FUSE

Fungal Systematics and Evolution

VOLUME 4
DECEMBER 2019
PAGES 21–31

doi.org/10.3114/fuse.2019.04.03

Holocarpic oomycete parasitoids of red algae are not *Olpidiopsis*

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Key words:
basal oomycetes
new combinations
Olpidiopsis
phylogeny
Pontisma
red algae
Saprolegnia
type species
10 new taxa

Abstract: *Olpidiopsis* is a genus of obligate holocarpic endobiotic oomycetes. Most of the species classified in the genus are known only from their morphology and life cycle, and a few have been examined for their ultrastructure or molecular phylogeny. However, the taxonomic placement of all sequenced species is provisional, as no sequence data are available for the type species, *O. saprolegniae*, to consolidate the taxonomy of species currently placed in the genus. Thus, efforts were undertaken to isolate *O. saprolegniae* from its type host, *Saprolegnia parasitica* and to infer its phylogenetic placement based on 18S rDNA sequences. As most species of *Olpidiopsis* for which sequence data are available are from rhodophyte hosts, we have also isolated the type species of the rhodophyte-parasitic genus *Pontisma*, *P. lagenidioides* and obtained partial 18S rDNA sequences. Phylogenetic reconstructions in the current study revealed that *O. saprolegniae* from *Saprolegnia parasitica* forms a monophyletic group with a morphologically similar isolate from *S. ferax*, and a morphologically and phylogenetically more divergent species from *S. terrestris*. However, they were widely separated from a monophyletic, yet unsupported clade containing *P. lagenidioides* and red algal parasites previously classified in *Olpidiopsis*. Consequently, all holocarpic parasites in red algae should be considered to be members of the genus *Pontisma* as previously suggested by some researchers. In addition, a new species of *Olpidiopsis*, *O. parthenogenetica* is introduced to accommodate the pathogen of *S. terrestris*.

Effectively published online: 10 May 2019.

INTRODUCTION

The *Oomycota* are heterotrophic filamentous organisms of the kingdom *Straminipila* (also informally referred to as stramenopiles) consisting of two classes, *Peronosporomycetes* and *Saprolegniomycetes*, as the crown group, as well as several lineages branching before them, which have not been formally assigned to class level (Dick 2001, Beakes & Thines 2017). While the crown group contains the bulk of known species and has been widely studied, the basal clades are rather poorly known (Karling 1942, 1981, Sparrow 1960, Alexopoulos *et al.* 1996, Thines 2014). The known species of the basal clades are obligate endobiotic holocarpic parasites of algae, invertebrates, and aquatic phycomycetes (Karling 1981, Sparrow 1960). Despite their widespread nature and assumed high diversity, little is known about their role in natural ecosystems, seasonal occurrence, and phylogeny. Comprehensive accounts of the basal holocarpic Oomycetes were published by Karling (1942, 1981), Sparrow (1943, 1960), and Dick (2001). No molecular phylogenetic information was included in these studies and, as morphological features are limited in holocarpic oomycetes, their phylogenetic relationships remained mostly speculative. More recently, several holocarpic oomycetes have been included in phylogenetic investigations (Sekimoto *et al.* 2008, 2009, Fletcher *et al.* 2015, Klochkova *et al.* 2015, 2017, Thines *et al.* 2015, Kwak *et al.* 2017, Buaya *et al.* 2017, 2019, Badis *et al.* 2018, Buaya & Thines 2019), but the type species of major genera, such as *Ectrogella* and *Olpidiopsis*, have not been included in phylogenetic investigations, leaving the taxonomy of the basal oomycetes fraught with uncertainty.

The genus *Olpidiopsis*, erected in the 19th century (Cornu 1872), is currently the largest genus of holocarpic oomycetes, with more than 60 species (Sparrow 1960) that are parasites of phylogenetically divergent groups: *Chlorophyta*, *Rhodophyta*, *Phaeophyta*, *Bacillariophyta*, *Dinoflagellata*, *Chytridiomycota*, and *Oomycota* (Karling 1981, Sparrow 1960, Dick 2001). Originally, Cornu described the genus to accommodate five holocarpic isolates, which were all parasites of members of the *Saprolegniales* (Cornu 1872). In three of his isolates, he observed thick-walled resting spores to which one or more, smaller empty vesicles were attached. Although he believed that there is a sexual relation between the two cells types forming the resting spores, this has not been proven to date. Cornu did not indicate the presence of resting spores as a generic character of *Olpidiopsis*, but some later researchers who studied the group indicated their potential use for genus delimitation (e.g. Barrett 1912). Besides *Olpidiopsis* species, the morphologically slightly more complex genus *Pontisma* has been described from red algae (Petersen 1905). The thallus of *Pontisma* consists of a series of olpidiopsis-like thallus segments, which form independent discharge tubes. Its only species *Pontisma lagenidioides* has been recorded as infecting several

Ceramium spp. (Karling 1942, Sparrow 1960). Karling (1942) considered *Pontisma* to be synonymous with another obligate marine pathogen, *Sirolpidium*, due to similarities in terms of thallus morphology and development. However, Sparrow (1960) and most other researchers did not support merging the genera because of differences in thallus branching and fragmentation. Resting spores have not been observed in either *Pontisma* or *Sirolpidium*, and neither genus has been included in phylogenetic investigations as yet.

To date, most species of *Olpidiopsis* that have been phylogenetically investigated are pathogens of marine rhodophyte algae. These include *O. porphyrae, O. bostrychiae, O. feldmanni,* and the invalidly described species *O. heterosiphoniae*, *O. muelleri*, *O. palmariae*, and *O. pyropiae*, which we validate in this manuscript (Sekimoto *et al.* 2008, 2009, Fletcher *et al.* 2015, Klochkova *et al.* 2015, 2017, Kwak *et al.* 2017, Badis *et al.* 2018). In addition, a single marine diatom parasite *O. drebesii* and the related freshwater diatom parasitoid, *O. gillii*, have sequence data available (Buaya *et al.* 2017, 2019). Already with molecular data for few species, *Olpidiopsis* seems to be polyphyletic, consisting of at least two groups, one in red algae and the other one in diatoms (Buaya *et al.* 2017). However, the type species of the genus *Olpidiopsis* is *O. saprolegniae*, a freshwater holocarpic parasite first seen in species of *Saprolegnia*. So far, no sequence data are available of this type species, hindering a taxonomic assessment of the genus *Olpidiopsis*. In the current study, *Olpidiopsis* isolates from three *Saprolegnia* species, as well as *P. lagenidioides* were investigated for their molecular phylogeny to resolve the taxonomy of the genus *Olpidiopsis*.

MATERIALS AND METHODS

Isolation, culture and microscopy

Japanese strains

Olpidiopsis saprolegniae s.lat. parasitic in *S. ferax* was isolated from a soil sample collected on 20 January 2007 on the campus of the University of Tsukuba, Tsukuba city, Ibaraki prefecture (Japan). *Olpidiopsis* sp. parasitic in *S. terrestris* was isolated from a soil sample collected on 17 June 2006 at the Sugadaira Research Station, Mountain Science Center, University of Tsukuba, Ueda city, Nagano prefecture (Japan). Dual cultures of the hosts and parasites were obtained using a hemp-seed-baiting method (Seymour 1970). About 8 g (wet weight) of soil sample was put into a plastic cup and 30 mL of sterilised distilled water (SDW) was added. After stirring, two autoclaved hemp seed halves (Seymour & Fuller 1987) were floated on the surface of the suspension as baits. The cup was incubated for about 1 wk at 20 °C until outgrowth of *Saprolegnia* was detected from the baits. Subsequently, baits were transferred into a 15 mL Petri dish with 8 mL SDW, and incubation was continued until endobiotic parasite thalli were observed in the host hyphae using an inverted light microscope (Eclipse E200, Nikon, Japan). Pure cultures of the host *Saprolegnia* spp. were established by a single-spore isolation technique (Inaba & Tokumasu 2002) and maintained on cornmeal agar (CMA, Nissui, Tokyo, Japan) plates. The hosts were identified from hemp-seed water cultures as outlined by Seymour (1970). Briefly, sterilised hemp seed halves were placed, cut-surface down, on the edge of colonies of the hosts growing on CMA plates for about 36 h at 20 °C. The infested hemp seed was transferred to a new Petri dish with SDW and incubated at 15 °C until mycelium was visible around them. The host species was identified based on the morphological features of asexual and sexual reproductive organs formed (Seymour 1970). To establish axenic dual cultures of the host and the parasite, the glass-ring method (Raper 1937) was used (Seymour & Fuller 1987). In brief, sterilised glass rings of 10 mm diam were embedded in CMA plates to a depth of about 1–2 mm. An actively growing hyphal tuff from the seeds with thalli of the parasite was cut from the baits and placed inside the glass ring. The plate was incubated at 20 °C and observed under the light microscope daily. After a few days of incubation, host hyphal tips including parasite thalli were growing outside of the ring. A hyphal tip infected with a single zoosporangium of the parasite was transferred to a Petri dish with SDW and incubated at 20 °C. After zoospore release from the zoosporangium was observed, host mycelium growing on half a hemp seed was added into the Petri dish and incubated until the newly provided hyphae of the host were visibly infected by zoospores of the parasite.

German and Norwegian strains

Olpidiopsis saprolegniae parasitic in *Saprolegnia parasitica* was isolated in May 2018 from two lakes in the state of Hessen (Germany), the Aartalsee at Niederweidbach (N50°41’32.2”, E8°28’43.3”) and the Trais-Horloffer See at Inheiden (N50°27’19”, E8°54’23”), but only for isolates from the former sequence data could be obtained.. About 1 L of lake water containing mixtures of filamentous algae, decaying twigs, floating organic debris and mineral sediment was collected at each site using plastic bottles. Subsequently, 10 mL of water samples were poured into 15 mL Petri dish in six replicates per site. About 10 split sesame seeds (Alnatura, Bickenbach, Germany) were added as baits on each plate and subsequently, plates were incubated in a climate chamber (CMP 6010, Conviron, Canada) at 16 °C and 12 °C for 14 h and 10 h in light (1000 lx, Narva, bio-vital, Germany) and darkness, respectively. The plates were incubated for 1–2 wk or until outgrowths of *Saprolegnia* were detected from the seed baits. Hyphal segments were screened for the presence of the endobiotic parasite using either an inverted compound light microscope (AE31, Motic, China) or a dissecting microscope (SZT 300, VWR, Belgium). When an endobiotic thallus of a parasite was detected on a hyphal strand, infected and uninfected hyphae were carefully removed using sterile forceps (3C-SA, rubis, Switzerland), washed multiple times in sterile distilled water until free from attached contaminants, and immediately transferred into double autoclaved lake water with mixture of 50 µg/mL ampicillin (Carl Roth GmbH, Germany) and sterile split sesame seeds. In this manner, both host and the parasite were propagated and bulked up. Infected sporangia were isolated by picking them individually for DNA extraction. Mature *O. saprolegniae* thalli were dissected out of the host hyphae by carefully splitting open the host hyphal wall under an inverted compound light microscope using either a heat-flamed, sharp, fine, self-produced glass needle (Shanor 1939) or by forcing out the thalli with a 10 µL pipette tip (Sarstedt, Germany). The isolated sporangia were washed twice in sterile distilled water, examined under a compound inverted microscope and immersed in either 0.5 mL of RNA*later* (Invitrogen, Thermo Fisher, Lithuania) in a 2 mL plastic vial (Sarstedt, Germany) or placed directly into 5 µL molecular grade water (Life Technologies, USA) in a PCR vial (Sarstedt, Germany), for subsequent nucleic acid extraction or direct PCR, respectively. Approximately 40 sporangia were collected per 2 mL tube for DNA extraction and 10 sporangia for each direct PCR amplification.

Pontisma lagenidioides on its red algal host *C. rubrum* was isolated in September 2017 from Oslo Fjord in Drøbak, Norway (N59°39'31", E10°37'47"). Samples were collected at two sites in the intertidal zone by plucking algae from their substrate and subsequently immersing them in 1 L plastic bottles containing fresh seawater. Subsequently, algal segments were transferred into 15 mL Petri-dishes filled with seawater and immediately screened for the presence of *Pontisma lagenidioides*, using either an inverted compound light microscope or a dissecting microscope. Infected segments of the algae were carefully removed using forceps and scalpel, washed multiple times in autoclaved seawater using 10 µL micropipette and immersed in 0.5 mL RNA*later* (Invitrogen, Thermo Fisher, Lithuania) or 70 % ethanol (VWR, France) for subsequent DNA extraction. Approximately 30 pieces containing parasite thalli were collected for nucleic acid extraction as described for *O. saprolegniae*.

Isolated infected hyphae or thallus segments were mounted on microscopic slides using sterile distilled water for *O. saprolegniae* and autoclaved seawater for *P. lagenidioides* for life cycle observations, morphological characterisation and DIC micrographs using a light microscope (Imager2, Carl Zeiss, Göttingen, Germany) equipped with a Zeiss Axiocam MRc5 (Carl Zeiss, Göttingen, Germany). The thalli of the parasites were also stained with zinc-iodine chloride solution (Carl Roth GmbH, Germany) to detect the presence of cellulose in sporangial walls. *Olpidiopsis saprolegniae* and *P. lagenidioides* were preserved in 70 % ethanol and deposited in the herbarium collection of the Senckenberg Museum of Natural History, Cryptogams Section, Frankfurt am Main under the herbarium accession numbers FR0046109 (*O. saprolegniae* OSE), FR0046110 (*O. saprolegniae* OS1), FR0046111 (*O. saprolegniae* OS2), and FR0046112 (*P. lagenidioides*).

DNA extraction, PCR and phylogenetic analyses

Japanese strains

For sequencing of Japanese *Olpidiopsis* spp., a direct PCR method was performed. About 20 to 30 zoospores released from a single zoosporangium of the axenic dual cultures were used as PCR template. PCR was performed in 50 µL reaction volumes containing 7 µL of distilled water, 1 µL of KOD-Fx (Toyobo, Oosaka, Japan), 25 µL of 2× PCR buffer for KOD-Fx, 10 µl of dNTP solution, 1 µL of each primer (10 pmol/µL), and 5 µL of the zoospore suspension as a template. Primers used were 18-F (5'-ATCTGGTTGATCCTGCCAGT-3') and 18-R (5'-GATCCTTCCGCAGGTTCACC-3') (Ueda-Nishimura & Mikata 1999). Amplification was conducted in a GeneAmp PCR System 9700 (Applied Biosystems, Foster, CA, USA) with the following conditions: an initial denaturation at 94 °C for 120 s, 30 cycles at 98 °C for 10 s, 61 °C for 30 s, and 68 °C for 90 s, and a final extension at 68 °C for 10 min. The amplified DNA was purified with a QIAquick PCR Purification Kit (QIAGEN) according to the instructions provided with the kit. For sequencing 18S rDNA in both directions, the primers 18-F, NS2, NS3, NS4, NS5, NS6, NS7 (White *et al.* 1990) and 18-R were used. Sequencing reactions were conducted using a BigDye Terminator v. 3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems), following the instructions of the manufacturer, in a Biometra T-Gradient Cycler (Biometra, Göttingen, Germany). The reaction products were purified using a CleanSEQ kit according to the instructions of the manufacturer (Agencourt Bioscience Corporation, Beverly, MA, USA). DNA sequences were obtained by capillary electrophoresis and fluorescence detection in an ABI PRISM 3730 DNA Sequencing System (Applied Biosystems).

German and Norwegian strains

For DNA extraction, samples were centrifuged at 19 000 *g* for 2 min at 22 °C to pellet the cells. Subsequently, RNA*later* or 70 % ethanol were carefully removed by pipetting and 400 µL SLS buffer of the innuPREP Plant DNA Kit (Analytik Jena AG, Germany) was added. To each 2 mL vial with cell suspension approximately 100 mg of sterile 0.1 mm Silica Glass Beads (Carl Roth GmbH, Germany) were added for *O. saprolegniae* and 10–15 steel beads (1 mm) for *P. lagenidioides.* Subsequently, samples were homogenized at 25 Hz for 5 min in a Retsch Mixer Mill MM 200 (Retsch GmbH, Germany). Extraction of DNA was carried using the innuPREP Plant DNA Kit following the protocol provided by the manufacturer. PCR for *O. saprolegniae* was carried out using Mango DNA Polymerase (Bioline, UK) with each 20 µL reaction mix containing 1× Mango Reaction buffer (Bioline, UK), dNTP (200 µM), $MgCl_2$ (2 mM), 0.8 µg/µL bovine serum albumin (Carl Roth GmbH, Germany), EUK422-445 (0.4 µM) forward primer, EUK1422-1440_R (0.4 µM) reverse primer (both from Wang *et al.* (2014)), 0.5 U Mango-Taq DNA Polymerase (Bioline, UK) and 5 µL DNA extract. PCR cycling was carried out on an Eppendorf Mastercycler proS (Eppendorf AG, Germany) equipped with a vapo.protect lid, with an initial denaturation at 95 °C for 4 min, 40 cycles at 95 °C for 20 s, 58 °C for 20 s and 72 °C for 60 s, and concluding with a final elongation at 72 °C for 8 min. PCR amplicons were sent for sequencing to the laboratory centre of the Senckenberg Biodiversity and Climate Research Centre (Frankfurt am Main, Germany) using the PCR primers used for PCR. In addition, direct PCRs were done as described for extracted DNA, except that isolated parasite thalli were directly added to 5 µL of molecular grade water (Life Technologies, USA), to which the other components were added. For confirmation of the host identity partial 18S rDNA of *Creamium rubrum* was amplified using Ranger DNA Polymerase (Bioline, UK) with each 20 µL reaction mix containing 1x Ranger Reaction buffer (Bioline, UK), EUK422-445 (0.4 µM) forward primer, EUK1422-1440_R (0.4 µM) reverse primer, 1 U of Ranger DNA Polymerase (Bioline, Germany) and 5 µL of molecular grade water with the isolated thalli. Amplification conditions were set to an initial denaturation at 95 °C for 3 min, 40 cycles at 98 °C for 10 s, 56 °C for 20 s and 72 °C for 60 s, and a final elongation at 72 °C for 4 min. Two positive amplification reactions (one for the ethanol and one for the RNA*later*-preserved samples) were mixed at equal volume and diluted by a factor of ten. Subsequently, the mixture was cloned into *Escherichia coli* using a CloneJET PCR Cloning Kit (Thermo Scientific, Germany), following the instructions of the manufacturer. Single bacterial colonies were picked into 20 µL molecular grade water and colony PCR was carried out with the Mango DNA Polymerase applying same conditions as described above, except that pJET1.2 plasmid primers were used. The amplification conditions were set to an initial denaturation at 95 °C for 3 min, 25 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s, and concluding with a final elongation at 72 °C for 4 min. Positive clones were sent for sequencing to the laboratory centre of the Senckenberg Biodiversity and Climate Research Centre (Frankfurt am Main, Germany) using pJET1.2 plasmid primers. The final consensus sequences were prepared using Geneious Pro v. 5.6 with forward and reverse sequences.

Phylogenetics

Sequences obtained from *O. saprolegniae* and *P. lagenioides* were added to the dataset of Buaya *et al.* (2017). The partial 18S (rDNA) sequences obtained in this study were deposited in GenBank under the accession numbers MK253535 (*O. saprolegniae* OSE), MK253527 (*O. saprolegniae* OS1), MK253534 (*O. saprolegniae* OS2), (*O. saprolegniae* ITM0011), (*O. parthenogenetica* ITM0012), and MK253530 (*P. lagenidioides*). Alignments were done using the Q-INS-i algorithm of MAFFT (Katoh & Stadley 2013) on the TrEase webserver (http://thines-lab.senckenberg.de/trease/), which was also used for Maximum Likelihood inference using the standard settings of the server. Phylogenetic analyses using the Minimum Evolution algorithm were done using MEGA v. 6 (Tamura *et al.* 2011) as described in Buaya *et al.* (2017).

RESULTS

Parasite detection

Freshwater samples collected during the summer of 2018 from two lakes in Hessen Germany, Aartalsee and Trais-Horloffersee yielded abundant colonies of aquatic oomycetes growing on sesame seed baits. About 90 % of the *Saprolegnia* colonies screened were infected by *Olpidiopsis saprolegniae.* Also, the strains obtained from the Japanese soil samples were highly infective on the hosts from which they were isolated. Due to the conspicuous early stages (Fig. 1A) infections were detected within a few days after the appearance of the host, and it was noted that young host hyphal segments were more frequently infected than older, mature parts. Already at 40× magnification using a stereomicroscope, bright specks were observed on the outer third of infected host colonies. Closer examination of these specks using an inverted microscope at 100× magnification revealed that they corresponded to hypertrophied hyphae with early developmental stages of the parasite.

Individuals of the red alga *Ceramium rubrum* were collected during autumn of 2017 from the Drøbak area on the Oslo Fjord, Norway. About 30 % of the algae collected were parasitised by *Pontisma lagenidioides*. Infection was often located on older thallus parts, localised between nodes. In rare instances younger thalli showed restricted infections, and infections were not observed occurring in developing tetraspores. After a period of 2–3 wk of incubation in a climate chamber with a cycle of 16 °C and 12 °C for 14 h and 10 h in light and darkness, respectively, infested hosts incubated in 15 mL seawater showed new infections and the growth of the parasite was faster than on fresh samples. Attempts to cultivate the parasite on agarised medium were not made.

Morphology and life cycle

The development of the parasite sporangia until zoospore release was followed using specimens of *O. saprolegniae* from *Saprolegnia parasitica* (Fig. 1), *O. saprolegniae s.lat*. from *S. ferax* (Fig. 2), *Olpidiopsis* sp. from *S. terrestris* (Fig. 3), and *P. lagenidioides* (Fig. 4).

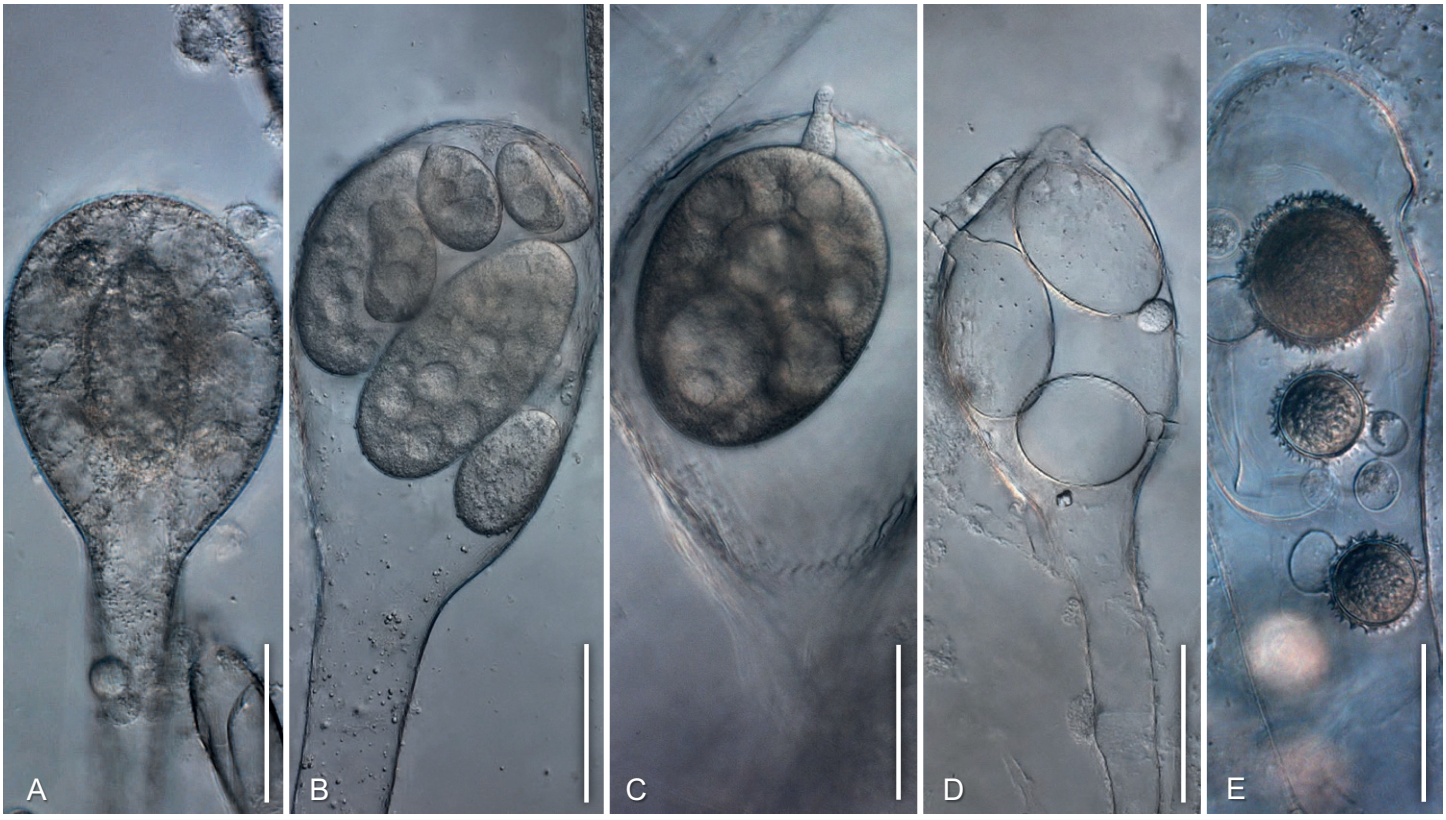

Fig. 1. DIC-light microscopy of *Olpidiopsis saprolegniae* at different life cycle stages on hypertrophied terminal hyphae of *Saprolegnia parasitica*. **A.** Single young thallus surrounded by a dense layer and radiating strands of host cytoplasm. **B.** Several asexual thalli, each with numerous vacuoles. **C.** Single mature vacuolated asexual thallus with developing single discharge tube. **D.** Empty parasite thallus with single discharge tube. **E.** Three mature echinulate resting spores each with attached empty antheridium. Scale bar = 50 µm in all photos.

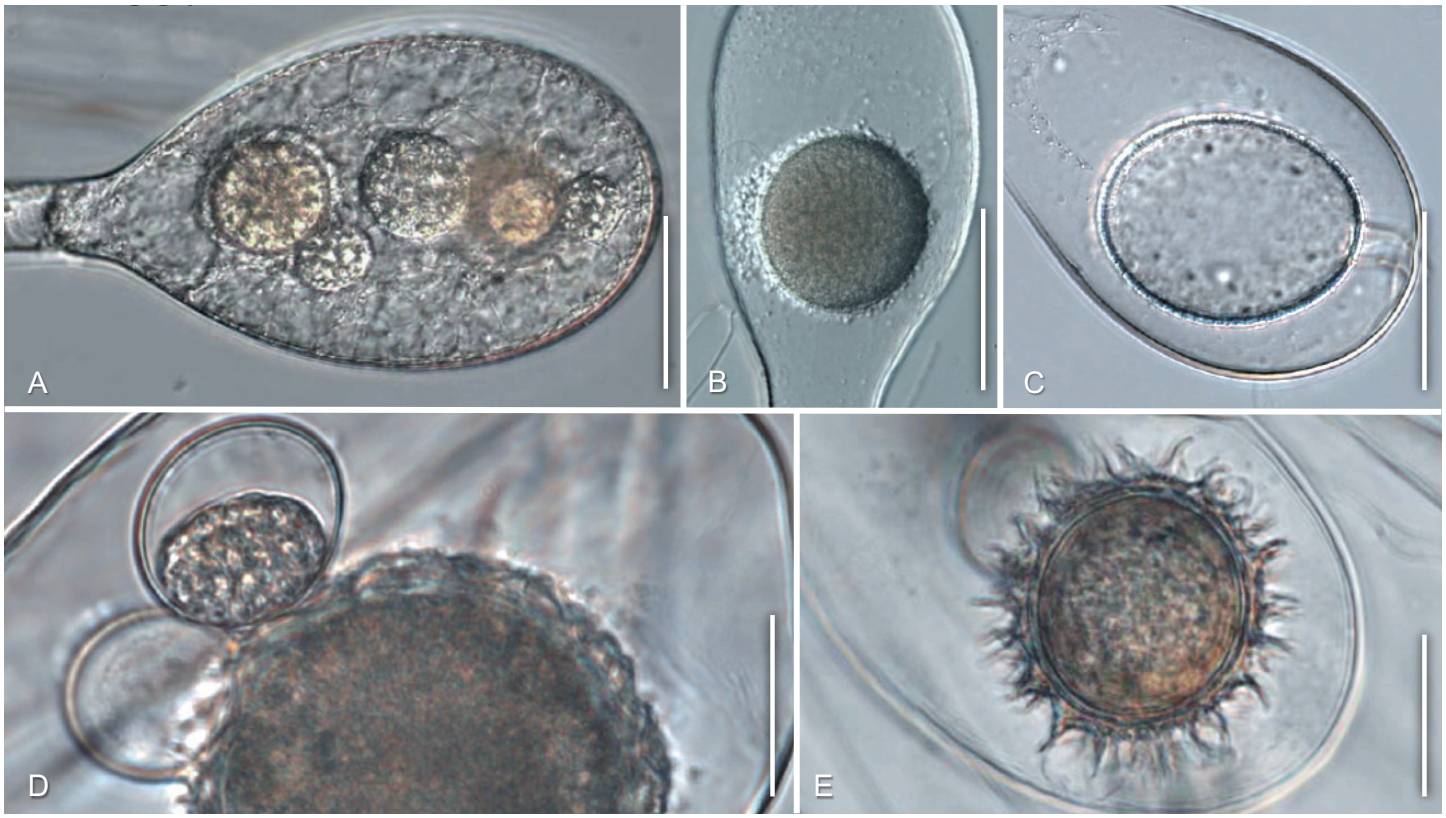

Fig. 2. Light microscopy of *Olpidiopsis saprolegniae s.lat*. at different life cycle stages on hypertrophied terminal hyphae of *Saprolegnia ferax*. **A.** Several young thalli with beginning differentiation into asexual and sexual thalli. **B.** Mature asexual thallus. **C.** Empty asexual thallus single discharge tube. **D.** Developing zygote with attached empty antheridial thallus and incompletely developed thallus with some granular cytoplasm. **E.** Mature, echinulate resting spore, each with long spines and attached empty antheridium. Scale bar = 50 µm in A–C and 20 µm in D and E.

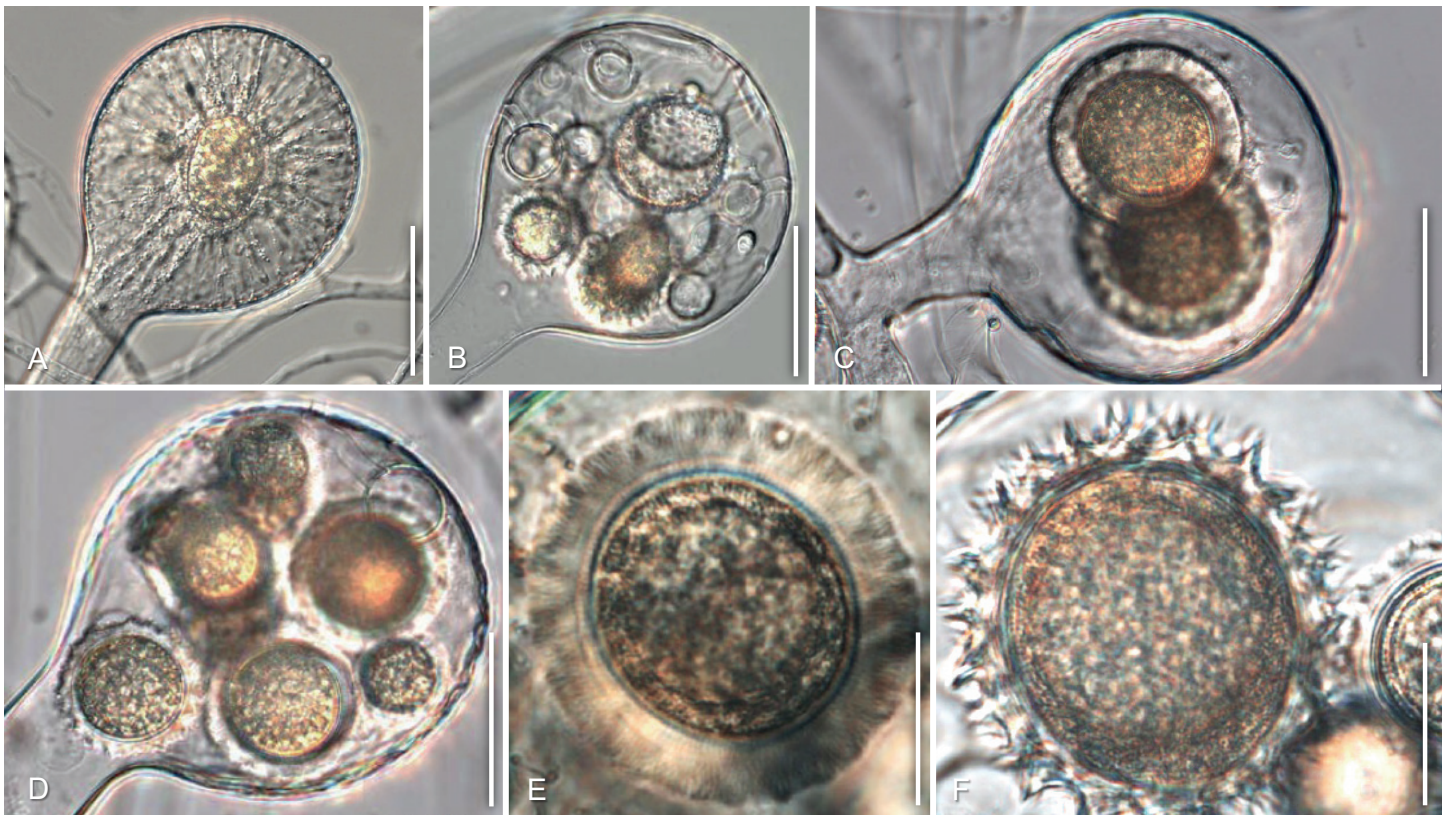

Fig. 3. Light microscopy of *Olpidiopsis* sp. at different life cycle stages on hypertrophied terminal hyphae of *Saprolegnia terrestris*. **A.** Single young thallus surrounded by a dense layer and radiating strands of host protoplasm. **B.** Sexual and asexual thalli at different developmental stages. **C.** Developing resting spores without apparent antheridial cell. **D.** Several resting spores at different developmental stages. **E.** Resting spore with thick, uniform fibrillose exospore layer. **E.** Resting spore with thick, spiny exospore layer. Scale bar = 50 µm in A–D and 20 µm in E and F.

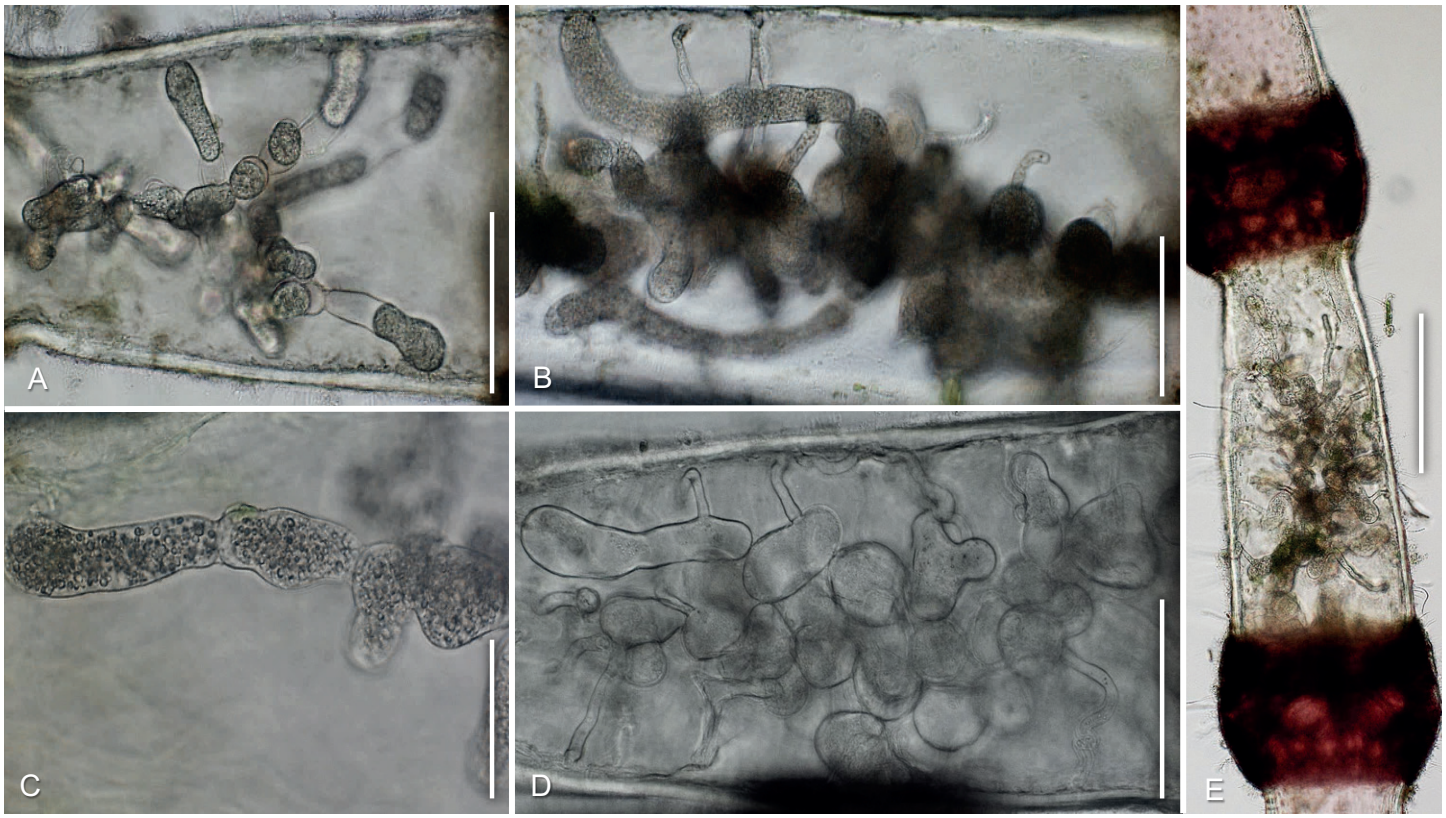

Fig. 4. DIC-light microscopy of *Pontisma lagenidioides* at different life cycle stages in *Ceramium rubrum*. **A.** Irregularly shaped, mature parasite thallus with multiple constrictions. **B.** Multiple mature tubular segments each with a developing single discharge tube. **C.** Segment containing undifferentiated zoospores. **D.** Multiple empty thallus segments and small individual thalli. **E.** Overview a mature parasite thallus growing on the internode of the host alga. Scale bar = 100 µm in A, B, and D, 50 µm in C, and 200 µm in E.

Olpidiopsis saprolegniae ex S. parasitica

Hyaline thalli were found single to several in hypertrophied host hyphae, mostly in terminal, sometimes in intercalary parts, and were mostly ovoid or ellipsoidal 8–190 × 5–140 µm in diameter. The walls of the thalli were colourless, thin, and smooth (Fig. 1B, C). A single discharge tube was formed per thallus, penetrating the host wall, which was cylindrical and of variable length (Fig. 1C, D). Zoospores were numerous and matured inside the thallus, they were oval to elongate, 2–4 µm in length, with two oppositely directed, subapical flagella. Antheridial thalli were mostly single, had a mostly subglobose shape, and were 20–45 µm diam, with a thin, smooth, and colourless wall. Oogonial thalli were globose to subglobose, 40–100 µm diam, initially with a smooth, colourless wall that became ornamented during the fertilisation process. Resting spores with globular content (Fig. 1E) developed from oogonial thalli, spherical to subspherical, and 40–100 µm diam, with a yellowish brown tint of varying intensity and a thick endospore wall. The exospore wall consisted of densely grouped, colourless, concavely tapering spines about 2–10 µm in height and width. The germination of the resting spores was not observed.

Olpidiopsis saprolegniae s.lat. (ITM0011) *ex S. ferax* (ITA2457)

Hyaline thalli were found single to several in hypertrophied host hyphae, usually in terminal, occasionally in intercalary parts. Smaller thalli were smooth, larger ones were rarely covered with a hair-like ornamentation. Thalli were variable in size and shape, spherical, 17–165 µm diam, or ovoid to ellipsoid, 22–140 × 19–120 µm. One to four cylindrical discharge tubes of variable length, either straight or contorted, were formed per thallus. The end of the discharge tubes was flush with the surface of the host hypha or extended beyond it. Zoospores matured within the sporangium and were slightly kidney-shaped or ovoid, 3–4 µm long, biflagellate. Usually, one to two hyaline, smooth antheridial thalli were observed per oogonial thallus. Antheridial thalli were globose to ellipsoidal, and measured 10–30(–37) µm diam. Upon resting spore formation antheridial thalli became occasionally embedded in the spiny ornamentation of the resting spore. Resting spores with globular content developed from oogonial thalli and were hyaline to brownish, globose to ellipsoidal, 17–68 µm diam. Their endospore wall was thick and the exospore wall was rarely smooth, but generally consisting of slender or broad, acutely tapering, spines, 1–8 µm in thickness at the base. The germination of the resting spores was not observed.

Olpidiopsis sp. (ITM0012) *ex S. terrestris* (ZSF0059)

Hyaline thalli usually numerous in hypertrophied host hyphae, usually in terminal, occasionally in intercalary parts. Smaller thalli were smooth, larger ones were covered in small spines. Thalli were variable in size and shape, spherical, 9–100 µm diam, or ovoid to ellipsoid, 12–75 × 10–65 µm. One to four short cylindrical discharge tubes were formed per thallus, from both smooth and spiny types. Zoospores matured within the sporangium and were slightly kidney-shaped or ovoid, 3–4 µm in long, biflagellate. Resting spores with globular content generally formed parthenogenetically, lacking antheridial thalli, and were hyaline to brownish, globose to ellipsoidal, 21–65 µm diam. Their endospore wall was thick and the exospore wall was

sometimes represented by a thick, smooth or unevenly dented layer, but mostly consisted of slender or broad, acutely tapering, spines, 3–11 µm in thickness at the base. The germination of the resting spores was not observed.

Pontisma lagenidioides ex Ceramium rubrum
The hyaline thallus was usually composed of a series of somewhat irregularly cylindrical, sausage-like segments (Fig. 4A–D) measuring 20–120 × 10–35 µm each separated by constrictions. The overall thallus network sometimes extended over more than 300 µm (Fig. 4E). Thallus segments usually formed a single, narrow cylindrical, bending discharge tube of variable length, some more than 100 µm long (Fig. 3B, D). The zoospores matured within the thallus and were irregularly reniform 4–7 × 2–3 µm, with two short lateral, oppositely directed flagella, swarming internally in the sporangium before emerging through the discharge tube. Resting spores were not observed.

Molecular phylogeny

In the phylogenetic tree (Fig. 5) based on partial 18S rDNA sequences *O. saprolegniae s.str.* isolates from *S. parasitica* in Germany (OS1, OS2, OSE) were grouped together with moderate to strong support. *Olpidiopsis saprolegniae s.lat*. isolated from *S. ferax* in Japan, ITM0011) was the sister linage to this group and together with it formed a monophyletic clade with maximum support. *Olpidiopsis* sp. (isolated from Japan, ITM0012 formed the sister lineage to *O. saprolegniae* with maximum support. *Olpidiopsis s.str*. grouped with *Miracula* with low support, forming the earliest diverging oomycete group. *Eurychasma* and *Haptoglossa* were grouped together with moderate to strong support, forming the next-diverging oomycete lineages, even though the branching order did not receive support. *Anisolpidium ectocarpii* and *Olpidiopsis drebesii*, both from phaeophyte hosts, grouped together with low support. *Pontisma lagenidioides* was within rhodophyte-infecting members of *Olpidiopsis*, forming a monophyletic clade without support. *Haliphthoros* and *Halocrusticida* grouped together with varying support as an unsupported sister group to the crown oomycetes, the *Peronosporomycetes* and *Saprolegniomycetes*, which were grouped together with strong to maximum support.

TAXONOMY

Olpidiopsis saprolegniae (A. Braun) Cornu, *Monogr. Saprolegniées*: 127. 1872.
Basionym: *Chytridium saprolegniae* A. Braun, *Abh. K. Preuss. Akad. Wiss. Berlin*: 61. 1856.

Type: **Germany**, *A. Braun, Abh. K. Preuss. Akad. Wiss. Berlin*: plate 5, fig. 23. 1856, **lectotype** designated by Cejp (1959). **Germany**, Hessen, Aartalsee, May 2018, A.T. *Buaya & M. Thines*, OS1 (**epitype** designated here FR0046110, MBT386914).

Notes: The identification of the type host for *Chytridium saprolegniae* A. Braun (the basionym of *O. saprolegniae* (A. Braun) Cornu) as *S. ferax* (Gruith.) Kütz. by Braun (1856) has to be interpreted in the light of the knowledge available at that time and, thus, the actual species parasitised is unclear. Also Dick (2001) gives the type host of *O. saprolegniae* as *Saprolegnia* sp., in line with this. The isolates from *S. parasitica* (OS1, OS2, OSE) most closely match *O. saprolegniae* as pictured by Braun (1856), and are thus considered to represent this species. Consequently, the isolate OS1 is considered typical and designated as epitype of *Chytridium saprolegniae*.

As the type species of *Olpidiopsis*, *O. saprolegniae*, is largely unrelated to the parasites of red algae assigned to the same genus, the parasites of red algae cannot be treated as members of *Olpidiopsis*. Dick (2001) transferred the *Olpidiopsis* species parasitic in red algae to the genus *Pontisma*, which is in line with the placement of the type species of *Pontisma* in the current study (without support) and the monophyly of parasites of red algae in the study of Fletcher *et al.* (2015) (with strong support). The branching thalli with constrictions might reflect a special situation in the type species, where infections occur in the large intercalary regions of *Ceramium*. However, the long, curved discharge tubes typical for *Pontisma* have also been observed in other species, such as in the olpidiopsis-like parasites in *Pyropia* (Klochkova *et al.* 2015, Kwak *et al.* 2017). It seems likely that the rhodophyte-infecting olpidiopsis-like parasites are monophyletic, with *Pontisma* being the oldest available generic name. Thus, the recently described, olpidiopsis-like parasites of red algae, which were not already transferred to *Pontisma* by Dick (2001), are here transferred to this genus. In addition, the order *Pontismatales* is described to accommodate *Pontisma*. The species *O. heterosiphoniae*, *O. muelleri*, *O. palmariae*, and *O. pyropiae* have not been validly described as their authors did not comply with the formal rules for describing fungal-like species and are thus validated here. In addition, the lectotype of *O. saprolegniae* is epitypified to fix its application.

Pontisma bostrychiae (Sekimoto *et al.*) Buaya & Thines, ***comb. nov.*** MycoBank MB830697.
Basionym: *Olpidiopsis bostrychiae* Sekimoto *et al.*, *Phycologia* **48**: 463. 2009. MB830684.

Pontisma heterosiphoniae (G.H. Kim & T.A. Klochkova) Buaya & Thines, ***comb. nov.*** MycoBank MB830702.
Basionym: ***Olpidiopsis heterosiphoniae*** G.H. Kim & T.A. Klochkova ***sp. nov.*** MycoBank MB830685.
Synonym: *Olpidiopsis heterosiphoniae* G.H. Kim & T.A. Klochkova, *Algal Res*. **28**: 267. 2017. MB830766. *Nom. inval*., Art. F.5.1 (Shenzhen).

Description: See Kim & Klochkova, *Algal Res.* **28**: 267. 2017.

Typus: Herbarium specimen of infected *Heterosiphonia japonica* from Wando, Korea; collected on the 17th of May 2006 by Kim G.H. and preserved at the Kongju National University.

Pontisma muelleri (Y. Badis & C.M.M. Gachon) Buaya & Thines, ***comb.*** **nov.** MycoBank MB830703.
Basionym: ***Olpidiopsis muelleri*** Y. Badis & C.M.M. Gachon, ***sp. nov.*** MycoBank MB830686.
Synonym: *Olpidiopsis muelleri* Y. Badis & C.M.M. Gachon, *J. Appl. Phycol.* **31**: 1249. 2018. MB828568. *Nom. inval*., Art. F.5.1 (Shenzhen).

Description: See Badis & Gachon, *J. Appl. Phycol.* **31**: 1249. 2018.

Typus: BM01222128, preserved at the National History Museum, London (BM).

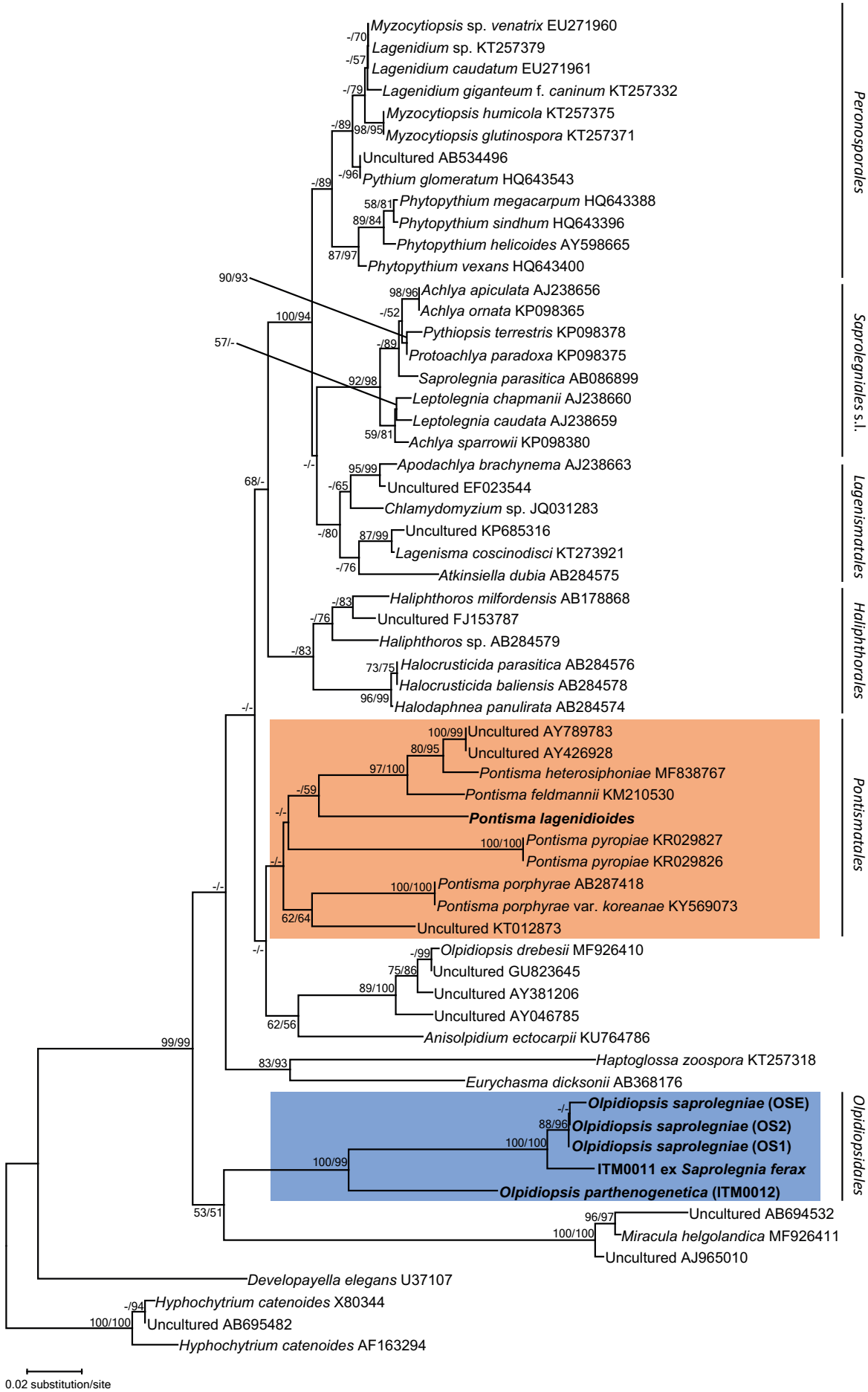

Fig. 5. Molecular phylogenetic reconstruction from Minimum Evolution analyses inferred from 18S rDNA sequences. Numbers on branches denote bootstrap values from maximum likelihood and minimum evolution analyses, in the respective order. A minus sign indicates less than 50 % bootstrap support.

Pontisma palmariae (Y. Badis & C.M.M. Gachon) Buaya & Thines, ***comb.* nov.** MycoBank MB830704.
Basionym: ***Olpidiopsis palmariae*** Y. Badis & C.M.M. Gachon, ***sp. nov.*** MycoBank MB830687.
Synonym: *Olpidiopsis palmariae* Y. Badis & C.M.M. Gachon, *J. Appl. Phycol.* **31**: 1249. 2018. MB828565. *Nom. inval*., Art. F.5.1 (Shenzhen).

Description: See Badis & Gachon, *J. Appl. Phycol.* **31**: 1249. 2018.

Typus: BM001222129, preserved at the National History Museum, London (BM).

Pontisma porphyrae (Sekimoto, *et al.*) Buaya & Thines, ***comb. nov.*** MycoBank MB830707.
Basionym: *Olpidiopsis porphyrae* Sekimoto *et al.*, *Mycol. Res*. **112**: 369. 2008. MB511288.

Pontisma pyropiae (G.H. Kim & T.A. Klochkova) Buaya & Thines, ***comb. nov.*** MycoBank MB830709.
Basionym: ***Olpidiopsis pyropiae*** G.H. Kim & T.A. Klochkova, ***sp. nov.*** MycoBank MB830688.
Synonym: *Olpidiopsis pyropiae* G.H. Kim & T.A. Klochkova, *J. Appl. Phycol.* **28**: 78. 2015. MB830767. *Nom. inval*., Art. F.5.1 (Shenzhen).

Description: See Kim & Klochkova, *J. Appl. Phycol.* **28**: 78. 2015.

Typus: CUP-068041, preserved at the Cornell Plant Pathology Herbarium (CUP).

Pontismatales Thines, *ord. nov.* MycoBank MB830689.

Description: Thallus simple or irregularly branched, holocarpic, exit tubes of variable length, one to several per thallus, zoospores without pronounced diplanetism, with two flagella. Parasitic in *Rhodophyta*.

Type genus: *Pontisma* H.E. Petersen.

Based on the formation of resting spores without conspicuous antheridial thalli and its phylogenetic position, a new species of *Olpidiopsis* is introduced here.

Olpidiopsis parthenogenetica S. Inaba, ***sp. nov.*** MycoBank MB830690. Fig. 3.

Etymology: Referring to the parthenogenetic formation of resting spores in this species.

Description: *Thalli* hyaline, usually numerous in hypertrophied host hyphae, mostly in terminal, occasionally in intercalary parts, smooth if small, covered in small spines if large, variable in size and shape, spherical, 9–100 µm diam, or ovoid to ellipsoid, 12–75 × 10–65 µm, discharge tubes 1–4 per thallus, short, cylindrical, formed from both smooth and ornamented vegetative thalli, zoospores maturing within the sporangium, slightly reniform to ovoid, 3–4 µm long, biflagellate, resting spores generally formed parthenogenetically, lacking antheridial thalli, with globular content, hyaline to brownish, globose to ellipsoidal, 21–65 µm diam, endospore wall thick, exospore wall sometimes as a smooth or unevenly dented thick layer, but mostly consisting of slender or broad, acutely tapering spines, 3–11 µm in thickness at the base, germination of resting spores not observed.

Typus: Fig. 3, depicting ITM0012. The specimen depicted in Fig. 3 was observed in hyphae of *Saprolegnia terrestris* grown from soil collected on the 17th of June 2006 at the Sugadaira Research Station, Mountain Science Center, University of Tsukuba, Ueda city, Nagano prefecture, Japan.

Type host: *Saprolegnia terrestris*.

Known distribution: Japan.

DISCUSSION

Despite its widespread occurrence and being the largest genus of the early-diverging oomycetes, the taxonomy of *Olpidiopsis* has been poorly resolved. The first report of *Olpidiopsis* was apparently published by Nägeli (1844) who mistook sporangia formed inside hypertrophied cells of *Achlya prolifera* as endogenous cell formations of the host. About a decade later, Braun (1855) described *Chytridium saprolegniae*, which was later placed in a genus of its own by Cornu (1872). Subsequently, parasites and parasitoids with simple, holocarpic thalli infecting algae were added (*e.g.* Zopf 1884). After some confusion regarding the generic treatment of olpidiopsis-like species (Fisch 1884, Schröter 1886, Fischer 1892), Sparrow (1960) came back to a rather broad circumscription of the genus.

Currently, *Olpidiopsis* contains 66 species, mostly parasitic to *Saprolegniales* and a few parasitic to *Pythiales* (Cornu, 1872, Maurizio, 1895, Barrett 1912, Coker 1923, Tokunaga 1933, Shanor 1939, McLarty 1941, Karling 1942, 1949, Whiffen 1942). Other members of the genus are parasites of freshwater green algae (Zopf 1884, de Wildeman 1896, Scherffel 1925, Sparrow 1936), marine red algae (Aleem 1952, Feldmann 1955, Sekimoto *et al.* 2008, 2009, Fletcher *et al.* 2015, Klochkova *et al.* 2015, 2017, Kwak *et al.* 2017, Badis *et al.* 2018) and few occur in diatoms (Friedmann 1952, Buaya *et al.* 2017, 2019). The degree of host specificity of olpidiopsis-like species is mostly speculative, but Shanor (1940) conducted large-scale cross infection experiments with twenty-five *Olpidiopsis* lineages parasitic to freshwater *Saprolegniales* and revealed rather high host specificity, often below the genus level.

The host specificity of the holocarpic parasites of red algae has been less well-documented, even though the observations of West *et al.* (2006), Sekimoto *et al.* (2009), and Klochkova *et al.* (2012) hint at somewhat wider host ranges, with potential hosts scattered throughout several host genera. *Pontisma* is known to infect members of the marine rhodophyte genus *Ceramium* (Petersen 1905, Sparrow 1936, Hönk 1939, Aleem 1950, Kobayashi & Ookubo 1953), but due to the high variability of. *P. lagenidioides*, it cannot be ruled out that the species forms more simple, olpidiopsis-like thalli in other hosts. *Pontisma* bears some similarity to the genus *Petersenia*, which usually has less clearly constricted, saccate and lobed, rarely more or less subspherical thalli (Sparrow 1960), a feature shared with some species of *Sirolpidium*. Because of these similarities, Karling (1942) synonymised *Pontisma* and *Petersenia* with *Sirolpidium*, but Sparrow (1960) did not follow this view and Dick (2001) expanded *Pontisma* to include all olpidiopsis-like species parasitic to red algae. This step seems to be justified in the light

of the high support found for the monophyly of olpidiopsis-like species on red algae by Fletcher *et al.* (2015) and the placement of *P. lagenidioides* among those parasites in the current study. Thus, we have followed this up by combining the recently-described olpidiopsis-like parasites of red algae into *Pontisma*. Whether those species of the rather heterogeneous genus *Petersenia*, of which the type species, *P. lobata* also parasitizes red algae, also belong here, needs to be clarified by targeted collections in future studies. We are aware that the phylogenetic relationships among the holocarpic parasites of red algae are not fully resolved, but we feel that the current assignment of all olpidiopsis-like species to *Pontisma*, following Dick (2001), is probably the most conservative approach, as it is likely that, if at all, only minor changes will become necessary, once sequence data become available for *Petersenia* and *Sirolpidium*.

There seems to be the possibility that the larger clades of olpidiopsis-like oomycetes are specific to certain host groups, such as *Pontisma* on red algae. The pathogens of phaeophyte algae, with the exception of *Miracula*, also grouped together. Whether or not the subclade that contains *O. drebesii* represents *Ectrogella* cannot be clarified at present, as sequence data for the type species of *Ectrogella* are missing. Given the rather clear diplanetism in the type species of *Ectrogella*, *E. bacillariacearum*, it seems unlikely that this species is closely related to *O. drebesii*, but renders an affinity between *Ectrogella* and early-diverging *Saprolegniomycetes*, such as *Lagenisma*, more likely. However, this assumption can only be clarified once sequence data are available.

ADDENDUM

In a recent manuscript (Bennett & Thines 2019), an invalid new combination was proposed, which is herewith validated.

Salisapilia tartarea (Nakagiri & S.Y. Newell) Hulvey, Nigrelli, Telle, Lamour & Thines, ***comb. nov.*** MycoBank MB830714.
Basionym: *Halophytophthora tartarea* Nakagiri & S.Y. Newell, *Mycoscience* **35**: 224. 1994. MB363474.
Synonyms: *Salisapilia tartarea* (Nakagiri & S.Y. Newell) Hulvey *et al.*, *Persoonia* **25**: 114. 2010. MB517468. *Nom. inval.*, Art. 41.5 (Melbourne).
Salisapilia tartarea (Nakagiri & S.Y. Newell) Hulvey *et al.*, *Fungal Syst. Evol.* **3**: 180. 2019. MB830653. *Nom. inval.*, Art. F.5.1 (Shenzhen).

ACKNOWLEDGEMENTS

Anthony T. Buaya is grateful to Katholischer Akademischer Ausländer-Dienst (KAAD) for a PhD scholarship. We would also like to thank ForBio-Research School in Biosystematics, Natural History Museum-University of Oslo, for micro-algae taxonomy training and fieldwork assistance to AB in Norway.

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