy mildews are obligate biotrophic pathogens parasitic to a broad number of angiosperm hosts (Thines and Choi 2016). Traditionally, they have been placed in a family of its own, the *Peronosporaceae*, which was usually opposed to the cultivable *Pythiaceae*, which members have a similar sexual reproduction, but less differentiated sporangiophores (Dick 2001). The genus *Phytophthora* was included within the latter family, as the vast majority of species can be cultivated and most do not produce highly differentiated sporangiophores. However, already early phylogenetic investigations (Cooke et al. 2000; Riethmüller et al. 2002) have revealed a very close relationship of *Phytophthora* and downy mildews and multigene phylogenies have demonstrated that the downy mildews are indeed nested within the genus *Phytophthora* (Göker et al. 2007), rendering the genus *Phytophthora* paraphyletic (Runge et al. 2011a). Thus, the classification of the *Peronosporaceae* was revised to include the genus *Phytophthora* and other related genera (*Calycofera*, *Halophytophthora*, *Globisporangium*,

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*Nothophytophthora*, *Phytopythium*) to avoid an inflation of ill-delineated taxa on the family level within the *Peronosporales* (Hulvey et al. 2010; Beakes and Thines 2017).

To delineate genera in downy mildews, sporangiophore and haustorium morphology are key features that have enabled the identification of monophyletic lineages (Constantinescu 1989, 1998; Constantinescu and Fatehi 2002; Göker et al. 2003; Voglmayr et al. 2004; Constantinescu et al. 2005; Thines et al. 2006, 2007, 2015; Telle and Thines 2012). All of the 20 currently accepted genera of downy mildews are monophyletic and well characterised (Telle and Thines 2012; Thines et al. 2015; Thines and Choi 2016).

Downy mildew diseases are characterised by an interaction, in which the plant usually shows some chlorotic discolouration as a result of infestation, and which remains biotrophic throughout the entire asexual life cycle, from the establishment of the infection to the sporulation of the pathogen through the stomata of the host. Many species of downy mildew can cause severe economic losses, such as *Bremia lactucae* in lettuce (Lebeda et al. 2008; Wroblewski et al. 2007), *Peronospora belbahrii* in basil (Belbahri et al. 2005; Thines et al. 2009), *Peronospora effusa* in spinach (Choi et al. 2015), *Plasmopara destructor* in balsamines (Görg et al. 2017), *Plasmopara viticola* in grapes (Salinari et al. 2006; Fontaine et al. 2013), and *Pseudoperonospora cubensis* in cucurbits (Lebeda and Cohen 2011; Runge et al. 2011b).

Species currently classified in the genus *Phytophthora* establish necrotrophic to hemibiotrophic interactions with their hosts (Erwin and Ribeiro 1996; Lee and Rose 2010; Jupe et al. 2013). Several of these species are causing destructive diseases, such as potato late blight caused by *Phytophthora infestans* (Haas et al. 2009, Yoshida et al. 2013) and sudden oak death caused by *Phytophthora ramorum* (Rizzo et al. 2002; Grünwald et al. 2019). Of special concern are some species with broad host ranges that affect a variety of crops, such as *Phytophthora capsici* (Hausbeck and Lamour 2004; Lamour et al. 2012), *Phytophthora cinnamomi* (Burgess et al. 2017), and *Phytophthora palmivora* (Brasier and Griffin 1979; Ali et al. 2017). While it seems clear that *Phytophthora* is paraphyletic or polyphyletic with respect to the downy mildews (Göker et al. 2007; Runge et al. 2011a; Sharma et al. 2015), the relationships of various well-defined monophyletic clades are still not fully resolved (Kroon et al. 2004; Blair et al. 2008; Martin et al. 2014; Yang et al. 2017), and the search for synapomorphies that could be used for delineation from other clades is difficult, none of the characters previously used for delineating *Phytophthora* groups (Waterhouse 1963) fully coincide with the phylogenetic structure of the genus (Cooke et al. 2000; Kroon et al. 2004; Blair et al. 2008). Thus, while it is clear that *Phytophthora* requires taxonomic revision (Runge et al. 2011a, b), only a minor step towards this has been taken until now (Thines 2023), since the infrageneric relationships of *Phytophthora* are unresolved (Martin et al. 2014; Yang et al. 2017).

The question of whether the downy mildews are monophyletic or not has been the subject of much dispute over the past years, as phylogenomic approaches with a limited taxon sampling in *Phytophthora* have often, but not always, supported downy mildew polyphyly (McCarthy and Fitzpatrick 2017; Yang et al. 2017; Bourret et al. 2018; Fletcher et al. 2019).

As there are still rather few genomes of *Phytophthora* species and downy mildew pathogens available, it was the aim of the current study to test the hypothesis that downy mildews are monophyletic by thorough multigene phylogenetic reconstructions. For this, we included all major oomycete lineages and in addition *Kawakamia cyperi*, an obligate biotrophic pathogen that causes downy mildew-like symptoms on various sedge species (Miyabe 1904), yet had been usually included in *Phytophthora* (Erwin and Ribeiro 1996). Furthermore, we also included *Peronophythora litchi*, a unique pathogen that is cultivable and had been included in *Phytophthora* (Göker et al. 2007), yet forms sporangiophores with considerable complexity (Chen 1961; Ye et al. 2016). To add another line of evidence, we performed AU analyses in addition to phylogenetic reconstructions to test the topology of major associations found in this study.

## Materials and methods

### Oomycete material and microscopy

Material of *Kawakamia cyperi* from *Cyperus esculentus* was collected from fields in Idaho in August 2012. Samples were dried between tissue paper and deposited in the herbarium Senckenbergianum (FR) under the accession number FR-0046142.

For microscopy, specimens were hydrated with 5% chloral hydrate solution and sporangia and sporogenous hyphae carefully scraped off the surface using a scalpel blade. The oomycete material was transferred to 5% chloral hydrate solution on a slide and covered with coverslips. Microscopic observations were made in DIC at × 400 magnification using a Zeiss Imager2 (Zeiss, Oberkochen, Germany) equipped with a Zeiss Axiocam with 3.2 Mp resolution. Measurements were done from images taken after calibration with the Zeiss Axiovision software. Measurements are reported as (maximum –) mean minus standard deviation – mean – mean plus standard deviation (– maximum).

## DNA, extraction, PCR, and sequencing

DNA of *Kawakamia cyperi* was extracted using the innuPREP Plant DNA Kit (Analytik Jena GmbH, Jena, Germany) as outlined in Telle and Thines (2008). Seven genes (*COX*2, nrLSU, *HSP90*, *EF1A*, *60S*, *ENOLASE*, and *ß-TUB*) were amplified and sequenced using the primers given in Table 1. For four genes new primers were designed. PCR reactions were conducted using MangoTaq reaction buffer (colourless), 200 µM dNTPs, 2 mM $MgCl_2$, 0.75 U MangoTaq polymerase (Bioline, Luckenwalde, Germany), 0.8 mg/mL BSA (bovine serum albumin), and 400 µM of each primer (Merck KGaA, Darmstadt, Germany) in an Eppendorf mastercycler ProS with vapo.protect lid (Eppendorf, Hamburg, Germany) using the programs given in Table 2. Because the qualities of the sequences of three genes (*HSP90*, *ENOLASE*, and *ß-TUB*) were poor, the PCR products were cloned in *Escherichia coli* using the CloneJET PCR cloning kit (ThermoFisherScientific, Waltham, USA) following the instructions provided with the kit. The inserts were amplified and sequenced using the provided plasmid primers. All PCR products were sequenced at the Senckenberg Biodiversity and Climate Research Centre (SBiK-F). For the cloned genes, forward and reverse sequences of at least three clones were used to assemble a consensus sequence to avoid PCR artefacts.

## Phylogenetic reconstructions

The reference sequences for all *Pythium* and *Phytophthora* specimens were obtained from the study of Blair et al. (2008). Furthermore, the alignment was complemented by the addition of sequences extracted from the published genomes of *Peronophythora litchii* (Ye et al. 2016, referred therein as *Phytophthora litchii*) and *Phytophthora podocarpi* (Studholme et al. 2015), as well as the sequences of *Ph. arenaria* (CBS 127950) and *Ph. alticola* (CBS 121939) available as individual genes on NCBI (https://www.ncbi.nlm.nih.gov/). The sequence data of *Pseudoperonospora cubensis* originate from Runge et al. (2011a, b); those of *Hyaloperonospora thlaspeos-perfoliati* were extracted from preliminary genome data; and those of the downy mildews *Sclerospora graminicola* (Kobayashi et al. 2017), *Plasmopara halstedii* (Sharma et al. 2015), *Plasmopara muralis* and *Plasmopara viticola* (Dussert et al. 2019), *Peronospora effusa* (Klein et al. 2020), *Peronospora belbahrii* (Thines

**Table 1** Primers with references or sequences used in this study

| Gene | Forward primer | | Reverse primer | |
|---|---|---|---|---|
| *COX2* | cox2F | Hudspeth et al. (2003) | cox2R | Hudspeth et al. (2003) |
| nrLSU | LR0R-O | Moncalvo et al. (1995) | LR6-O | Riethmüller et al. (2002) |
| *EF1A* | EF1A_for | Kroon et al. 2004 | EF1A_rev | Kroon et al. 2004 |
| *HSP90* | HSP90_F1 | Blair et al. 2008 | HSP90_SP_R* | 5′-TCCTTCATNAGCTTGCACA |
| *60S* | 60S_SP_F* | 5’-TGYTAYCGTTTCCARAACAAGAA | 60S_SP_R* | 5′-GGRAATTTRAACTTRGCACG |
| *ENOLASE* | Enl_FY | Blair et al. 2008 | Enl_SP_R* | 5′-GCRATRAACGTGTCCTC |
| *ß-TUB* | Btub_SP_F2* | 5’-GCYGGTAAYAAYTGGC | Btub_SP_R2* | 5′-CCARTGCAAGAAWGCYTTACGA |

*Primers designed in this study

**Table 2** PCR programs used in this study

| | *COX2* | nrLSU | *60S, EF1A, ß-TUB* | *HSP90, ENOLASE* |
|---|---|---|---|---|
| 1 | 96 °C/4:00 min | | 96 °C/10:00 min | |
| 2 | 96 °C/0:20 min | | 96 °C/0:20 min | |
| 3 | 51 °C/0:40 min | 56 °C/0:40 min | 53 °C*/0:40 min | 60 °C*/0:40 min |
| 4 | 72 °C/0:40 min | 72 °C/1:00 min | 72 °C/2:00 min | |
| | Repeat steps 2–4 36 times | | Repeat steps 2–4 10 times | |
| 5 | 72°C/4:00 min | | 96 °C/0:20 min | |
| 6 | | | 48 °C/0:40 min | 55 °C/0:40 min |
| 7 | | | 72 °C/2:00 min | |
| | | | Repeat steps 5–7 30 times | |
| 8 | | | 72 °C/10:00 min | |
| Hold at 10 °C | | | | |

*temperature was decremented by 0.5 °C per cycle

et al. 2020), and *Peronospora tabacina* (Derevnina et al. 2015) were extracted from published genomes. A summary of the data used and the corresponding accession numbers is provided in Supplementary File 1. Despite the availability of additional genomes, full-length high-quality (i.e. without frameshift mutations in core genes or a high number of ambiguities) data of all genes used in this analysis could not be obtained, which is why those data were not included. The seven genes present for all species included were aligned separately using the Mafft webserver applying the G-INS-i algorithm for the protein-coding genes and Q-INS-i for the nrLSU gene. All further parameters were set to default (https://mafft.cbrc.jp/alignment/server/; Katoh et al. 2019). The seven alignments were manually trimmed to remove leading and trailing gaps and subsequently merged to a final alignment with a length of 5240 positions. A partitioning file was prepared including codon-based gene partitioning for all genes except for the non-protein coding nrLSU gene resulting in 19 partitions. This was optimized using PartitionFinder 2 (Lanfear et al. 2016) applying the greedy search algorithm (Lanfear et al. 2012) and setting the branch length parameter to unlinked. Phylogenetic reconstructions were done using the maximum likelihood (ML) algorithm as well as minimum evolution (ME). Maximum likelihood analyses was conducted using IQ-TREE (version 2.2, Nguyen et al. 2015) applying a partitioned analysis (Chernomor et al. 2016) with the best scheme from PartitionFinder 2. All other parameters were set to default and bootstrapping (Felsenstein 1985) was performed with 1000 non-parametric replicates. Minimum evolution analysis was conducted with MEGA (version 7, Kumar et al. 2016) applying the minimum evolution method (Rzhetsky and Nei 1992) with 1000 bootstraps (Felsenstein 1985) and the Tamura-Nei algorithm (Tamura and Nei 1993). The gamma distribution shape parameter was estimated by the tool provided by MEGA and set to 0.2695. The patterns among lineages parameter were set to different (heterogenous).

In order to test the phylogenetic affiliations of the downy mildew subclades with itself and with the clades of *Phytophthora*, the approximately unbiased (AU) test (Shimodaira 2002) was applied (Table 3). For this, the site-wise log-likelihoods for 94 associations were calculated using IQtree (Nguyen et al. 2015) with a reduced alignment containing 35 specimens including one downy mildew species from every genus and representatives of the clades and subclades of *Phytophthora*, according to Yang et al. (2017). The AU-test using the TREEASS program of the CONSEL package (Shimodaira and Hasegawa 2001) was conducted as described in Runge et al. (2011a, b) with *Ph. boehmeriae* set as outgroup. All files used in the analyses are provided in Supplementary File 2.

To satisfy a request during review, we also downloaded the alignment of Bourret et al. (2018) for analysis. From that dataset, we removed leading and trailing ends, sequences

**Table 3** Results of the site-wise log-likelihoods generated for associations previously observed in phylogenomic studies and multigene phylogenies

| Possible association | OBS | AU | NP | BP | PP | KH | SH | WKH | WSH |
|---|---|---|---|---|---|---|---|---|---|
| (DMall, 4, 1) | 0 | 0.823 | 0.138 | 0.003 | 0.077 | 0.507 | 1.000 | 0.507 | 1.000 |
| (DMall) | 0 | 0.516 | 0.481 | 0.487 | 0.436 | 0.469 | 1.000 | 0.494 | 1.000 |
| (DMall, 4) | 0 | 0.439 | 0.114 | 0.113 | 0.207 | 0.507 | 1.000 | 0.507 | 1.000 |
| (DMall, 1) | 4.4 | 0.413 | 0.062 | 0.060 | 0.001 | 0.262 | 0.990 | 0.262 | 0.962 |
| (DMall, 5) | 20.5 | 0.174 | 0.032 | 0.030 | 0 | 0.105 | 0.857 | 0.105 | 0.696 |
| (DMall, 4, 5) | 28.0 | 0.042 | 0.002 | 0.002 | 0 | 0.025 | 0.740 | 0.024 | 0.290 |
| (Kacy, DM2, 4) | 104.7 | 0 | 0 | 0 | 0 | 0.001 | 0.015 | 0 | 0.002 |
| (Kacy, DM2, 1) | 110.1 | 0 | 0 | 0 | 0 | 0 | 0.005 | 0 | 0 |
| (Kacy, DMPH, 4) | 114.2 | 0 | 0 | 0 | 0 | 0 | 0.004 | 0 | 0 |
| (DM2, 4) | 114.4 | 0 | 0 | 0 | 0 | 0 | 0.004 | 0 | 0 |
| (Kacy, DMPH, 1) | 116.8 | 0 | 0 | 0 | 0 | 0 | 0.007 | 0 | 0 |
| (Kacy, DM2, 2) | 118.3 | 0.001 | 0 | 0 | 0 | 0 | 0.003 | 0 | 0.001 |
| (DM2,1) | 118.4 | 0 | 0 | 0 | 0 | 0 | 0.003 | 0 | 0 |
| (DM2, 5) | 129.7 | 0 | 0 | 0 | 0 | 0 | 0.002 | 0 | 0 |
| (Kacy, DMPH, 5) | 129.8 | 0 | 0 | 0 | 0 | 0 | 0.002 | 0 | 0 |

Columns show the observed log-likelihood differences of the edges (OBS) and support values for the following tests: approximately unbiased (AU), bootstrap probability (NP, BP; and PP), Kishino-Hasegawa (KH), Shimodaira-Hasegawa (SH), weighted Kishino-Hasegawa (WKH), weighted Shimodaira-Hasegawa (WSH). Kacy: *Kawakamia cyperi*, DMPH: *Plasmopara halstedii*, *Bremia lactucae*; DM2: *Sclerospora graminicola*, *Hyaloperonospora thlaspeos-perfoliati*, *Peronospora tabacina*; *Pseudoperonospora cubensis*; DMall: Kacy, DMPH, DM2; clade 1: *Phytophthora cactorum*, *Ph. nicotianae*, *Ph. clandestina*, *Ph. infestans*; clade 2: *Ph. bisheria*, *Ph. multivesiculata*, *Ph. citricola*, *Ph. capsici*, *Ph. colocasiae*; clade 3: *Ph. psychrophila*, clade 4.1: *Ph. quercina*; clade 4.2: *Peronophythora litchi*; clade 5: *Ph. heveae*

with large amounts of missing data, and gapped sites. This filtering was done to remove the larger part of noise incited by bad quality sequences, and the resulting alignment can be retrieved from Supplementary File 3. Subsequently, the alignment was subjected to ME analysis in MEGA7, using default parameters, except for choosing the Tamura-Nei substitution model (most complex standard model offered by MEGA7) and 500 bootstrap replicates. In addition, ML and ME were done in FastTree2 and RAxML, as implemented on the TrEase webserver (www.thines-lab.senckenberg.de/trease), using 500 and 1000 bootstrap replicates, respectively. This set was meant to be used only for a rough checking, if the signal found in our detailed analyses would also be retrieved from the dataset of Bourret et al. (2018), if only a rather simple quality enhancement would be done.

## Results

### Morphology

*Kawakamia cyperi* was observed to have irregular sporangiophores with a slightly thickened wall (Fig. 1 A–D). Sporangia were often clustered (Fig. 1 M), similar to *Sclerophthora*, with a conspicuous papilla that was visible already early during sporangial development (Fig. 1 D, E) and mostly with a broad pedicel, tapering towards the ultimate branchlet (Fig. 1 F–J). Sporangia ($n = 100$) were (24.5–)31.5–36.5–41.5(–48) µm long and (16.2–)19.5–22.5–25.5(–28.5) µm broad, with a length to breadth ratio of (1.33–)1.46–1.63–1.8(–2.09). Haustoria were pyriform to broadly lobed, often more than one per host cell (Fig. 1 K, L). Antheridia were diclinous or monoclinous, mostly paragynous. Oospores ($n = 14$) were pale ochre to pale brown, thick walled, aplerotic (Fig. 1 N–Q), (23–)25.5–28–30.5(–32.5) µm in outer diameter, inner diameter (17–)19–21.5–23(–25.5) µm, the ratio of the outer to the inner diameter was (1.22–)1.26–1-32–1.38(–1.5), and the wall thickness was (2.5–)3–3.5–4(–4.5) µm.

### Phylogeny

The phylogeny of the individual loci (*COX2*, nrLSU, *HSP90*, *EF1A*, *60S*, *ENOLASE*, and *ß-TUB*) did not yield support for conflicting topologies and, thus, all loci were concatenated for phylogenetic analysis, resulting in an alignment of 5240 positions. Phylogenetic reconstructions in maximum likelihood (ML) and minimum evolution (ME) resulted in largely congruent topologies with no support for conflicting topologies. Thus, only the tree from the ML analysis is shown in Fig. 2, with bootstrap support (BS) from ML and ME inference added on the branches in the respective order.

In all phylogenetic analyses, the downy mildews, including *Kawakamia cyperi*, formed a monophyletic clade with maximum support. Within this clade the *Plasmopara* specimens and *Bremia lactucae* were grouped together with strong support in both analyses. Together with *Sclerospora graminicola* and *Kawakamia cyperi*, they formed a subclade that received some support in ML. *Pseudoperonospora cubensis*, belonging to one of the two genera with coloured conidia, was inferred as the sister lineage to all other downy mildew genera, even though without support.

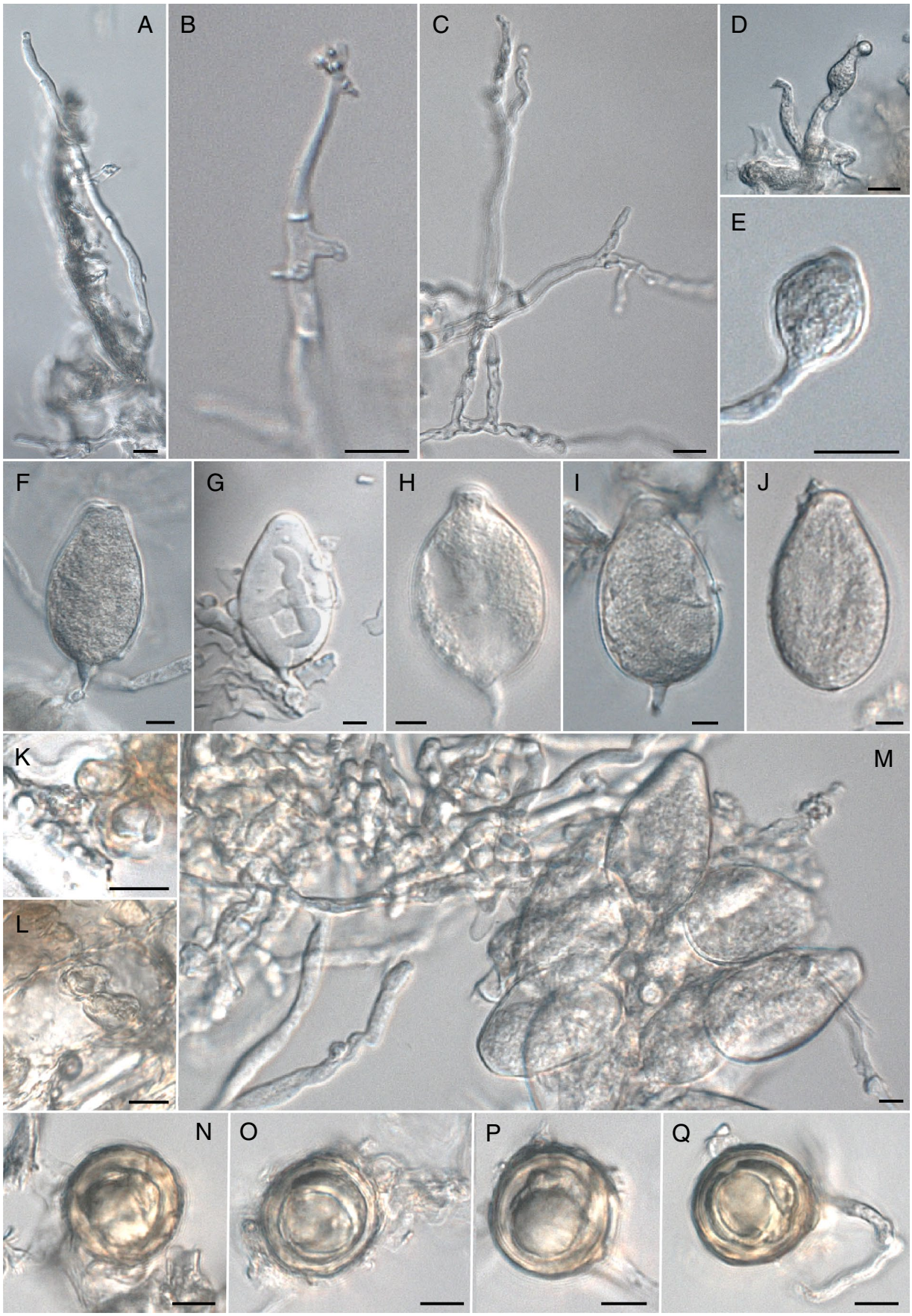
Clade 4 of *Phytophthora s.*l., including *Peronophythora litchii*, the pathogen causing litchi downy blight in East Asia, was resolved as monophyletic with considerable support (80% and 97% BS) and found to be the sister group to clade 12 with no to moderate support (89% BS in ME). *Phytophthora* clade 1, which contains *Phytophthora infestans*, the type species of the genus, was resolved with strong support (99% and 92% BS). The monophyly of clade 2 also received strong support (99% and 94% BS). While the relationships of clades 1, 2, and 4, and the downy mildews could not be resolved, they collectively formed a monophyletic group with no to moderate support (84% BS in ME).

Clades 3, 5, and 6 received maximum support for their monophyly, while the monophyly of clade 7 received maximum to high support (100% and 99% BS). Collectively, these formed an unsupported group together with *Phytophthora podocarpi*. The remaining groups were clade 8 and a group consisting of species of clades 9 and 10, which both received good support for their monophyly (99% and 81% BS, as well as 100% and 99% BS, respectively). The relationships of the monophyletic groups to each other could not be resolved.

Phylogenetic analyses of the dataset of Bourret et al. (2018) after removing some missing data and bad quality sequences resulted in resolving the downy mildews as monophyletic in ME analyses, with maximum support in the reconstruction with FastTree2, and moderate support in the reconstruction done with MEGA7 (Supplementary File 4). However, in ML the DM were split into two clades (see discussion of the signal erosion artefact known from ML analyses).

### Approximately unbiased (AU) testing

As the multigene phylogeny with high sampling from existing sequence information in both *Phytophthora* and the downy mildews revealed downy mildew monophyly in contrast to all phylogenomic investigations with low taxon sampling, except Ye et al. (2016), AU testing was carried out. In the AU testing the topology found in the current multigene phylogeny was tested against diverging topologies found in phylogenomic studies with limited sampling in both *Phytophthora* and downy mildews. These were a splitting of downy mildews, as observed in Sharma et al. (2015), McCarthy and Fitzpatrick (2017), Kobayashi et al. (2017), Bourret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◂**Fig. 1** Microscopic features of *Kawakamia cyperi* on *Cyperus esculentum*. **A**–**C** sporangiophores; **D**, **E** developing sporangia; **F**–**J** mature sporangia with variable pedicels; **K**, **L** haustoria; **M** clustered sporangia; **N**–**Q** oogonia with oospores. Bar = 5 µm in all pictures, except for N–Q, where they are 10 µm

et al. (2018), Fletcher et al. (2019), Dussert et al. (2019), and Klein et al. (2020), an association of *Plasmopara* (here in DMPH) with clade 1 (Sharma et al. 2015; McCarthy and Fitzpatrick 2017; Bourret et al. 2018; Fletcher et al. 2019; Dussert et al. 2019; Klein et al. 2020), an association of some downy mildews with clade 5 of *Phytophthora* (Bourret et al. 2018), an association of clade 1 with *Hyaloperonospora* (here in DM2), and a grouping of clade 4 not with downy mildews (Ye et al. 2016). The AU testing conducted in this study (Table 3) refuted all associations that would not result in a monophyly of the downy mildews or a grouping of all downy mildews with clades 1 or 4 (Table 3).

## Taxonomy

A new combination for *Phytophthora cyperi-bulbosi* in *Kawakamia* is made here, as all morphological features and the obligate biotrophy of the pathogen support this placement.

***Kawakamia cyperi-bulbosi*** (Seethal. & K. Ramakr.) Thines, **comb. nov.**, MycoBank MB847402

*Basionym*: *Phytophthora cyperi-bulbosi* Seethal. & K. Ramakr., Curr. Sci. 22(5): 150 (1953)

## Discussion

### Evolution of downy mildews

Even though there is now a general agreement that the genus *Phytophthora* is closely related to the downy mildews and thus needs to be treated in the *Peronosporaceae* instead of the *Pythiaceae* (Cooke et al. 2000; Riethmüller et al. 2002; Göker et al. 2003, 2007; Thines 2009; Hulvey et al. 2010; Beakes and Thines 2017), the relationship between downy mildews and members of the genus *Phytophthora* s.l. has been elusive. While some studies inferred a monophyly of the downy mildews (Göker et al. 2007; Ye et al. 2016), others have concluded that the downy mildews are polyphyletic (Sharma et al. 2015; McCarthy and Fitzpatrick 2017; Bourret et al. 2018; Dussert et al. 2019; Fletcher et al. 2019; Klein et al. 2020). In this study, which included a broad sampling of *Phytophthora* based on Blair et al. (2008) and all downy mildew lineages, the downy mildews formed a monophyletic clade with maximum support in all analyses. This result was confirmed by the AU analyses, which were not performed in previous studies inferring a polyphyly of downy mildews, and which rejected all topologies observed in previous studies that suggested a potential polyphyly of the downy mildews.

That phylogenomic studies yielded topologies inconsistent with a monophyly of the downy mildews has been seen as evidence that obligate biotrophy and downy mildew characteristics have evolved at least twice in the *Peronosporaceae*. However, high bootstrap support only means that the same topology is produced when alignment columns are randomly sampled, which is to be expected in case of extremely large datasets, as the randomisation effect is low. The problem with phylogenomic investigations is that due to the very large amount of data, phylogenetic signal heterogeneity is too equally distributed to be readily picked up by bootstrapping resulting in very high support values for almost any given branch. For example, this can be observed in a recent study by Winkworth et al. (2022), where the topology observed by Bourret et al. (2018) and characterised as discordance between mitochondrial and nuclear loci is highly supported based on bootstrapping of a dataset with complete mitochondrial genomes. Quartet sampling, which is less prone to saturation artefacts, is a potential alternative to bootstrapping, but is still not widely used (Klötzl and Haubold 2016; Pease et al. 2018). However, the topologies of such phylogenies can change significantly, if additional taxa are included, especially in a situation where deep branching is difficult to resolve and incomplete lineage sorting at major splits might have occurred (Mishra et al. 2018). This means that broad taxon sampling is crucial in phylogenomic analyses, which is complicated by the still low amount of genomes available. In addition, especially when including diverse lineages with highly divergent mutation rates, alignments are often not straightforward, leading to incorrect assignment of homology. A way to avoid this is to focus on highly conserved genes that can be unambiguously aligned, and to exclude alignment parts where homology assignment is low (Philippe et al. 2000; Kück et al. 2010, 2014; Wu et al. 2012; Mishra et al. 2018). This approach will lead to a massive reduction in data, but avoids various artefacts, thus providing more credible phylogenetic reconstructions. It is notable that a strict filtering of genomic data will result in datasets with rather few high confidence genes, not too far from "normal" multigene analyses. Thus, multigene phylogenies using well-characterised and alignable loci on a comprehensive taxon sampling are probably better suited for inferring relationships difficult to disentangle than phylogenomic analyses using a large amount of ill-characterised loci on a comparatively small dataset. However, once comprehensive amounts of genomes of downy mildews and all *Phytophthora* lineages have become available, the approaches mentioned above are likely to provide a more detailed picture of the relationships between downy mildew and *Phytophthora* s.l. lineages.

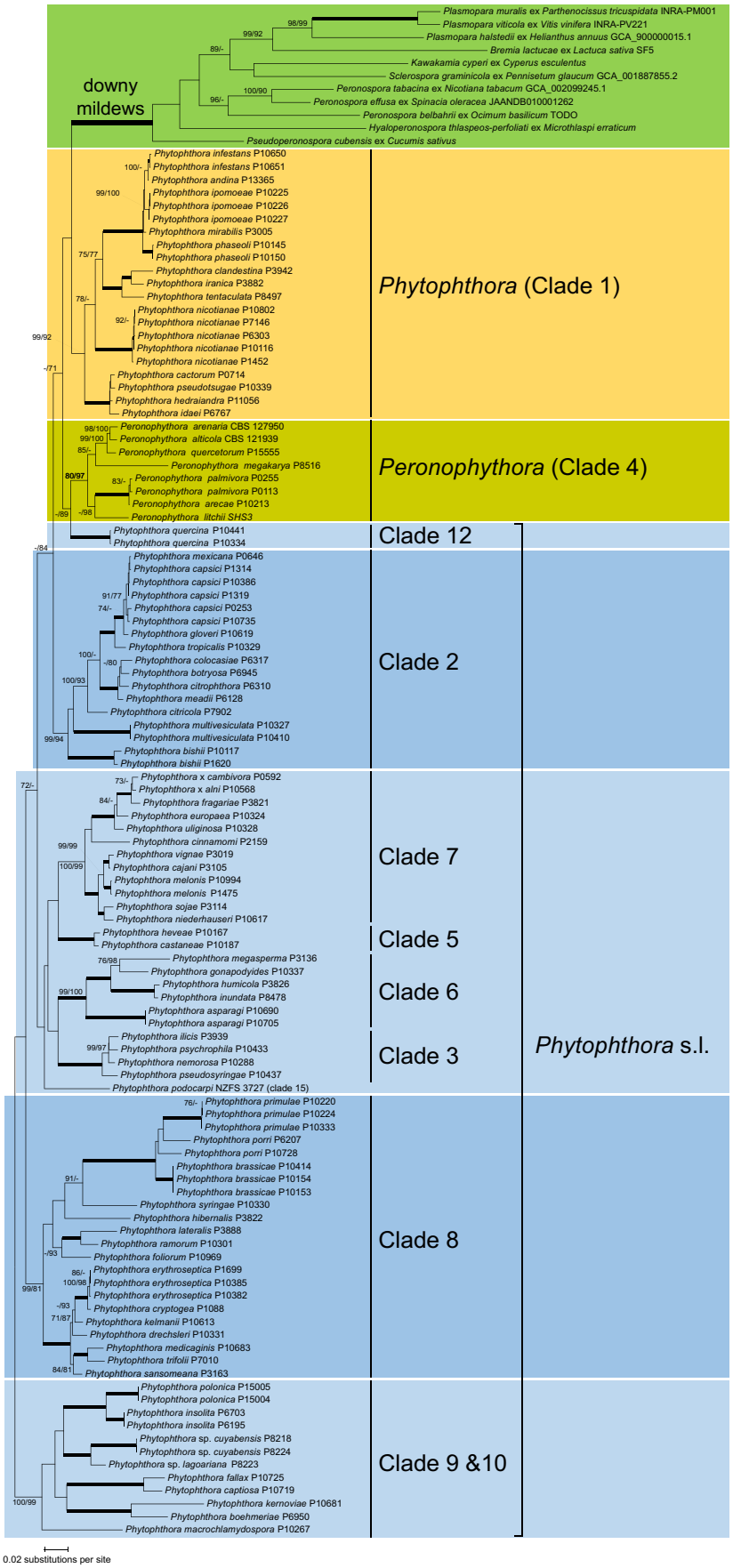


**Fig. 2** Phylogenetic reconstruction in maximum likelihood, with support values from maximum likelihood and minimum evolution, in the respective order. A minus sign denotes lack of support for the displayed or a conflicting topology. Thickened branches indicate maximum support in both analyses

It should be noted that for an unknown reason, the addition of *Ph. podocarpi* to the dataset is a major cause of disturbance in the phylogenetic signal. Without its inclusion, the monophyly of the downy mildews and their sister-group relationship to clade 4 is supported with maximum and high support values (data not shown). With its inclusion, the monophyly of downy mildew still received maximum support, but the support for grouping the different clades of *Phytophthora* and to position of the monophyletic downy mildews within them becomes unresolved. We assume that the cause of this might be incomplete lineage sorting due to a very quick radiation of *Phytophthora* lineages at the time downy mildews emerged. However, this clearly needs further careful evaluation once even more genomes become available. In any case, phylogenetic reconstructions based on lineages with largely differing mutation rates, such as the downy mildews and *Phytophthora*, can lead to significant artefacts in likelihood analyses (Struck et al. 2014; Redmond and McLysaght 2021; Susko and Roger 2021), even when a correct model is assumed (Kück et al. 2012), a problem which, alongside various other assumptions (Bromham 2019), has so far not been widely taken into consideration. One of the major issues with the likelihood assumption is that signal erosion can lead to a shifting of long branches to the inclusion of various short branches in between (Kück et al. 2012) or a grouping of long branches with short branches sharing some sequence similarities (Fleming et al. 2023). Thus, as both morphology and minimum evolution phylogenetic reconstructions support a monophyly of the downy mildews, also when using a quality-filtered dataset from Bourret et al. (2018), we assume that the splitting of the downy mildews in ML observed when the dataset of Bourret et al. 2018, but not in the current dataset, is the result of a branch-length heterogeneity artefact.

It is noteworthy that *Kawakamia cyperi*, introduced by Miyabe (1904), and characterised by sporangia with a unique pedicel, somewhat differentiated sporangiophores and thick-walled oospores, was found to be nested within the downy mildews with strong support. The genus was not accepted widely, and both Waterhouse (1963) and Ho (1990) considered *Kawakamia cyperi* to be a member of *Phytophthora*. The conspicuous pedicel that broadens towards the sporangium was the key feature Miyabe (1904) based the description of the genus on. However, Ho and Chang (1992) did not observe this feature in the type specimen, suggesting that it might not preserve well. In the current study, the pedicel as described by Miyabe was clearly visible. *Kawakamia* has mostly not been accepted as a genus independent from *Phytophthora*, even though it is obligate biotrophic and causes symptoms resembling downy mildew infections. In line with the assumed obligate biotrophic nature (Seethalakshmi 1953; Erwin and Ribeiro 1996), trials to cultivate the species failed an all media tried (CMA, OMA, PCA, PDA, SAM, V8; Thines, unpublished results), supporting previous reports that the phytophthora-like downy mildew species from *Cyperaceae* are not cultivable (Seethalakshmi 1953; Erwin and Ribeiro 1996). Thus, phylogenetic relationships, lack of cultivability, and the unique morphology all support treating *Kawakamia* as an independent genus. If the several species of *Kawakamia* described on *Cyperaceae* belong to several species or just one or two needs to be clarified in future studies. However, on the basis of its tuberculatae oogonia, *Kawakamia cyperi-bulbosi* is distinct from the other species described form *Cyperus* (Seethalakshmi 1953; Seethalakshmi and Ramakrishnan 1953). *Kawakamia cyperi* was also reported from *Digitaria* (Ho et al. 2004), which would extend the host range of the genus to *Poaceae*. However, the strongly plerotic oospores and the lack of the characteristic pedicel suggest that this was a different species. In any case, the presence of another phytophthora-like graminicolous downy mildew provides further support for the hypothesis that the downy mildews might have evolved from hosts in *Poaceae* (Thines 2009).

The concept of what exactly constitutes a downy mildew probably needs to be updated in the light of the current study. Already Thines (2009) emphasised that some downy mildew genera on *Poaceae* exhibit some features that could be seen as phytophthora-like, e.g. the continuous outgrowth of the sporangiophores in *Viennotia*, die somewhat successive formation of sporangia and the lack of well-differentiated sporangiophores in *Sclerophthora*, and the intracellular mycelium observed in *Poakatesthia* (Thines 2009). Even though it can currently not be unambiguously clarified, if the rather undifferentiated sporangiophores observed in *Kawakamia* and *Sclerophthora* represent an ancestral state or a derived reduction, it is noteworthy that in the clades probably most closely related to downy mildews (clades 1 and 4), there are several species with rather complex sporangiophores, suggesting that a reduction is the more likely scenario. Considering this, the most clear-cut synapomorphy of downy mildews with respect to *Phytophthora* seems to be their obligate biotrophic nature, while the complex sporangiophores are a secondary trait that is variable and has probably faced reduction in *Kawakamia* and *Sclerophthora*.

In line with a previous study (Ye et al. 2016), the downy mildews were found to be monophyletic and associated with *Phytophthora* s.l. clades 1 and 4 in this study. Clade 4 also contains *Peronophythora litchi*, a pathogen causing downy blight of litchi. Members of clade 4 produce great amounts of aerial, papillate sporangia that are borne on somewhat differentiated sporangiophores and usually produce aplerotic oospores. The highest degree of differentiation is found in *Peronophythora litchii*, where sporangiophores superficially resemble sporangiophores of *Peronospora* (Chen 1961; Ye et al. 2016). A first step to resolving the polyphyly of

*Phytophthora* has been taken by Thines (2023), who transferred the members of clade 4 to the genus *Peronophythora*.

The evidence that downy mildews had evolved more than once from within phytophthora-like ancestors (which would render *Phytophthora* polyphyletic, not paraphyletic in the logically concise definition given by Nelson (1971)) is not strong and probably the result of a branch-heterogeneity artefact (Kück et al. 2012; Fleming et al. 2023). It is refuted by the current study that includes only complete data (using a matrix without missing data) of high quality, a balanced selection of taxa within downy mildews, and AU testing as a second line of evidence. In the AU analysis only the monophyly of the downy mildews, the sister-group relationship of downy mildews with *Peronophythora* and clade 1, as well as this group with clade 1, received meaningful support. In contrast, none of the previously inferred scenarios for a splitting of the downy mildews (e.g. Bourret et al. 2018; Fletcher et al. 2019) received any support. Thus, it seems highly likely that obligate biotrophy, the hallmark of downy mildew physiology, has evolved just once in *Phytophthora*, giving rise to the highly diverse downy mildew lineage. As Brasier et al. (2022) emphasised that already phytophthora-like species are diverse and species-rich. This is even surpassed by downy mildews, which have a much higher degree of morphological differentiation, e.g. spanning the complete spectrum from phytophthora-like to highly complex sporangiophores, and are likely to be much more species-rich than phytophthora-like groups (Thines and Choi 2016). It will be an interesting subject for future studies which evolutionary innovation has triggered this differentiation from the ancestral lineage, which possibly gave rise to all downy mildew lineages currently recognised.

## Taxonomic considerations arising from the findings of this study

Recently, Brasier et al. (2022) proposed that *Phytophthora* should remain as a paraphyletic genus. However, several of their considerations are debatable. For example, the statement is incorrect that the genus concept in downy mildews would be in a way that some genera were more closely related to each other than closely related *Phytophthora* species. In fact, the genetic distances between downy mildew genera and species are considerable (e.g. Göker et al. 2007; Runge et al. 2011a, b; Bourret et al. 2018; this study). Only the genera *Bremia*, *Novotelnova*, and *Protobremia* are rather closely related and probably will need to be merged into *Bremia* to avoid an inflation of genera (Choi and Thines 2015). It is noteworthy that the several major clades of *Phytophthora* have remained stable entities over the past two decades, as highlighted by Brasier et al. (2022), and which seem to render it possible to achieve a stable classification with establishing new genera to resolve the paraphyly of *Phytophthora*. As the relationships of the various potential genus-level groups to each other are not fully resolved, we refrain from formally introducing new genera or reactivating old genus names, such as *Phloeophthora* (Klebhahn 1906), with its type species *Phloephthora syringae* for clade 8, as the largest possible groups should be named to avoid unnecessary taxonomic changes among species now included in *Phytophthora*. Also it seems to be recommendable to use names relatable to *Phytophthora* when introducing new genus names. Apart from the already described names, *Peronophythora* and *Phloeophthora*, this could be names like *Paraphytophthora*, *Pseudophytophthora*, or *Phytophthoropsis*, to keep the communications barrier low, as also recommended by Brasier et al. (2022). The phylogenetic reconstruction presented in this study suggests that it might be necessary to introduce or reactivate five genus names for clade 12; clade 2; clades 3, 5, 6, and 7; clade 8; and clades 9 and 10, while clade 1 with the type species, *Phytophthora infestans*, would retain the name *Phytophthora*. However, before formal taxonomic changes can be made, it needs to be ascertained that clade 12 remains isolated and that clades 3, 5, 6, and 7 form a monophylum. Therefore, broadly sampled phylogenomic studies, including also some genera closely related to *Phytophthora*, are warranted for this.

The rather general and descriptive terms used so far to characterise phytophthora-like species have their use in identifying species, but are hardly informative for grouping phylogenetically related species (Brasier et al. 2022). This is not surprising, considering that many of the widely recognised characters rather refer to ecological adaptations, host range, or mode of dissemination, which will be relevant to the adaptation to a certain environment, but are unlikely to carry a phylogenetic signal. Thus, the pairings of unrelated phytophthora-like species with similar phenotypic traits as presented by Brasier et al. (2022) cannot be used as an argumentation against recognising genus-level groups as genera. Their conclusion that there are "often considerable biological and phylogenetic distances between *Phytophthora* and the DMs [downy mildews]" (Brasier et al. 2022) is refuted by other parts of the discussion in which they state that are some downy mildew genera that have retained phytophthora-like traits and that clade 4 has several downy-mildew-like features. This is in line with this study and previous considerations by Thines (2009), who showed that the traditional divide between *Phytophthora* and downy mildews is artificial, and that obligate biotrophy is probably the uniting synapomorphy of the downy mildew genera.

Accepting paraphyly is not representing the scientific consensus. In fact, much progress in understanding evolutionary processes has been due to recognising lineages in previously paraphyletic taxa as independent, e.g. in the

case of *Reptilia* that are paraphyletic with respect to birds (e.g. Card et al. 2023). Even with abandoning the formal use of "*Reptilia*", the informal designation "reptiles" to refer to groups that share some plesiomophic traits is still useful for communication. The same applies also to the term "phytophthora-like", for organisms with traits that before were thought to be characteristic of a monophylum. In this conjunction, it should also be noted that there are several other phytophthora-like groups that have been previously separated from *Phytophthora*, e.g. *Halophytophthora* and the earlier-diverging genera *Salisapilia* and *Halophytophthora* (Hulvey et al. 2010; Bennett et al. 2018; Bennett and Thines 2019), often primarily triggered by on phylogenetic. Together with the phylogenetic investigations, a re-evaluation of morphological characters was also done, leading to the appraisal of seemingly minor morphological traits, such as the "cigarette-ash-like" plugs typical for *Salisapilia* (Hulvey et al. 2010; Bennett and Thines 2019) as synapomorphic features. Even in the absence of morphological features, it seems to be reasonable to recognise the genus-level groups currently represented by clades as genera, to reflect evolutionary relationships. This has also been done by Jung et al. (2017) when formally describing *Nothophytophthora* with the statement "*Nothophytophthora* is morphologically similar to *Phytophthora* and phylogenetically constitutes a monophyletic sister genus of *Phytophthora*". The same argument is applicable also for the other lineages of phytophthora-like organisms.

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**Author contribution** MT conceived the study; BM analyzed the unpublished genome of *Hyaloperonospora thalspeos-perfoliati* and provided sequence data; SP conducted the phylogenetic reconstructions and AU analysis; MT did the microscopy work; MT wrote the manuscript with major contributions from SP; all authors read and approved the manuscript.

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**Availability of data and materials** Sequence data have been deposited in GeneBank.

## Declarations

**Ethics approval and consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Competing interests** MT is the Editor-in-Chief of *Mycological Progress*, but was not involved in the editorial processes associated with handling this manuscript.

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