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Adding a missing piece to the puzzle of oomycete phylogeny: the placement of *Rhipidium interruptum* (*Rhipidiaceae*)

I. Tsai[1,2*], M. Thines[1,2]

[1]Evolutionary Analyses and Biological Archives, Senckenberg Biodiversity and Climate Research Centre (SBiK-F), Senckenberganlage 25, 60325 Frankfurt am Main, Germany
[2]Department of Biological Sciences, Institute of Ecology, Evolution and Diversity, Goethe University Frankfurt am Main, Max-von-Laue-Str. 13, 60438 Frankfurt am Main, Germany

*Corresponding author: ichen.tsai@senckenberg.de

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Abstract: Oomycetes are a group of fungus-like organisms, which phylogenetically comprise early diverging lineages that are mostly holocarpic, and two crown classes, the *Peronosporomycetes* and *Saprolegniomycetes*, including many well-investigated pathogens of plants and animals. However, there is a poorly studied group, the *Rhipidiales*, which placement amongst the crown oomycetes is ambiguous. It accommodates several taxa with a sophisticated vegetative and reproductive cycle, as well as structural organisation, that is arguably the most complex in the oomycete lineage. Despite the remarkable morphological complexity and their notable perseverance in the face of faster-growing saprotrophic oomycetes and fungi, the knowledge on *Rhipidiales* is limited to date, as the most complex members are not easily cultured, even by targeted approaches. This also leads to inadequate sequence data for the order, which was sourced from only the two least complex out of seven introduced genera, *i.e. Sapromyces* and *Salispina*. In the present study, *ex-situ* baiting was done using various fruit substrates, and naturally-shed twigs or fruits acquired from water bodies were examined. As a result of these efforts, the species *Rhipidium interruptum* was obtained and gross cultivation was accomplished using poplar (*Populus nigra*) twigs as substrate, which allowed further documentation of both asexual and sexual reproduction. This enabled phylogenetic and detailed morphological study, as well as an epitypification of the species. Phylogenetic analyses based on *cox*2 and nrLSU sequences revealed *Rhipidium* as the sister genus of *Sapromyces*. The morphological studies done support a conspecificity of *R. interruptum* and *R. continuum*, which might in turn be conspecific with *R. americanum*. Though several further studies will be required to fit the scattered missing pieces of knowledge on *Rhipidiales* together revealing a more complete picture of oomycete evolution, we hope that the current study can serve as a cornerstone for future investigations in the group.

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INTRODUCTION

Oomycetes are eukaryotic, fungus-like, heterotrophic microorganisms that are ubiquitous in marine, brackish, freshwater, as well as terrestrial habitats, and comprise both saprotrophs and pathogens of various hosts (Sparrow 1960, Howard & Johnson 1969, Choi *et al.* 2008, Thines 2014, Bennett *et al.* 2018, Bennett & Thines 2019, Hassett *et al.* 2021). Destructive oomycete pathogens are responsible for several devastating diseases (Erwin & Ribeiro 1996, Bruno *et al.* 2011), the most notorious being *Phytophthora infestans*, which causes potato late blight and led to the historical Great Famine in Ireland (Yuen 2021). On animal hosts, saprolegniosis caused by *Saprolegnia* species can cause ecological damage by infesting and killing amphibians, fish, crustaceans, and several other aquatic beings, and cause enormous economic losses in aquaculture (Bly *et al.* 1992, Czeczuga *et al.* 1999, Hussein & Hatai 2002, Costa & Lopes 2022). Apart from these rather well-known groups, some holocarpic oomycetes have high ecological relevance, as they have been found to fatally infect diverse species of diatoms and algae that play a vital role by forming the basic energy source of the aquatic food web (Jensen 1993, Serôdio & Lavaud 2020). For example, in Iceland, *Pontisma blauvikense* was found parasitic to brown algae (Buaya *et al.* 2023), *Lagena ausuennarstadhirensis* and *Miracula einbuarlaekurica* were reported as endoparasitoids in pennate freshwater diatoms (Buaya & Thines 2022a, Thines & Buaya 2022), and *M. blauvikense* and *M. islandica* were found to parasitise marine diatoms (Buaya *et al.* 2021a, Buaya & Thines 2022b). Other holocarpic oomycetes are obligate parasites of other oomycetes, *e.g. Olpidiopsis verrucosa* was found parasitic to *Achlya glomerata* (Johnson 1955), and *O. incrassata* pathogenic to several *Saprolegniaceae* species (Slifkin 1961). In

addition to the pathogenic species, a multitude of saprotrophic oomycetes are omnipresent, both in *Peronosporales* and *Saprolegniales* (Marano *et al.* 2016, Beakes & Thines 2017). Also, there are a variety of saprotrophic oomycetes not belonging to either order and that have attained much less attention despite their widespread occurrence. These are organisms previously included in one order, the *Leptomitales* (Sparrow 1960), but are now placed in separate orders. The first order is *Leptomitales*, which includes *Leptomitus lacteus* (*Leptomitaceae*), commonly found in polluted water, thus called "sewage fungus" (Dix & Webster 1995). The second order is *Rhipidiales*, which is the subject of the present study. Examples from the latter order are *Salispina hoi* (*Salispinaceae*), isolated from decaying mangrove leaf litter (Bennett *et al.* 2018), and *Aqualinderella fermentans* (*Rhipidiaceae*), which was acquired from various species of juicy fruits or nuts as baiting substrates in stagnant water, and was notably observed thriving in anaerobic conditions (Emerson & Weston 1967, Emerson & Held 1969, Czeczuga *et al.* 2004). The most complex thallus structure in *Rhipidiales* is found amongst *Rhipidium* species. *Rhipidium* includes the species *R. americanum*, *R. compactum*, *R. interruptum* (*europaeum*), *R. parthenosporum*, and *R. thaxteri*, which were recovered from a number of submerged plant substrates (Cornu 1871, Thaxter 1896, Kolkwitz *et al.* 1915, von Minden 1916, Kanouse 1927b, Matthews 1936, Sparrow 1960). Compared to the study on pathogenic oomycetes, saprotrophs are generally less studied, even though they are increasingly gaining attention in recent years (Nakagiri 2000, Hulvey *et al.* 2010, Marano *et al.* 2016, Bennett & Thines 2020).

The phylum *Oomycota* is usually sorted into three groups – the early diverging lineages with several holocarpic species, and the two "crown clades" that comprise the majority of known oomycete diversity, namely the classes *Peronosporomycetes* and *Saprolegniomycetes* (Beakes *et al.* 2014, Thines 2014). The order *Rhipidiales* is probably sister to the clade of all other taxa in *Peronosporomycetes* (Thines 2014) and comprises two families, *Rhipidiaceae* and *Salispinaceae* (Bennett *et al.* 2018, Bennett & Thines 2020), with six genera included in the former, *Rhipidium*, *Sapromyces*, *Aqulinderella*, *Mindeniella*, *Nellymyces*, *Araiospora* (Sparrow 1960, Emerson & Weston 1967, Batko 1971), and only *Salispina* in the latter (Bennett *et al.* 2018). Amongst the seven genera of *Rhipidiales*, sequence data are exclusively available from two of them, *viz. Sapromyces* (*Sapromyces elongatus* of the *Rhipidiaceae*) and *Salispina* (*Salispina hoi*, *Salispina intermedia*, *Salispina lobata*, and *Salispina spinosa* of the *Salispinaceae*) (Petersen & Rosendahl 2000, Jesus 2015, Bennett *et al.* 2018).

There are only few phylogenetic studies involving rhipidialean members based on either single or concatenated loci (Hudspeth *et al.* 2000, Petersen & Rosendahl 2000, Riethmüller *et al.* 2000, Hudspeth *et al.* 2003, Beakes *et al.* 2012). In these studies, contradicting or poorly resolved topologies based on different loci were observed. Riethmüller *et al.* (2000) analysed the large ribosomal subunit DNA (nrLSU) of several oomycetes, and inferred that the subclass *Rhipidiomycetidae* was closer related to *Saprolegniomycetidae* than to *Peronosporomycetidae*, with maximum bootstrap support. In contrast, Petersen & Rosendahl (2000), also based on nrLSU sequences they studied, could not explicitly resolve the placement of *Rhipidiales* due to low bootstrap support (74 % and 67 %, depending on the analysis). Hudspeth *et al.* (2000) inferred a phylogeny based on partial mitochondrial cytochrome c oxidase subunit II (*cox*2) sequences of representative species from *Peronosporomycetidae* and *Saprolegniomycetidae* (currently recognised as *Peronosporomycetes* and *Saprolegniomycetes*, respectively), and found strong bootstrap support for a clade accommodating *Peronosporales* and *Rhipidiales*. In all of these studies, it was emphasised that *Sapromyces elongatus* was the only species representing *Rhipidiales*, thus, additional representatives were required to validate the monophyly of *Rhipidiales*. Furthermore, the conflicting topologies found highlighted the need for including more sequence data and taxa (Hudspeth *et al.* 2000, Petersen & Rosendahl 2000, Riethmüller *et al.* 2000, Dick 2001). Bennett *et al.* (2018) reconstructed the peronosporomycete phylogeny based on a concatenation of nrLSU, *cox*1 and *cox*2, thereby inferring a monophyletic *Rhipidiales*, including both *Salispinaceae* and *Rhipidiaceae*, However, the phylogenetic reconstruction did not contain an outgroup outside the crown groups, thus not clarifying if *Peronosporomycetes* or *Saprolegniomycetes* should include *Rhipidiales*. In addition, none of the studies mentioned above included a member of *Rhipidium*. Due to the lack of sequence data for *Rhipidium*, the placement of this group has remained obscure.

Studies into the morphologically more complex members of the *Rhipidiaceae*, such as members of the genus *Rhipidium*, which feature a well-developed, branched rhizoid, a well-differentiated basal cell, and filamentous branches carrying reproductive organs, have been hampered by the difficulty in cultivating them. Though short gross or pure culture maintenance of species with restricted thallus growth (*Rhipidium*, *Araiospora*, and *Aqualinderella*) was achieved, they could not be sustained as growth ceased after some time (von Minden 1916, Emerson 1950, Emerson & Weston 1967). Detailed studies regarding physiology, life cycle, ecological interaction, and other experiments requiring a constant supply of vital material are limited as a result of difficulties in long-term axenic cultivation. Considering that *Rhipidiales* are arguably the structurally most complex group of oomycetes, and appears to occupy a peculiar ecological niche that renders them competitive in the face of faster-growing substrate competitors, this seems to be a critical obstacle towards understanding the evolutionary plasticity of oomycetes. In addition, such cultures would also enable the sequencing of multiple loci or even high-quality genomes that could help understanding the evolution of complex thallus structures from simple mycelial forms. An increased knowledge on *Rhipidiales* would also enable in-depth ecological and evolutionary comparisons to the equally diverse but also understudied *Leptomitales*, which often occur in similar environments and have some similar features, such as constricted hyphae.

Considering the research gaps mentioned above, the aim of the present study was to isolate the type species of *Rhipidium* and achieve gross culture for at least one strain over prolonged time to obtain sequence data for a phylogeny based on both nuclear and mitochondrial sequences that might resolve the ambiguous placement of *Rhipidiales*.

MATERIALS AND METHODS

The species discussed in this study was acquired with various approaches to sampling from different water bodies in three European countries (Fig. 1), with submerged plant substance collection (natural baits), *ex-situ* mud-baiting, multi-pustule sub-baiting, and gross cultivation.

Sampling and baiting

Denmark

***Sampling date*:** 16 Nov. 2021. ***Location*:** The sampled ditch was near the intersection of Vesterhavsvej and Gammelgabvej, in Nørre Nebel (55.795356°N, 8.233995°E). ***Description of the environment*:** The ditch had a shallow, reddish brown muddy bed, with some leaf litter (*Malus sylvestris* and *Quercus petraea*) (Fig. 1A), and was surrounded with pastures and fields. Above the sampling site, trees arranged into a hedge, which included *Malus sylvestris* and *Quercus petraea* were observed, and along the ditch several additional tree species were present. ***Natural bait collection*:** Fallen apples submerged in the ditch were collected, with one of them (fruit code GVap01 for **G**ammelgabvej-**V**esterhavsvej **ap**ple **01**) conspicuously covered by loosely distributed whitish grey or light greyish brown pustules on parts of its surface (Fig. 2A, appearance of the fruit GVap01 on the third day after collection). The fruits were kept in tap water in a plastic bucket when not examined. ***Multi-pustule sub-baiting*:** The sub-baiting was carried out on 19 Nov. 2021, by picking 10 pustules with fine-end tweezers from GVap01, to a container with tap water and three apples (collected from one of the fruiting apple trees nearby the sampled ditch, and stored at room temperature before use). The baiting set was incubated at room temperature in Blåvand, Denmark, without a fixed light period, with daylight through windows for around eight hours, subsequently indoor fluorescent lamp light for roughly another eight hours, and darkness for the rest of the day. The baiting set was incubated under this condition for 4 d, transported by car for approx. 8 h, to be again incubated at room temperature (*circa* 20 °C) in a laboratory room at the Biodiversity and Climate Research Centre in Frankfurt am Main, Germany, again without specific settings of the light cycle.

Italy

***Sampling date*:** 31 Dec. 2021. ***Location*:** The sampled ditch was on the side of the street Via Pralboino, which connects the towns Pralboino and Gottolengo, in the Province of Brescia (45.275659°N, 10.241645°E). ***Description of the environment*:** The ditch had a shallow, greyish brown muddy bed, with a large amount of leaf litter and shedded twigs from *Populus nigra*, both in the water and on the banks of the ditch. Living and decaying grasses and herbs covered the banks on both sides of the ditch, and plants of *Arum maculatum* were growing along. Some widely spaced trees (*Populus nigra* var. *italica*) were growing above the ditch. A wide area of durum wheat fields was surrounding the ditch that was next to an unpaved road. **Ex-situ *mud-baiting*:** About 500 g of wet mud was collected from the ditch bed into a BPA-free, plastic food storage container (Lock & Lock, Seoul, South Korea), and brought to the laboratory after the end of the sampling trip. The mud baiting was carried out 6 d after sampling, on 6 Jan. 2022. A selection of various fruits, namely apples, olives, tomatoes (small fruit variety), and *Cotoneaster* spp., was employed as baiting substrates in water above the mud. The baiting set was incubated at 10 °C with 12/12 h light/dark period to simulate the natural conditions at the time of sampling. On 16 Feb. 2022, after 40 d of baiting, one of the olive fruits had developed pustules that were used for sub-baiting on other fruits, two tomatoes were heavily infested by fungi thus removed, and the remaining set including the olive showing pustules (simplified fruit code given: VPolv01) were transferred (with another two olives from the original container added 6 d later that had been overlooked during the first transfer) into a 370 mL Weck jar (J. WECK GmbH & Co. KG, Wehr-Öflingen, Germany) containing fresh tap water mixed with some muddy water from the original container, and incubated without a sealing ring. The water was replaced whenever turbid since then. All following examinations, on fruits apart from VPolv01, were carried out based on this set of baits.

Germany

***Sampling date*:** 28 May 2022. ***Location*:** Samples were taken from the river Lahn close to its bank near a platform reaching into the river, where the water was running slower in comparison to the main stream (50.572778°N, 8.641806°E), and from the Lake Silbersee at its southern shore (50.575694°N, 8.640194°E), both near Gießen (Hesse). ***Natural bait collection*:** Submerged twigs were collected from the water, with some of them already showing small, yet suspicious, pustules on the surface. The sampled twigs were from several tree species, including *Salix* sp. and *Populus* sp., while some of them could not be readily identified. ***Description of the environment*:** Ducks and geese were active around the riverside of Lahn, with willow and poplar trees growing on the banks, and several shedded twigs were observed in the water. Abundant in number and comprised

Fig. 1. Sampling sites. **A.** Ditch near the intersection of Gammelgabvej and Vesterhavsvej in Nørre Nebel, Denmark. **B.** Ditch near Via Pralboino in Pralboino, Province of Brescia, Italy. **C.** River Lahn in Gießen, Hesse, Germany. **D.** Lake Silbersee in Gießen, Hesse, Germany.

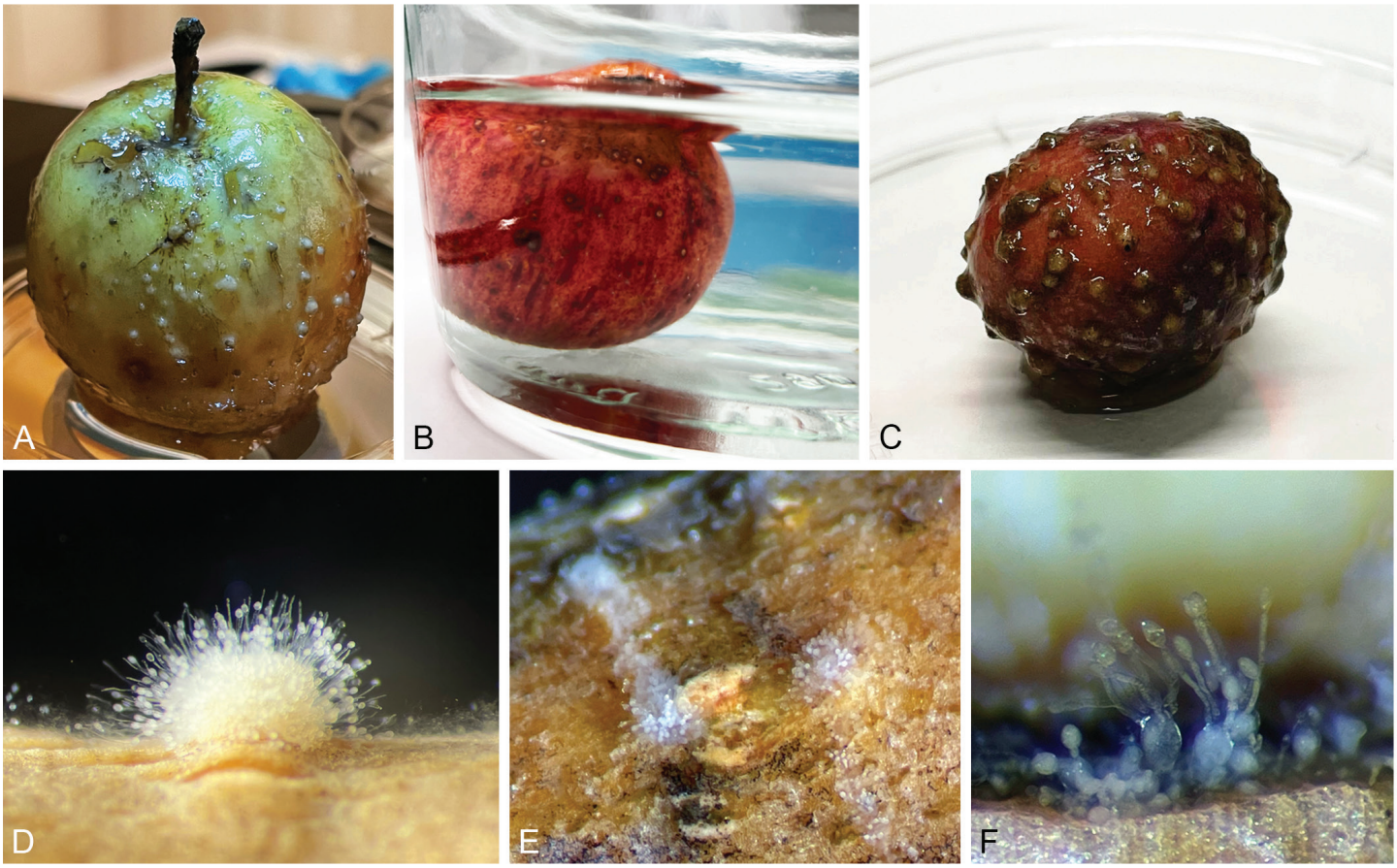

Fig. 2. Substrates showing growth of rhipidialean pustules. **A.** Pustules growing on a small apple, fruit GVap01 (Denmark). **B.** Pustules starting to grow on another apple fruit as the substrate for multi-pustule sub-baiting (Denmark), and from which FR-0046164 was obtained. **C.** Pustules on an olive, fruit VPolv01 (Italy), from which FR-0046165 was acquired. **D.** A pustule growing on poplar twig in the gross cultivation of the Lahn strain (Germany). **E, F.** Pustule(s) on poplar twig in the gross cultivation of the Silbersee strain (Germany).

of various species, trees were growing around the shore of Silbersee, a shallow lake derived from the extraction of gravel. ***Material maintenance*:** The sampled twigs were rinsed and placed in 142 mm (diam) × 20 mm (depth) polystyrene Petri dishes (Sarstedt, Nümbrecht, Germany) with fresh tap water after having been brought to the laboratory, and incubated at 10 °C with a 12/12h light/dark rhythm. The twigs were rinsed once per week (roughly), meanwhile the water in dishes was renewed, to avoid an overly flourishing population of protists and their food, to slow down the speed of decay of the twigs.

Gross cultivation

The gross cultivation was performed on twig samples collected from the river Lahn and the lake Silbersee, by co-incubating the sampled twigs with new twigs in 142 mm × 20 mm polystyrene Petri dishes (Sarstedt, Nümbrecht, Germany) in tap water. For this, twigs of different tree species were employed. Twigs of alder (*Alnus glutinosa*) and poplar (*Populus nigra*) were successively applied on 13 and 20 Jun. 2022 to the dishes and co-incubated with the sampled twigs.

The alder twigs were artificially wounded at multiple positions with sterile surgical blades, after serendipitously observing pustules growing from a natural bark opening close to the end of one twig. However, the pustules on alder twigs did not progress as well as those thriving on poplar. Therefore, the sub-cultivation on alder twigs was discontinued after a few rounds of successive weekly addition of twigs.

Due to the quick development of various decay-associated organisms on natural poplar twigs, twigs added subsequently were subjected to a mild pasteurisation in a hot water bath from respectively the 23rd and 25th of Jun. 2022 for gross cultures from Lahn and Silbersee. The hot bathing at 60 to 65 °C for 2–3 h drastically reduced potential contaminants from the twig materials, and was fixed to 2 h at 65 °C after a few experiments. When many twigs were bathed at once, twigs not directly used for baiting were stored at -20 °C until needed. Additional hot-bathed poplar twigs were applied to the gross cultivation in an interval of 1–2 wk, depending on the condition of pustule growth, meanwhile all the new and old twigs were rinsed and water replaced as mentioned before.

Material examination and specimen preparation

The plant substrates were screened, and suspicious pustules growing on the substrate surface were carefully extracted from the plant tissue and dissected by using fine-tipped tweezers, assisted by a fine iron needle, under a dissecting microscope (Zeiss SteREO Discovery). The pustules were then mounted onto microscopic slides using tap water and covered with coverslips for further documentation using differential interference contrast light microscopy on compound microscope (Zeiss Axio Imager2) equipped with Zeiss Axiocam MRc5 camera operated by AxioVision Rel. 4 for photography and measurements. All the microscopy-related equipment and software were acquired from Carl Zeiss, Oberkochen, Germany.

The processing of materials differed between the initial phase of the investigation and the later phase, as outlined below. The initial phase encompasses the handling of samples from Denmark and Italy. For this, a single pustule was dissected into two halves, one for specimen preparation, and the other collected into a drop of 10 µL sterile deionized distilled water in a 2 mL SafeSeal microtube (Sarstedt, Nümbrecht, Germany), and frozen at -20 °C for later DNA extraction and sequence analyses. Another one or more pustules on the same fruit with similar appearance, occurring adjacent to the previously dissected one, was/were subsequently detached from the substrate and used for microscopy. However, if the pustule selected for specimen preservation and DNA extraction is distinct from the one used for microscopy, this method always leads to an indirect connection between the morphological and phylogenetic conclusions. Therefore, this practice was abandoned after the authors realised the issue. This means that only the sequence data were considered unambiguous for samples derived from the earlier method, while morphological features, even though in line with the results from the later method, were not considered for the morphological characterisation. To solve the matter, in the later phase the procedure from the initial phase was modified and applied to the samples from Germany (Lahn and Silbersee). Instead of cutting single pustule into halves, it was divided into three parts, one for obtaining a specimen, a second for microscopy, and a third for molecular analyses. Thereby, all the materials used for a single isolate could be directly connected.

The specimens of single pustule isolates were stored in approximately 1 mL of TE-buffered ethanol solution (94–96 % ethanol, 0.3–0.5× TE), in 1.8 mL screw-capped CryoPure tubes (Sarstedt, Nümbrecht, Germany). Specimens of the isolates included in the phylogenetic analyses of this study were deposited in the Herbarium Senckenbergianum (Frankfurt am Main), with the Herbarium codes: FR-0046161, FR-0046163 to FR-0046166 (Table 1).

DNA extraction and sequence analyses

Genomic DNA was extracted using the innuPREP Plant DNA Kit (Analytikjena AG, Jena, Germany), following the manufacturer's instruction. Polymerase chain reaction (PCR) was performed to amplify nrLSU and *cox*2 of the obtained rhipidialean isolates, using a Mango*Taq*™ DNA polymerase kit (Meridian Bioscience, Inc., Cincinnati, Ohio, USA), with each single reaction performed in a 25 µL volume containing 1× of Mango*Taq* colourless reaction buffer, 2 mM of $MgCl_2$, 0.8 mg/mL of bovine serum albumin, 200 µM of dNTPs, 400 µM of each forward and reverse primers, 0.15 µL of Mango*Taq*™ polymerase, 11.35 µL of sterile deionised distilled water, plus 2 µL of DNA template. To amplify nrLSU, the primers LR0R-O and LR6-O (Moncalvo *et al.* 1995, Riethmüller *et al.* 2002), and for *cox*2, cox2-F and cox2-RC4 were used (Hudspeth *et al.* 2000, Choi *et al.* 2015). The PCR cycling programs were run on an Eppendorf Mastercycler proS equipped with a vapoprotect lid (Eppendorf AG, Hamburg, Germany). For amplifying nrLSU, PCR was initiated with a denaturation at 95 °C for 2 min, followed by 40 or 36 cycles at 95 °C for 20 s, 53 or 54.5 °C for 20 s, and 72 °C for 2 min, and terminated with a final elongation at 72 °C for 7 min; for *cox*2, PCR started with an initial denaturation at 96 °C for 6 min, followed by 36 cycles at 96 °C for 20 s, 50 °C for 40 s, and 72 °C for 40 s, and a final elongation at 72 °C for 6 min. The PCR amplicons were subsequently mixed with home-made DNA loading buffer (AG Thines, Senckenberg Biodiversity and Climate Research Centre (SBiK-F), Frankfurt am Main, Germany) and loaded on an 1 % agarose gel stained with ethidium bromide, flanked by the HyperLadder™ 1kb standard (Meridian Bioscience, Inc., Cincinnati, Ohio, USA), and visualised after electrophoresis by UV illumination. Each of the successfully amplified products were diluted to slightly lower than 4 ng/µL with sterile water and sent with the fitting forward and reverse primer solution (diluted freshly from 100 mM to approximately 5 mM with molecular grade water) for sequencing at the laboratory centre of SBiK-F (Frankfurt am Main, Germany).

Consensus sequences were obtained via Geneious Pro v. 5.6.7 (Biomatters, Inc., Auckland, New Zealand) by an editing based on forward and reverse sequences, assisted by a sequence chromatography visualisation in Chromas v. 2.6.6 (Technelysium Pty. Ltd., South Brisbane QLD, Australia) for judging on the reliability of basecalls. The final consensus sequences were aligned after the addition of sequences for the loci investigated from previous publications (cited in Table 1) and from an unpublished genome of the *Lagenisma coscinodisci* strain LgC2 (Isla, Buaya *et al.* 2019a) using the MAFFT v. 7 online server (Katoh *et al.* 2019; https://mafft.cbrc.jp/alignment/server/. Accessed on 20 Feb. 2023). The aligned sequence datasets of the single loci were trimmed to remove leading and trailing gaps via MEGA v. 7.0.26 (Kumar *et al.* 2016), followed by concatenating both loci via FASconCAT, v. 1.11 (Kück & Meusemann 2010). The phylogenetic analyses of the concatenated dataset were performed using the TrEase webserver (Mishra *et al.* unpublished; http://thines-lab.senckenberg.de/trease/. Accessed on 20 Feb. 2023), executing FastTree2 (Price *et al.* 2010) for minimum evolution (ME) trees with the generalised time-reversible (GTR) model and 1 000 bootstrap replicates, RAxML v. 8 (Stamatakis 2014) for maximum likelihood (ML) reconstruction applying the GTRGAMMA model and 1 000 bootstrap replicates, and MrBayes v. 3.2 (Ronquist *et al.* 2012) for Bayesian inference (BI) with the 6 GTR model run for 1 000 000 generations, sampling every 1 000th tree, and discarding the 30 % of the trees for ensuring sampling of trees from the stationary phase. The phylogenetic trees were displayed, rooted and adjusted to stepwise-up with FigTree v. 1.4.0 (Rambaut 2012) and MEGA v. 7.0.26 (Kumar *et al.* 2016), and subsequently annotated in MS PowerPoint 2019 (Microsoft Inc., Redmond, U.S.A.).

The sequences obtained in this study were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/), with accession numbers given in Table 1. The alignments used for generating the trees can be retrieved from Supplementary File 1.

RESULTS

Baiting and cultivation

Danish samples — multi-pustule sub-baiting

White pustules were discovered after 12 d of baiting (on 1 Dec. 2021) on the surface of one of the three apples that served as baiting substrate (Fig. 2B, photographed on the first day when the pustules were found, before they grew more). A specimen (FR-0046164) was made with one of the halves of a pustule, and the other half from the same pustule was used for the phylogenetic inference of this study.

Table 1. Information on specimens used in the phylogenetic analyses of this study.

Species	Isolate number	Other isolate information	Loci		References[fp]	
			LSU rDNA	*cox*2	LSU rDNA	*cox*2
Albugo candida	HNB2590	–	EU826108	EU826092	Thines *et al.* (2009)	
Haliphthoros milfordensis	NJM 0131	–	AB178869	AB178870	Sekimoto *et al.* (2007)	
Halophytophthora batemanensis	CBS 679.84[ex]	DNA isolation No. MG 25-3, 33-5	DQ361227	DQ365703	Göker *et al.* (2007)	
Halophytophthora vesicula	NBRC 32216[ex]	= CBS 393.81 = IFO 32216	KT455418	MF991427	Jesus *et al.* (2016)	Bennett *et al.* (2018)
Haptoglossa zoospora	LEV6507	–	KT257316	KT257467	Spies *et al.* (2016)	
Hyaloperonospora erophilae	MG 19-4 (DNA isolation No.)	Collection No. MG 1961	AY271998	DQ365705	Göker *et al.* (2003)	Göker *et al*. (2007)
Hyaloperonospora parasitica	MG 19-1 (DNA isolation No.)	Collection No. MG 1964	AY271996	DQ365710	Göker *et al*. (2003)	Göker *et al.* (2007)
Lagenisma coscinodisci	**LgC2**	–	**OQ717007**	**OQ729759**	**this study[ds]**	
Peronospora alpicola	P2706	Herbarium No. JK-F0514	MH730852	KJ654286	Thines *et al.* (2019)	Choi *et al.* (2015)
Peronospora ficariae	P0974	Herbarium No. GLM630333	MH730844	KJ654239	Thines *et al.* (2019)	Choi *et al.* (2015)
Phytophthora boehmeriae	CBS 291.29[ex]	= PD 00181 = P6950	HQ665190	PD_00181	Robideau *et al.* (2011)	Phytophthora Database
Phytophthora ramorum	CBS 101553[ex]	= PD 00065 = P10103	HQ665053	PD_00065	Robideau *et al.* (2011)	Phytophthora Database
Phytopythium helicoides	CBS 286.31	–	HQ665186	MF397926	Robideau *et al.* (2011)	Bennett *et al.* (2017b)
Phytopythium vexans	CBS 119.80	–	HQ665090	EF426547	Robideau *et al.* (2011)	Belbahri *et al.* (2016)[ds]
Pythium capillosum	CBS 222.94	–	HQ665164	KJ595360	Robideau *et al.* (2011)	Hyde *et al.* (2014)
Pythium inflatum	CBS 168.68	–	HQ665140	KJ595352	Robideau *et al.* (2011)	Hyde *et al.* (2014)
Rhipidium interruptum	**FR0046161[ep]**	**= isolate LNunk-sub-pn01**	**OQ685023**	**OQ680192**	**this study**	
	FR0046163	**= isolate SSGsv-sub-pn01**	**OQ685024**	**OQ680193**	**this study**	
	FR0046164	**= isolate GVap01sub**	**OQ685022**	**OQ680191**	**this study**	
	FR0046165	**= isolate VPolv01**	**OQ685025**	**OQ680194**	**this study**	
	FR0046166	**= isolate VPolv05**	**OQ685026**	**OQ680195**	**this study**	
Salisapilia sapeloensis	LT6440[ex]	= CBS 127946 = NBRC 108756	HQ232457	KJ654178	Hulvey *et al.* (2010)	Choi *et al.* (2015)
Salisapilia tartarea	CBS 208.95[ex]	–	HQ232464	MF598484	Hulvey *et al.* (2010)	Bennett *et al.* (2017a)
Salispina hoi	USTCMS 1611[ex]	–	MG385863	MF991430	Bennett *et al.* (2018)	
Salispina spinosa	CBS 591.85[ex]	= NBRC 32593 = IFO 32593 = ATCC 28294	KT920434	MF991428	Jesus (2015)	Bennett *et al.* (2018)
Saprolegnia ferax	F4257	= AR6 = DNA isolation No. MG 53-4	EU826118	EU826103	Thines *et al.* (2009)	
Saprolegnia parasitica	CBS 223.65	–	HQ665165	NW012157837	Robideau *et al.* (2011)	Jiang *et al.* (2013)
Sapromyces elongatus	CBS 213.82	–	AF235950	KT257452	Petersen & Rosendahl (2000)	Spies *et al.* (2016)

Italian samples — in-situ *mud-baiting*

Suspicious pustules were found after 24 d of baiting (on 30 Jan. 2022), growing on the olive VPolv01 and some tomatoes. Pustules on fruit VPolv01 already grew large by 14 Feb. 2022, each roughly 1–2 mm diam, densely covering the fruit surface. They appeared somewhat slimy, with a colour ranging from very light grey to light yellowish brown (Fig. 2C). The specimen FR-0046165 was made from VPolv01 from one half of a pustule with the other half serving as material for sequence analyses.

Pustules appeared on the other olive and tomato fruits, and were collected 9 and 10 d, respectively, after the container was re-baited as described earlier in the methods section. The specimen FR-0046166 was made in the same way as FR-0046165, from one of the olive fruits (fruit code: VPolv05) co-incubated with VPolv01.

Lahn and Silbersee samples — gross cultures

Pustules appearing to have been formed by a *Rhipidium* species were first seen on a natural wound located at one of the ends of an alder twig. Though the alder twigs were artificially wounded subsequent to this observation, and more pustules were found on those wounds, the pustules on alder twigs did not thrive as those on the poplar twigs. Therefore, the cultivation on alder twigs was terminated, and the pustules emerging on poplar twigs, which were first found on 30 Jun. 2022 (7 and 5 d after addition of hot-water-treated twigs to Lahn and Silbersee cultures), were subcultured for further study.

These gross cultures were later used for documentation of asexual and sexual reproduction of the obtained *Rhipidium* species. The specimens, FR-0046161, FR-0046162, and FR-0046163, were derived from this culture as described before, and enabled a direct correlation of sequences and morphology. The gross cultures of Lahn could be sustained to date (late May, 2023), while the cultivation of the Silbersee isolate was as first considered not sustainable due to an absence of newly appearing pustules over a few months. However, shortly after the initial manuscript draft was submitted, *Rhipidium* pustules were again found from the Silbersee cultures, with successive growth and reproduction until today (9 Jun. 2023).

Growth habit and morphology

The description here is based on the isolates FR-0046161 to FR-0046163, and the two isolates without preserved fungarium specimens LNunk-sub-pn03 and LNunk-sub-pn04, all from Lahn or Silbersee sub-cultures using poplar twigs as substrate. All the isolates measured in the morphological description were confirmed having identical *cox*2 sequences. Their specific characteristics are given below, and microscopic features presented in Fig. 3.

Pustules thrived on the surface of poplar twigs, were usually widely spaced, usually emerging from natural cracks of the twigs, or right at or near the budding points. Well-developed pustules were nearly semi-spherical (Fig. 2D), with a refractive granular surface, colour white, off-white, to light orange-pink, or very pale brown, like seashell and bisque (hexadecimal colour code #fff5ee and #f2d2bd, respectively), when observed with naked eyes or under low magnification through a dissecting microscope (Fig. 2D, E). Pustules were formed by several (often about 30) individual plants (*i.e.* structure consisting of rhizoid, basal cell, and filamentous branches with or without reproductive organs) in a cluster (Fig. 3N–Q). *Rhizoids* were arbuscular, branched monopodially, hyaline or stained with the colours of the consumed plant substrates, often with thicker "main roots" (Fig. 3S), and decreasing strongly in diameter at the second or third branching order, followed by fine branches in similar width, with blunt or round distal ends (Fig. 3U); occasionally a single "main root" from which distinctly thinner "side roots" branched was formed (Fig. 3T). *Basal cells* were cylindrical, trumpet-like, or clavate, varying in size, 463–992 µm long × 43–110 µm diam (n = 19, as basal cells were difficult to detach in a way that their shape would remain unspoiled), hyaline, normally carrying granules containing consumed plant substances in the respective colours. The basal cells were swollen towards the top, or doubly to quintuply lobed (Fig. 3N, O), but sometimes also branched (Fig. 3P, Q), rarely with secondary or higher order lobes/branches. In case of simple swellings these were often flattened like a platform. The wall of the basal cells was refractive and thickened throughout, but generally thicker towards the apical ends. A constricted site at the basal cell joint towards the rhizoid was often observed, but this feature was also frequently absent. *Filamentous branches* were tubular, cylindrical or clavate, slightly swollen near the distal ends, not tapering towards the bases, with primary filamentous branches varying in size, 39–609 µm long × 10–27 µm diam (n = 100), colourless, sometimes containing granules of plant substances in the colour of the substrate. The filamentous branches emerged from the distal ends of lobes or branches of the basal cell, or irregularly at the crown of the swollen section (or platform), sometimes with extreme differences in length (very long and short) observed on the same basal cell, growing singly (Fig. 3C) or with a second or more proliferations (often once, sometimes twice, Fig. 3D, E), constricted at both the base, beneath reproductive organs, and the distal ends, but not at the budding point of proliferating branches (Fig. 3D, E). Secondary (proliferated) filamentous branches 36–305 µm long × 11–19 µm diam (n = 11, as secondary branches tended to fold, rendering them difficult to measure unambiguously). Though in some cases long primary filamentous branches were observed, often they were rather short and stout, while secondary filamentous branches arising from them were rather long and slender. Without a clear-cut pattern, zoosporangia or oogonia were formed before the proliferation of filamentous branches and at their distal ends. However, at proliferation sites mostly zoosporangia were formed, while and at the most distal ends zoosporangia or oogonia were produced. *Zoosporangia* were prolate ellipsoidal (majority), prolate spheroidal, obovoid, globose, ovoid, narrowly obovoid, perprolate ellipsoidal, or obpyriform, 42–90 µm long × 28–61 µm diam (n = 100), hyaline, smooth-walled, mostly solely (Fig. 3C), or doubly (Fig. 3F), rarely triply at the interval or distal ends of filamentous branches, each always sitting on a constriction site (Fig. 3C–F), with a wide distal opening when empty, either with the earlier shape remaining or displaying a partially wrinkled wall. In that case empty zoosporangia displayed multiple fine streaks along the longitudinal axis. Discharge tubes or extending sheaths were not observed, but presumably formed and evanescent, as occasionally short and collapsed residues were observed at the orifice of the sporangia (Fig. 3D). *Zoospores* were not observed, as no stimulation method was found to trigger release. *Oogonia* were globose or subglobose, 38–69 µm × 40–68 µm (n = 53), hyaline when immature, hyaline, golden or cupper when containing an oospore, with a smooth wall on the outer surface, while the inner surface had an undulate contour due to an uneven

thickening of the oogonial wall (Fig. 3G). *Oospores* (Fig. 3G–J) were globose or oblate spherical, singly in each oogonium, not filling the oogonium completely. The oospore wall was unevenly thickened leading to sculptured surface with irregular ridges forming a broad reticulum (Fig. 3J), strongly reflective, leading to an asterisk-like (Fig. 3H) or undulate (Fig. 3G) appearance in mid-section view, with a reticulate pattern (Fig. 3I) when focussing between the outer and the inner wall. The size of the oospores was 31–55 µm × 34–55 µm (n = 50) when including the outer wall. Due to the refractive nature the distinction

Fig. 3. Morphological characteristics of *Rhipidium interruptum* in differential interference contrast. **A.** Constrictions at the bases of filamentous branches. **B.** Immature reproductive organs. **C.** Zoosporangia and their supporting filamentous branches. **D.** Extension of the basal filamentous branch (proliferation). **E.** Successive growth of zoosporangia on a single filament (second proliferation). **F.** Terminal growth of two zoosporangia, with a younger sporangium next to a mature one. **G.** Oogonium containing a single oospore with unevenly thickened outer wall, leading to an undulate contour. **H.** An asterisk-like oospore in section-view. **I.** Reticulate pattern formed by ridges of oosporic outer wall, when focusing in between the outer and the inner wall. **J.** Ridges on the outer wall of oospore. **K.** Branched antheridial stalk. **L.** Bending antheridial stalk. **M.** Antheridium arising immediately beneath an oogonium. **N.** Entire individual plant. **O.** Multi-lobed basal cell. **P.** Basal cell with asymmetrical distal branches. **Q.** Basal cell divided into three distal branches with higher order subdivisions. **R.** Constriction at the joint of a basal cell and a rhizoid. **S.** Rhizoid divided into two asymmetrical main branches. **T.** Fine branches growing out laterally from a main "root". **U.** Fine distal rhizoidal branches. Scale bars: A, G–J = 10 µm; B–F, K–M, R–U = 20 µm; N–Q = 100 µm. Isolates depicted: A–C, N, R–U = FR-0046161 (LNunk-sub-pn01), O = FR-0046162 (LNunk-sub-pn02), G–H, J–K LNunk-sub-pn03, I, L, M LNunk-sub-pn04, D–F, P–Q = FR-0046163 (SSGsv-sub-pn01).

between outer and inner wall layer was not always possible. The cell lumen of the oospores was globose or oblate spherical, with a smooth outline, 21–35 µm × 22–34 µm (n = 50), and appeared colourless. Tiny granules were sometimes observed outside the centre in a section-view (Fig. 3H). *Antheridia* were clavate, prolate ellipsoidal, obovoid, or reniform, 19–41 µm long × 14–18 µm diam (n = 7, as antheridia mostly had a deformed appearance after fertilisation), often with granular contents (colour cupper, not filling the entire antheridium), generally adhering to the lower part of oogonium close to the constriction site. Antheridial filaments were stout (Fig. 3M), slender and irregularly bending (Fig. 3L), or sometimes branching (Fig. 3K). The antheridia appeared to be both monoclinous and diclinous (Fig. 3 K–M), but the origins were mostly obscure, as antheridial filaments were rather thin-walled and did not retain shape or integrity well after fertilisation. *Constrictions* were located at the base of reproductive organs between them and their supporting branches, wall strongly thickened at the constrictions, refractive (Fig. 3A), forming a channel which was sometimes extremely thin and only visible as a faint line. Occasionally, a short plug-like structure (texture similar to the wall) was observed at one of the two ends of the channel.

Phylogeny and species delimitation

Only moderately supported conflicting topologies were observed when comparing phylogenetic reconstructions based on nrLSU and *cox*2 (Supplementary File 2). This is due to the well-known fact that *cox*2 is too variable to reveal higher level-relationships by itself (*e.g.* Choi *et al.* 2015, Buaya & Thines 2022b). However, the signal in the more conserved first and second codon positions can be harnessed when including a second, more conserved locus. Thus, a concatenated alignment of *cox2* and nrLSU was used for inferring the phylogenetic tree shown in Fig. 4. Apart from the five *Rhipidium* isolates derived from this study, 23 reference species, representative of the main orders of *Peronosporomycetes* and *Saprolegniomycetes*, were adopted in the phylogenetic analyses (14 members of *Peronosporales*, one of the *Albuginales*, two of the *Saprolegniales*, one of the *Leptomitales*, and three of the *Rhipidiales*), while two additional species, *Haliphthoros milfordensis* NJM 0131 and *Haptoglossa zoospora* LEV6507, representing the early-diverging oomycete lineages, served as outgroup. The phylogenetic tree shown in Fig. 4 is based on the topology of the ME reconstruction, with support values of all analyses (ME, ML, BI) added on the branches next to the nodes. The accession numbers of all sequences used in this study are listed in Table 1.

The five *Rhipidium* isolates, FR-0046161, FR-0046163–FR-0046166, formed a monophyletic clade without internal sequence variation in the concatenated tree, with maximum support in all analyses (Fig. 4). This clade was sister to *Sapromyces elongatus* (CBS 213.82). The clade accommodating *Rhipidium* and *Sapromyces* species was also highly supported in the three phylogenetic reconstructions (100 % bootstrap support (BS) in ME, 94 % BS in ML, and 1.0 posterior probability (PP) in BI). The *Rhipidiaceae*, with currently the above-mentioned two representative genera included, can, thus, be interpreted as monophyletic.

The two *Salispina* species, with sequences of *Salispina spinosa* (CBS 591.85) and *Salispina hoi* (USTCMS 1611) as representatives of the *Salispinaceae*, formed a clade that received maximum support values in all three reconstructions, and was placed sister to the clade representing *Rhipidiaceae*, thereby forming a monophyletic *Rhipidiales*. The monophyly of the *Rhipidiales* was strongly supported by all three reconstructions, with 100 % BS in ME, 99 % BS in ML, and 1.0 PP in BI.

The *Rhipidiales* were grouped together with *Albuginales* and *Peronosporales*, forming a large clade with strong support values – 93 % BS in ME, 98 % BS in ML, and 1.0 PP in BI. Therefore, the order *Rhipidiales* is confirmed as belonging to the class *Peronosporomycetes*. Furthermore, it was inferred that the *Saprolegniomycetes*, with representative species of both *Saprolegniales* and *Leptomitales*, formed a well-supported clade (maximum support in all reconstructions) sister to the *Peronosporomycetes*. The two crown clades of oomycetes are thus confirmed as monophyletic in the phylogenetic inference of this study.

TAXONOMY

Based on the strongly supported monophyletic clade formed by the *Rhipidium* isolates, with no internal variation, and considering the morphological similarity of the isolates, they are interpreted as belonging to the same species. Comparing to the systematic description of several *Rhipidium* species (Sparrow 1960), the species showed similarities with both *R. interruptum* and *R. americanum*. However, according to Thaxter (1896) these two species can be differentiated by the antheridial characteristics, as that the former is usually diclinous, with branching or contorted stalks, while the latter is monoclinous (or androgynous) (Thaxter 1896, Sparrow 1960). Thus, the species found in this study has been interpreted as *R. interruptum*, due to the observation of branching antheridial threads (Fig. 3K), and the sometimes-twisted antheridial stalk (Fig. 3L). As a first step towards stabilising the taxonomic treatment of *Rhipidium*, which species and morphological differentiation have been interpreted divergently, an epitype for its type species, *Rhipidium interruptum*, is given below.

Rhipidium interruptum Cornu, *Bull. Soc. Bot. France* **18**: 58. 1871.
Synonyms: *Rhipidium continuum* Cornu, *Bull. Soc. Bot. France* **18**: 58. 1871.
Rhipidium europaeum Minden, *Kryptogamenfl. Mark Brandenburg* **5**: 597. 1912 (1915).

Specimens examined: **Denmark**, Nørre Nebel, ditch, on an apple fruit, collected on 16 Nov. 2021, *I. Tsai & M. Thines*, sub-baiting on 19 Nov. 2021, on an apple fruit, isolated on 14 Jan. 2022, fungarium number FR-0046164. **Italy**, Province of Brescia, Pralboino, ditch, mud, collected on 31 Dec. 2021, *I. Tsai & M. Thines*, mud-baiting on 6 Jan. 2022, on an olive fruit, isolated on 14 Feb. 2022, fungarium number FR-0046165. Isolates derived from subculturing of the aforementioned source: from an olive fruit, isolated on 25 Feb. 2022, fungarium number FB-0046166; as well as from a tomato fruit, isolated on 26 Feb. 2022, laboratory isolate code VPtmt01. **Germany**, Gießen, river Lahn, on a twig, collected on 28 May 2022, *M. Thines*, gross culture established on 20 Jun. 2022, on a poplar twig, isolates obtained from the gross culture on 6 Jul. 2022, fungarium number FR-0046161, FR-0046162; as well as on 15 Sep. 2022, laboratory isolate codes LNunk-sub-pn03, LNunk-sub-pn04; Gießen, lake Silbersee, on a twig of *Salix* sp., collected on 28 May 2022, *M. Thines*, gross culture established on 20 Jun. 2022, on a poplar twig,

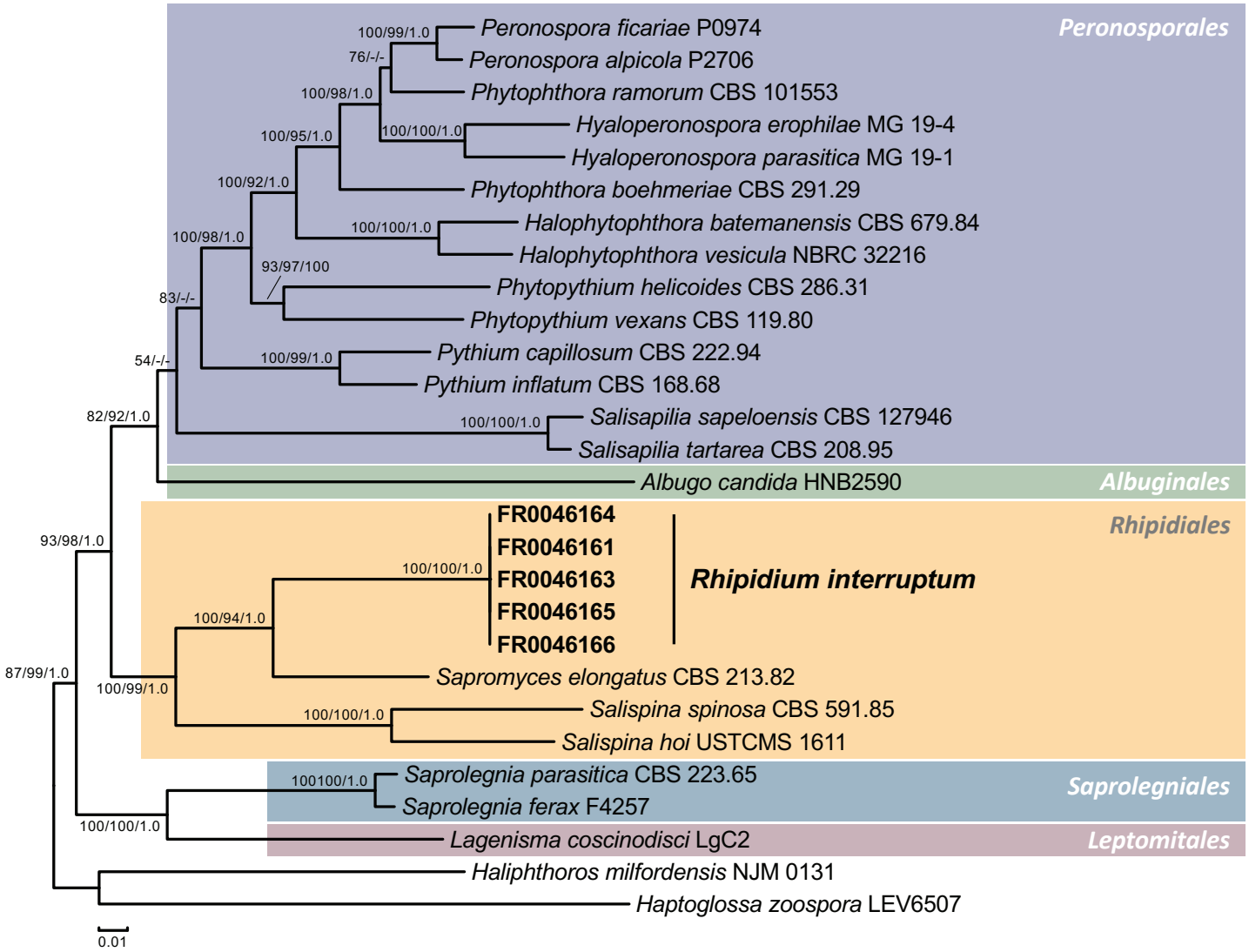

Fig. 4. Phylogenetic reconstruction (in Minimum Evolution) based on a concatenated sequence dataset of nrLSU and *cox*2. Bootstrap support (BS) values from Minimum Evolution and Maximum Likelihood, as well as posterior probabilities from the Bayesian Inference are given on the branches next to the nodes in the respective order. A minus sign denotes an alternate but not highly supported topology (BS < 80 % or PP < 0.98). The scale bar represents the number of substitutions per site.

isolate obtained from the gross culture on 6 Jul. 2022, fungarium number FR-0046163.

Typification: **Germany**, *M. von Minden*, *Mykolog. Untersuch. Berichte*, 1916; plate 2, fig. 9–20, **lectotype** (iconotype) designated by Cejp (1959). **Germany**, Gießen, Lahn, May 2022, *I. Tsai & M. Thines*, LNunk-sub-pn01 (**epitype** designated here, MBT10012614, voucher deposited in the Fungarium Senckenbergianum, accession number FR-0046161).

Notes: The observed characteristics of the isolates LNunk-sub-pn01 (FR-0046161), LNunk-sub-pn02 (FR-0046162), LNunk-sub-pn03, and LNunk-sub-pn04, matched the depiction of von Minden (1916), although zoospores release and germination could not be observed in the current study. However, based on all the accessible features, these isolates can be considered as representative for the species *R. interruptum*. As LNunk-sub-pn01 provided the best collection of characters amongst all the four isolates, its specimen (FR-0046161) was designated as the epitype of the species.

DISCUSSION

In several previous studies, it was suggested that there is a need to include more representatives of the *Rhipidiales* in future phylogenetic reconstructions (Riethmüller *et al.* 2000, Hudspeth *et al.* 2000, Petersen & Rosendahl 2000, Dick 2001). However, it took more than 15 yr, until Li *et al.* (2016) and Bennett *et al.* (2018) included *Salispina* species (*Salispinaceae*) into the oomycete phylogeny, and Bennett *et al.* (2018) could reclassify them to the *Rhipidiales* in their own family, *Salispinaceae*. However, *Sapromyces elongatus* remained the sole taxon representing *Rhipidiaceae*. With the acquisition of documentable *Rhipidium* isolates in this study, the *Rhipidiaceae* were finally represented by taxa of *Rhipidiaceae* that span the entire spectrum of the family, from the genus *Sapromyces* that features only weakly differentiated basal cells to the genus *Rhipidium*, which arguably forms the most pronounced differentiation between rhizoids, basal cells, filamentous branches, and reproductive organs. *Sapromyces* and *Rhipidium* together formed a well-supported monophyletic clade in this study, sister to anther well-supported lineage, the *Salispinaceae*. In line with Bennett *et al.* (2018) the *Rhipidiales*

was strongly supported as monophyletic. However, as Bennett *et al.* (2018) did not include early-diverging oomycete lineages that could serve as an outgroup, and no member of the *Leptomitales* was included, the question of whether or not *Rhipidiales* can be considered members of the *Peronosporomycetes* (Beakes & Thines 2017) could not be resolved in that study.

The phylogeny shown in this study is overall consistent with previous studies (Thines *et al.* 2009, Thines 2014, Li *et al.* 2016, Bennett *et al.* 2018), though based on a distinct or partially different dataset. The combination of nrLSU and *cox*2 seems to be highly suited to clarify oomycete phylogenetic relationships on various levels (Thines *et al.* 2009). Bennett *et al.* (2018) additionally included *cox*1 in their phylogenetic study, with a similar resolution as Thines *et al.* (2009). In line with Choi *et al.* (2015) we assume that both *cox*1 and *cox*2 show a high discriminatory power towards species level, while the resolution on higher levels still seems to be satisfactory. However, the wider phylogenetic sampling available for *cox*2 sequences renders them more suitable to resolve global phylogenetic patterns. Also nrSSU that was previously used, *e.g.* in the study of Li *et al.* (2016), and resulted in a reconstruction that inferred the monophyly of *Rhipidiales* with moderate to high support. Though the locus seems to provide a good resolution in delimitation of species of early diverging holocarpic oomycetes (Buaya *et al.* 2019b, 2021b, Buaya & Thines 2020), nrLSU sequences seem to be better suited to resolve relationships in the "crown group" of oomycetes, the *Peronosporomycetes* and *Saprolegniomycetes*, which is the reason they were used in the current study. In the present study, the closely related species *Salisapilia sapeloensis* and *Salisapilia tartarea*, as well as *Saprolegnia ferax* and *Saprolegnia parasitica* could be differentiated, while at the same time, no intraspecific variation was observed within *Rhipidium interruptum*, supporting that all isolates reported on in this study are representatives of a single species.

Rhipidium interruptum was first introduced by Cornu (1871), described with "*L'une [espèce] présente des filaments munis de nombreux étranglements* (the one [species] has filaments with numerous constrictions)", which was distinguished from another species, *Rhipidium continuum*, stating "*L'autre n'en a jamais qu'un seul [étranglement] à la base de chaque filament* (the other has but a single [constriction] at the base of each filament)". Apart from this, both species resembled each other, with a stellate oospore in mid-section-view. Thus, the two species were exclusively differentiated based on the formation of constrictions. Some years later, van Tieghem provided a sketch of *R. interruptum* (van Tieghem 1884, fig. no. 617, p. 1024). Subsequently, Thaxter (1896) found *Rhipidium* in North America and assigned his samples to a new species, *Rhipidium americanum*, based on non-branched monoclinous antheridia that attached to the base of the oogonium below which they originated. After two decades in which *Rhipidiales* did not receive much attention, von Minden (1916) scrutinised *Rhipidium* in Germany and proposed *Rhipidium europaeum* as a new name to accommodate and replace both *R. interruptum* and *R. continuum*, since the specimen he had found at that time featured filamentous branches both with and without multiple constructions. Thus, both names were no longer representative to the species, and the character was assumed to be influenced by environmental conditions (Kolkwitz *et al.* 1915, von Minden 1916). Kanouse (1927b) and Sparrow (1936) reported the occurrence of *R. europaeum* in Michigan and Cambridge, respectively, picking up the revision of von Minden (1916). Paradoxically, by reporting *R. europaeum* in North America, they rendered this name as ambiguous as von Minden (1916) had found *R. interruptum* ambiguous. A few decades later, Sparrows (1960) gave a general description of *R. interruptum* in his account *Aquatic Phycomycetes*, in which *R. europaeum* and its varieties as well as *R. continuum* were synonymised with *R. interruptum*. Cejp (1959) designated a lectotype (iconotype) for *R. interruptum*, based on von Minden's illustration (1916, plate 2), which was also recognised by Dick (2001) in his systematic account *Straminipilous Fungi*. However, heretofore no actual specimen had been designated as an epitype, leaving the interpretation of the reportedly highly variable type species of *Rhipidium* (Sparrow 1960) ambiguous. However, it is essential for the interpretation of variation and potentially existing similar species to pinpoint *R. interruptum* to a specimen for which sequence data are available, which is the reason why an epitype for *R. interruptum* was designated in this study.

It is noteworthy to mention that – when considering the observed variation in *R. americanum* and *R. interruptum*, the two species differ only in one character to distinguish them, which is in the development of antheridia. In the former they are monoclinous, and the latter diclinous with bending, twisting, or often branched stalks (Thaxter 1896, Kanouse 1927b, Sparrow 1936, 1960). In this study, the deterioration of antheridial structures after fertilisation rendered the unambiguous identification of the origin of antheridia difficult. Still, both openly branching (Fig. 3K) and unbranched bending (Fig. 3L) antheridial stalks were observed, necessitating the interpretation of the species as *R. interruptum*. However, it is noteworthy that Kanouse (1927b) illustrated a *Rhipidium* species she interpreted as *R. americanum* due to the formation of monoclinous antheridia arising directly below the oogonium. However, she showed that antheridia could also be branched instead of forming the typical short-stalked and unbranched form. Considering the high degree of variation observed in previous studies (*e.g.* von Minden 1916) and in the current one, it seems both possible that *R. americanum* is an independent species capable of shifting some characters, *e.g.* to cope with environmental conditions, or that *R. americanum* is conspecific with *R. interruptum*, with the formation of diclinous *vs* monoclinous antheridia being responses to different environmental conditions of the same species. Thus, it seems to be advisable to carry out further molecular and morphological studies including more isolates from both Europe and North America as well as testing variation incited by various environmental conditions (von Minden 1916), to investigate the intraspecific variation and environmental plasticity of *Rhipidium* species.

However, an investigation of *Rhipidiaceae* is still challenging due to the difficulty of gross and pure cultivation, which is additionally hampered by the difficulty of triggering zoospore release, which was rarely observed in the laboratory (Matthews 1936). Also, the low number of reports for some species and genera, *e.g. Mindeniella* (Kanouse 1927a, Sparrow & Cutter 1941), renders targeted sampling difficult, especially, as in many areas, land use has changed dramatically over the past century. Thus, while this study marks a first step into investigating *Rhipidiaceae* in more details by providing sequence data of the type of *Rhipidium*, *R. interruptum*, designating an epitype for it, and establishing a procedure for maintaining gross cultures with poplar twigs now surviving for almost 1 yr, it is clear that the current study is only the beginning of collecting the missing pieces of the puzzle of rhipidialean species evolution and their phylogenetic placements in the oomycete tree of life.

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Conflict of interest: The authors declare that there is no conflict of interest.

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Supplementary Material: http://fuse-journal.org/

Supplementary File 1. Sequence alignment of concatenated dataset. The full length of dataset is 1153 bp. Sections: ***cox*2** positions 1–437, **nrLSU** positions 438–1153.

Supplementary File 2. Phylogenetic reconstructions (in Minimum Evolution) for the individual loci. Bootstrap support values from Minimum Evolution and Maximum Likelihood, and posterior probabilities from Bayesian Inference are given on the branches next to the nodes in the respective order. A minus sign denotes an alternate but not highly supported topology (BS < 80 % or PP < 0.98). An "x" represents alternate highly supported topology (BS > 80 % or PP > 0.98). The isolates of *Rhipidium interruptum* derived from this study are marked in bold. The scale bars represent the number of substitutions per site.