# THE SIMPLE HOLOCARPIC OOMYCETES: TAXONOMY AND PHYLOGENETIC STUDY

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# ABSTRACT

The early-diverging oomycetes contain a large number of holocarpic obligate parasites of diatoms, algae, aquatic phycomycetes, and invertebrate animals. These organisms are diverse and widespread. However, taxonomic placement most of the early-diverging oomycetes remains provisional and unresolved, since many have not been sequenced and studied for molecular phylogeny. Here, we report the taxonomy and phylogeny of several holocarpic oomycetes that we have rediscovered and newly classified, including several new species combinations. Phylogenetic reconstructions revealed that the type species of genus *Ectrogella* (*E. bacillariacearum*) is a member of the early-diverging *Saprolegniales*, while the type species of *Olpidiopsis* (*O*. *saprolegniae*) and *Pontisma* (*P. lagenidioides*) grouped within the early-diverging lineage of oomycetes forming distinct clades. Since the monophyletic red-algae parasitoids are unrelated to the *Olpidiopsis*, these were reclassified to the genus *Pontisma*, while genus *Diatomophthora* was introduced to accommodate all the diatom parasitoids that were previously assigned to *Olpidiopsis*. In addition, four new oomycete parasitoids, *Miracula helgolandica*, *Miracula moenusica*, *Diatomophthora drebesii* and *Olpidiopsis parthenogenetica* and a single rediscovered species, *Diatomophthora gillii,* are also classified here, including eight new species combinations of red-algae parasites (*Pontisma bostrychiae*, *P. heterosiphoniae*, *P. muelleri*, *P. palmariae*, *P. porphyrae*, *P. pyropiae*) and diatom parasitoids (*Diatomophthora drebesii*, *D. gillii*). The results obtained in this study have further improved the resolution and expanded the knowledge on the phylogeny of the early-diverging oomycetes, leading to the establishment of three new orders (*Miraculales*, *Diatomophthorales*, *Pontismatales*) and one order (*Anisolpidiales*) being reintroduced.

## SUMMARY

The early-diverging oomycetes now include seven orders (*Miraculales*, *Olpidiopsidales*, *Eurychasmatales*, *Haptoglossales*, *Anisolpidiales*, *Pontismatales,* and *Haliphthorales*) and eight families (*Miraculaceae*, *Olpidiopsidaceae*, *Eurychasmataceae*, *Haptoglossaceae*, *Anisolpidiaceae*, *Diatomophthoraceae*, *Pontismataceae*, and *Haliphthoraceae*). Primarily holocarpic, these organisms are obligate biotrophic parasites of diatoms, algae, aquatic oomycetes and invertebrates. They are ubiquitous and can be found in almost all types of aquatic environments. However, little is known on the biology and ecology of these organisms, despite their widespread occurrence and importance in understanding the evolution of the oomycetes. Up to now, the taxonomy and phylogeny of the early-diverging lineage of oomycetes has remained largely unresolved as compared to *Saprolegniales* and *Peronosporales*, since the majority have not been sequenced or have been incompletely described. It is also becoming apparent that the classical systematics of the early-diverging oomycetes (Karling, 1942; Sparrow, 1960; Dick, 2001) are not supported by molecular phylogeny and, thus, require significant revision. However, resolving the phylogeny of the early-diverging lineage of oomycetes remains challenging since most species that were previously assigned to this clade remain unsequenced, including several of the type species (e.g. *Aphanomycopsis bacillariacearum*, *Ectrogella bacillariacearum*, *Olpidiopsis saprolegniae*, *Pontisma lagenidioides*, and *Sirolpidium bryopsidis*). The thesis, herein, presents the outcome of five different studies that aimed to resolve the phylogeny of the early-diverging lineage of oomycetes using molecular phylogeny.

In the first publication (Buaya *et al*., 2017), two new early-diverging oomycetes parasitoids (*Miracula helgolandica*, and *Olpidiopsis drebesii*) from two bloom-forming marine diatoms (*Pseudo-nitzschia pungen*s, and *Rhizosolenia imbricata*) were introduced and classified. *Oomycetes* parasitoids of diatoms are common, especially in the marine environments, seasonally recurring in parallel to phytoplankton blooms. However, the biology and ecology of these inconspicuous parasitoids are not well understood, despite their widespread nature and potential role in the breakdown of diatom blooms. For the past decades, most of the diatom-infecting oomycetes were mostly documented from freshwater while few studies were carried out on marine environments. A newly recognised holocarpic oomycetes, parasitising the poisonous bloom-forming diatom *Pseudo-nitzschia pungens*, has been reported from Prince Edward Island, Canada. Its life-cycle and morphology has been well characterised, but its taxonomic affiliation within the early-diverging oomycetes was obscure due to the unavailability of sequence data. This parasitoid was initially speculated to be related to either *Ectrogella* or *Olpidiopsis* because of close morphological similarities and host-range. Efforts were made to resolve the phylogeny of this new oomycetes, which was re-isolated in the Helgoland Reede (North Sea, Germany), together with another novel diatom-infecting species. Based on the distinctiveness of their morphology, development and molecular phylogeny, the isolates were classified as new species. Phylogenetic reconstruction shows that the parasitoid infecting *Rhizosolenia imbricata* is related to the *Olpidiopsis* genus and was described as *Olpidiopsis drebesii*, while the *Pseudo-nitzschia pungens* parasitoid was classified to a novel genus *Miracula* and named as *Miracula helgolandica*. Interestingly, a second *Miracula* species was discovered from freshwater and described as *Miracula*

*moenusica*. This new species is presented in the third publication (Buaya and Thines, 2019c); it is a virulent holocarpic parasitoid of the invasive centric diatom *Pleurosira laevis*. Phylogenetic reconstructions revealed in this study, show that this parasitoid is closely related to *M. helgolandica* with high support, together with other divergent sequences derived from environmental sequencing. The genus *Miracula*, described in this study, was assigned to its own family of *Miraculaceae* and this group is probably the earliest-diverging lineage of the oomycetes.

In the second publication (Buaya, Ploch and Thines, 2019a), the freshwater diatom parasitoid *Olpidiopsis gillii* was rediscovered from the River Main (Hesse, Germany) and its phylogenetic placement in the early-diverging lineage of oomycetes was resolved. *Olpidiopsis gillii* is the only known diatom-infecting parasitoid from the genus *Olpidiopsis*. Originally assigned to *Olpidium*, this parasitoid has often been synonymised to *Ectrogella bacillariacearum* and other early-diverging oomycetes parasitoids because of its similar life-cycle and host-range. However, zoospore diplanetism is absent in this species, in contrast to the diplanetic zoospores produced by *E. bacillariacearum* and other *Ectrogella* parasitoids (e.g. *E. monostoma*, and *E. licmophorae*). Molecular sequences obtained in this study revealed that *O. gillii* is a *bona fide* early-diverging oomycete and forms a monophyletic grouping with *Olpidiopsis drebesii*, another marine diatom-infecting oomycete and several other undescribed environmental sequences. However, phylogenetic placement of these simple holocarpic parasitoids into the early-diverging lineage of oomycetes still remains provisional because many of the described species remain unsequenced, especially the type species (e.g. *Aphanomycopsis bacillariacearum*, *Ectrogella bacillariacearum*, *Olpidiopsis saprolegniae*, *Pontisma*

*lagenidioides*, and *Sirolpidium bryopsis*). In order to clarify and settle the taxonomy of the early-diverging oomycetes, efforts were undertaken to isolate the type species of the early-diverging oomycetes, resulting in the resolution of the phylogenetic placement of the genera *Olpidiopsis*, *Pontisma,* and *Ectrogella*; these are reported in the fourth (Buaya *et al*., 2019d) and fifth publications (Buaya and Thines, 2020).

The genus *Olpidiopsis* contains the largest assemblage of early-diverging oomycetes that are parasites of *Chlorophyta*, *Rhodophyta*, *Phaeophyta*, *Bacillariophyt*a, *Dinoflagellata*, *Chytridiomycota*, and *Oomycota*. Most of the sequenced species of *Olpidiopsis* are from the marine *Rhodophyta*; this genus was initially speculated to be polyphyletic. However, phylogenetic placement of the *Olpidiopsis* type species, *O. saprolegniae,* from *Saprolegnia parasitica* revealed in this study, shows that *Olpidiopsidales* are monophyletic and are largely unrelated to the parasitoids of red-algae (Buaya *et al*., 2019d), while the phylogenetic placement of the *Rhodophyte* parasite *Pontisma* type species, *P. lagenidioides*, shows that it is largely unrelated to the *Olpidiopsidales* and forms a monophyletic grouping together with the parasites of marine red-algae (Buaya *et al*., 2019d). As the type species of *Olpidiopsis*, *O. saprolegniae*, is largely unrelated to the parasites of red-algae, all parasitoids of *Rhodophyte* algae were transferred to *Pontisma*. This taxonomic reappraisal has resulted in six new combinations of *Pontisma*: *Pontisma heterosiphoniae*, *P. feldmanii*, *P. pyropiae*, *P. porphyrae*, *P. porphyrae var. koreanae*, *P. bostrychiae*, *P. muelleri*, and *P. palmariae*. In addition, a new order *Pontismatales* has been introduced to accommodate the monophyletic clade containing parasites of *Rhodophyte* algae that were previously assigned to *Olpidiopsis* (Buaya *et al*., 2019d). Furthermore, a new *Olpidiopsis* species was also described in this study, *O.*

*parthenogenetica* from *Saprolegnia terrestris*, isolated from Japan (Buaya *et al*., 2019d). Aside from *Olpidiopsis* and *Pontisma*, the type species of the genus *Ectrogella*, *E. bacillariacerum*, was rediscovered from the River Main (Hesse, Germany) and its phylogenetic placement has also been reported here (Buaya and Thines, 2020). Genus *Ectrogella* comprises a heterogeneous group of obligate endoparasitoids, mostly of diatoms and filamentous algae. Despite their widespread occurrence, taxonomic affinities of most species in this genus remain unresolved and unclear. Initially, the genus was speculated to have an affinity to the early-diverging lineage of oomycetes because of close similarities in morphology, life-cycle and host-range. However, phylogenetic investigations of the type species *E. bacillariacearum* show that it is grouped among the early-diverging lineages of the *Saprolegniomycetes* with high support and is largely unrelated to the monophyletic olpidiopsis-like diatom parasitoids (Buaya and Thines, 2020). The genus *Diatomophthora* was introduced in order to accommodate the monophyletic grouping containing the diatom-infecting clade, which are neither related to the genus *Ectrogella* nor to *Olpidiopsis*, including the two sequenced species of diatom parasitoids, *Diatomopthora drebesii*, and *D. gillii* (Buaya and Thines, 2020). This study partially resolved the phylogeny and taxonomy of the early-diverging lineages of the oomycetes in five published papers. Findings obtained in this study further expanded the present knowledge on the taxonomy and phylogeny of these ubiquitous, but little-known, members of the phylum *Oomycota*.

# ZUSAMMENFASSUNG

Die früh divergierenden Oomyceten umfassen im Moment sieben Ordnungen (*Miraculales*, *Olpidiopsidales*, *Eurychasmatales*, *Haptoglossales*, *Anisolpidiales*, *Pontismatales*, und *Haliphthorales*) und acht Familien (*Miraculaceae*, *Olpidiopsidaceae*, *Eurychasmataceae*, *Haptoglossaceae*, *Anisolpidiaceae*, *Diatomophthoraceae*, *Pontismataceae*, und *Haliphthoraceae*). All diese primär holokarpen Organismen sind obligat biotrophe Parasiten von Diatomeen, Algen, aquatischen Oomyceten und Invertebraten. Sie sind ubiquitär verbreitet und können in fast allen aquatischen Ökosystemen gefunden werden. Jedoch ist, trotz ihrer weltweiten Verbreitung und ihrer Bedeutung für das Verständnis der Evolution der Oomyceten, nur wenig über die Biologie und Ökologie dieser Organismen bekannt. Verglichen mit den *Saprolegniales* und *Peronosporales* ist die Taxonomie und Phylogenie der früh divergierenden Linien von Oomyceten größtenteils ungeklärt, da die Mehrheit nicht sequenziert oder nur unvollständig beschrieben ist. Zudem zeichnet sich ab, dass die klassische Systematik der früh divergierenden Oomyceten (Karling, 1942; Sparrow, 1960; Dick, 2001) nicht durch molekulare Phylogenien gestützt wird, und somit dringend einer Revision bedarf. Da jedoch viele Arten, im Besonderen zahlreiche Typusarten, die zuvor dieser Gruppe zugeordnet worden sind, immer noch nicht sequenziert sind, bleibt die vollständige Aufklärung der phylogenetischen Beziehungen der früh divergierenden Linien der Oomyceten eine Herausforderung. Diese Dissertation präsentiert die Ergebnisse fünfer Studien, deren Ziel es war, die Phylogenie der früh divergierenden Linien der Oomyceten unter Verwendung molekularer Methoden aufzuklären.

In der ersten Veröffentlichung (Buaya *et al*., 2017) wurden zwei neue Arten von früh divergierenden Oomyceten (*Miracula helgolandica*, und *Olpidiopsis drebesii*) beschrieben und klassifiziert, die unterschiedliche, Algenblüten ausbildende, marine Diatomeen (*Pseudo-nitzschia pungens*, und *Rhizosolenia imbricata*) parasitieren. Diatomeen infizierende, parasitäre Oomyceten sind speziell in marinen Ökosystemen häufig und tauchen saisonal, parallel zu den Phytoplanktonblüten auf. Doch trotz ihrer weiten Verbreitung und ihrer vermuteten Rolle beim Zusammenbruch der Algenblüten sind die Biologie und Ökologie dieser unscheinbaren Parasiten nur unzureichend verstanden. Die meisten Berichte Diatomeen infizierender Oomyceten der letzten Jahrzehnte stammen von Süßwasserökosystemen, während nur einige wenige Untersuchungen an marinen Habitaten durchgeführt wurden. Ein bei Prince Edward Island, Kanada, neu entdeckter holokarper Oomycet, der die giftige, Algenblüten ausbildende Diatomee *Pseudo-nitzschia pungens* parasitiert, wurde zwar morphologisch gut beschrieben, aber seine Zugehörigkeit zu den früh-divergierenden Oomyceten bleibt aufgrund fehlender Sequenzdaten unklar. Anfangs wurde aufgrund starker morphologischer Ähnlichkeiten und ähnlichem Wirtsspektrum spekuliert, dass der Parasitoid entweder *Ectrogella* oder *Olpidiopsis* zuzuordnen ist. Daher wurden Anstrengungen unternommen diesen Oomyceten phylogenetisch zu charakterisieren und er konnte in der Helgoland Reede (Nordsee, Deutschland) zusammen mit einer anderen, unbeschriebenen Art wiedergefunden werden. Basierend auf den Unterschieden in Morphologie, im Lebenszyklus und in der molekularen Phylogenie wurden diese zwei Isolate als neue Arten beschrieben. Die phylogenetische Rekonstruktion zeigte dabei, dass die *Rhizosolenia imbricata* infizierende Art mit der Gattung *Olpidiopsis* verwandt ist

und daher als *Olpidiopsis drebesii* beschrieben wurde, während der Parasitoid von *Pseudo-nitzschia pungens* der neuerhobenen Gattung *Miracula* zugeordnet und *Miracula helgolandica* genannt wurde. Spannenderweise konnte im Süßwasser eine zweite Art der neuen Gattung *Miracula* entdeckt werden, die als *Miracula moenusica* beschrieben wurde. Diese Art wird in der dritten Publikation dieser Dissertation präsentiert (Buaya and Thines, 2019c) und ist ein virulenter, holokarper Parasitoid der invasiven, zentrischen Diatomee *Pleurosira leavis*. Die phylogenetischen Rekonstruktionen zeigen, dass dieser Parasitoid nah, und durch hohe Unterstützungswerte gesichert, mit *M. helgolandica* und weiteren, durch Sequenzierung von Umwelt-DNA gefundenen, unbestimmten Organismen verwandt ist. Daher wurde die hier beschriebene Gattung *Miracula* einer eigenen, neu erhobenen Familie (*Miraculaceae*) zugeordnet, die vermutlich die am frühesten divergierende Linie der Oomyceten darstellt.

Die zweite Arbeit (Buaya, Ploch und Thines, 2019a) beschreibt einen Parasitoiden von Süßwasserdiatomeen, *Olpidiopsis gillii*, wiederentdeckt im Fluss Main (Hessen, Deutschland), und charakterisiert seine phylogenetische Position innerhalb der früh divergierenden Linien der Oomyceten. *Olpidiopsis gillii* ist die einzig bekannte, Süßwasserdiatomeen infizierende Art aus der Gattung *Olpidiopsis*. Ursprünglich der Gattung *Olpidium* zugeordnet, wurde diese Art oft mit *Ectrogella bacillariacearum* und anderen früh divergierenden Oomyceten aufgrund von Ähnlichkeiten im Lebenszyklus und Wirtsspektrum synonymisiert. Jedoch gibt es im Gegensatz zu den diplanetischen Zoosporen, die von *E. bacillariacearum* und anderen *Ectrogella* Arten (z.B. *E. Monostoma*, und *E. licmophorae*) gebildet werden, bei dieser Art keinen Diplanetismus der Zoosporen. Die in dieser Arbeit generierten, molekularen Sequenzen lassen eine

Zuordnung von *O. gillii* zu den früh divergierenden Oomyceten *bona fide* zu und er bildet sogar eine monophyletische Gruppe mit *Olpidiopsis drebesii*, einem anderen, marine Diatomeen infizierenden Oomyceten, sowie weiteren, unbeschriebenen Umweltsequenzen. Jedoch musste die phylogenetische Gruppierung dieser einfachen holokarpen Parasiten in die früh divergierenden Linien der Oomyceten vorläufig bleiben, weil viele beschriebene Arten und besonders Typusarten (z.B. *Aphanomycopsis bacillariacearum*, *Ectrogella bacillariacearum*, *Olpidiopsis saprolegniae*, *Pontisma lagenidioides*, und *Sirolpidium bryopsis*) bisher nicht sequenziert sind. Um die Taxonomie der früh divergierenden Oomyceten umfassender aufzuklären, wurden Bemühungen unternommen diese Typusarten zu isolieren, was zur Aufklärung der phylogenetischen Positionierung der Gattungen *Olpidiopsis*, *Pontisma* und *Ectrogella* führte, und in der vierten (Buaya *et al*., 2019d) und fünften Publikation (Buaya and Thines, 2020) vorgestellt wird.

Die Gattung *Olpidiopsis* umfasst die größte Gruppe früh divergierender Oomyceten, welche Parasiten von *Chlorophyta, Rhodophyta, Phaeophyta, Bacillariophyta, Dinoflagellata, Chytridiomycota*, und *Oomycota* sind. Die meisten, der bereits sequenzierten Arten der Gattung *Olpidiopsis*, von welcher man anfänglich annahm, sie sei polyphyletisch, stammen dabei von marinen Rhodophyceen. Jedoch zeigte die phylogenetische Analyse der Typusart von *Olpidiopsis*, *O. saprolegniae* isoliert von *Saprolegnia parasitica*, dass die *Olpidiopsidales* monophyletisch und nicht mit den Parasiten von Rotalgen verwandt sind (Buaya *et al*., 2019d), während die phylogenetische Analyse der Rhodophyceen parasitierenden Gattung *Pontisma,* repräsentiert durch die Typusart *P. lagenidioides,* keine nähere Verwandtschaft zu den

*Olpidiopsidales* aufweist und selbst eine monophyletische Gruppe mit weiteren Parasiten mariner Rotalgen bildet (Buaya *et al*., 2019d). Da die Typusart von *Olpidiopsis*, *O. saprolegniae*, nicht näher mit den Parasiten der Rotalgen verwandt ist, wurden alle Parasitoiden der *Rhodophyta* in die Gattung *Pontisma* transferiert: *Pontisma heterosiphoniae*, *P. feldmanii*, *P. pyropiae*, *P. porphyrae*, *P. porphyrae* var. *koreanae*, *P. bostrychiae*, *P. muelleri*, and *P. palmariae*. Zusätzlich dazu wurde die neue Ordnung *Pontismatales* eingeführt, um der monophyletischen Natur der Parasiten der Rhodophyceen Rechnung zu tragen, die zuvor Olpidiopsis zugeordnet waren (Buaya *et al*., 2019d). Weiterhin wurde eine neue *Olpidiopsis* Art, *O. parthenogenetica* isoliert von *Saprolegnia terrestris* in Japan, in dieser Veröffentlichung beschrieben (Buaya *et al*., 2019d). Zusätzlich zu *Olpidiopsis* und *Pontisma* konnten auch die Typusarten von *Ectrogella*, *E. bacillariacerum*, im Fluss Main (Hessen, Deutschland) wiederentdeckt und ihre phylogenetische Position entschlüsselt werden (Buaya and Thines, 2020). Die Gattung *Ectrogella* umfasst eine heterogene Gruppe von obligaten Endoparasiten von Diatomeen und filamentösen Algen. Trotz ihrer weiten Verbreitung ist die taxonomische Zugehörigkeit der meisten Arten dieser Gattung weiterhin ungeklärt oder unsicher. Zuerst wurde vermutet, dass diese Gattung, wegen starker Ähnlichkeiten in Morphologie, Lebenszyklus und Wirtsspektrum zu den früh divergierenden Oomyceten gehört. Jedoch zeigten phylogenetische Untersuchungen der Typusart *E. bacilariacearum*, dass sie, gesichert durch hohe Unterstützungswerte, zu den früh divergierenden Linien der *Saprolegniomyceten* gehört und nicht näher mit der monophyletischen Gruppe von *Olpidiopsis*-ähnlichen Parasitoiden der Diatomeen verwandt ist (Buaya and Thines, 2020). Daher wurde die neue Gattung *Diatomophthora*, die die beiden in dieser Arbeit

sequenzierten Arten *Diatomophthora drebesii* und *D. gilii* beinhaltet, eingeführt, um der Monophylie dieser Diatomeen-infizierenden Gruppe, die weder zur Gattung *Ectrogella* noch zu *Olpidiopsis* gehört, Rechnung zu tragen (Buaya and Thines, 2020). Diese Arbeit konnte somit die Phylogenie und Taxonomie der früh divergierenden Linien der Oomyceten in fünf veröffentlichten Manuskripten tiefer beleuchten. Die Entdeckungen in dieser Dissertation erweiterten daher das vorhandene Wissen über die Taxonomie und Phylogenie dieser ubiquitär verbreiteten, aber wenig bekannten, Mitglieder des Phylums *Oomycota*.

# PUBLICATIONS

**Included in the thesis**

*Published*

1. **Buaya AT**, Ploch S, Hanic L, Nam B, Nigrelli L, Kraberg A and Thines M. Phylogeny of *Miracula helgolandica* gen. et sp. nov. and *Olpidiopsis drebesii* sp. nov., two basal oomycete parasitoids of marine diatoms, with notes on the taxonomy of *Ectrogella*-like species. *Mycological Progress*, Volume 16, Page 1041-1050, December 2017.

2. **Buaya AT**, Ploch S and Thines M. Rediscovery and phylogenetic placement of *Olpidiopsis gillii* (de Wildeman) Friedmann, a holocarpic oomycete parasitoid of freshwater diatoms. *Mycoscience*, Volume 60, Page 141-146, May 2019.

3. **Buaya AT** and Thines M. *Miracula moenusica*, a new member of the holocarpic parasitoid genus from the invasive freshwater diatom *Pleurosira laevis*. *Fungal Systematics and Evolution*, Volume 3, Page 35-40, January 2019.

4. **Buaya AT**, Ploch S, Inaba S and Thines M. Holocarpic oomycete parasitoids of red algae are not *Olpidiopsis*. *Fungal Systematics and Evolution*, Volume 4, Page 21-31, December 2019.

5. **Buaya AT** and Thines M. *Diatomophthoraceae*-a new family of *olpidiopsis*-like diatom parasitoids largely unrelated to *Ectrogella*. *Fungal Systematics and Evolution*, Volume 5, Page 113-118, June 2020.

**Not included in the thesis**

*Published*

6. **Buaya AT**, Kraberg A and Thines M. Dual culture of the oomycete *Lagenisma coscinodisci* Drebes and *Coscinodiscus* diatoms as a model for plankton/parasite interactions. *Helgoland Marine Research*, Volume 73, 2, April 2019.

7. Hassett B, Thines M, **Buaya AT**, Ploch S and Gradinger R. A glimpse into the biogeography, seasonality, and ecological functions of arctic marine Oomycota. *IMA Fungus*, Volume 10, 6, June 2019.

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9. Bode E, Heinrich AK, Hirschmann M, Abebew D, Shi YN, Vo TD, Wesche F, Shi YM, Grün P, Simonyi S, Keller N, Engel Y, Wenski S, Bennet R, Beyer S, Bischoff I, **Buaya A**, Brandt S, Cakmak I, Çimen H, Eckstein S, Frank D, Fürst R, Gand M, Geisslinger G, Hazir S, Henke M, Heermann R, Lecaudey V, Schäfer W, Schiffmann S, Schüffler A, Schwenk R, Skaljac M, Thines E, Thines M, Ulshöfer T, Vilcinskas A, Wichelhaus TA and Bode HB. Promoter Activation in *Δhfq* Mutants as an Efficient Tool for Specialized Metabolite Production Enabling Direct Bioactivity Testing. *Angewandte Chemie*, Volume 58, Page 18957-18963, November 2019.

*Submitted or under-review*

10. **Buaya AT** and Thines M. *Bolbea parasitica* gen. et sp. nov., a cultivable holocarpic parasitoid of the early-diverging Saprolegniomycetes. *Fungal Systematics and Evolution*, Submitted and Under-review.

11. **Buaya AT**, Ploch S, Kraberg A and Thines M. *Diatomophthora perforans* comb. nov. is a radiating, potentially plurivorous parasitoid of marine diatoms. *Mycological Progress*, Submitted and Under-review.

12. **Buaya AT** and Thines M. On the biology and phylogeny of the early-diverging oomycetes. *Philippine Journal of Systematic Biology*, Submitted and Under-review.

# 1 INTRODUCTION

## 1.1 Oomycetes

The *Oomycetes* are fungal-like heterotrophic organisms belonging to the Kingdom Straminipila (often informally referred to as “stramenopiles”) of the SAR (Straminipila, Alveolata, Rhizaria) Superkingdom (Baldauf *et al*., 2000) together with phototrophic organisms, such as the brown seaweeds and diatoms (Silberfeld, Rousseau and de Reviers, 2014). Sometimes, the kingdom Straminipila is also referred to as Chromista or Chromalveolata (Cavalier-Smith and Chao, 2006; Cavalier-Smith, 2018), but as the older kingdom concept ‘Chromista’ is not monophyletic and the Alveolata and Straminipila are both very deeply branching lineages, ‘Straminipila’ is the preferred kingdom-level designation. The *Oomycota* are sometimes referred to as *Pseudofungi* (Cavalier-Smith, 1997; Cavalier-Smith and Chao, 2006) but then also including the *Hyphochytriomycota* and *Labyrinthulomycota*. As the latter two groups are often described under the zoological, rather than the botanical Code of Nomenclature and some bacteriophagic unicellular protists seem to branch within the group (Tong, 1995; Kühn, Medlin and Eller, 2004), the phylum designation as *Oomycota* is preferable. The group is traditionally studied by mycologists, because of superficial similarities due to convergent evolution (Alexopoulos, Mims and Blackwell, 1996; Lévesque, 2011). However, there is no immediate phylogenetic relationship with the Mycota (Alexopoulos, Mims and Blackwell, 1996; Beakes and Sekimoto, 2009; Beakes, Glockling and Sekimoto, 2012; Beakes and Thines, 2017). Several characters set oomycetes apart from Mycota. Unlike Mycota, oomycetes produce diploid or polyploid thalli with meiosis occurring in the developing gametangia (Alexopoulos, Mims and Blackwell, 1996; Dick, 2001); asexual reproduction

is by means of conidia or conidiosporangia, which often produce heterokont zoospores with a whiplash flagellum directed backwards and longer tinsel flagellum directed forward that is ornamented with mastigonemes (Dick, 2001; Walker and van West, 2007); sexual reproduction is oogamous by means of gametangial fusion resulting in the production of thick-walled oospores (Alexopoulos, Mims and Blackwell, 1996; Dick, 2001); the cell wall is composed primarily of β-glucans and varying amounts of cellulose rather than chitin (Cooper and Aronson, 1967; Bartnicki-Garcia, 1968; Lin and Aronson, 1970; Wang and Bartnicki-Garcia, 1974; Myklestad and Granum, 2009; Beakes, Glockling and Sekimoto, 2012); cytoplasmic dense-body or “finger-print” vacuoles with storage of mycolaminarin polysaccharide phosphate (Traquair and McKeen, 1980; Bortnick, Powell and Bangert, 1985; Beakes, Glockling and Sekimoto, 2012); mitochondria with tubular cristae (Alexopoulos, Mims and Blackwell, 1996; Beakes, Glockling and Sekimoto, 2012) and a different biochemical pathway for the synthesis of amino acid lysine (Vogel, 1960; Vogel, 1961; Vogel, 1964).

The *Oomycota* are ubiquitous and can be found in almost all types of environments, in both aquatic (e.g. lakes, rivers, ponds, mangrove swamps, brackish waters, oceans, arctic, and Antarctica) and terrestrial ecosystems (e.g. soil, muds, and plant parasites) (Sparrow, 1936; Sparrow, 1960; Canter and Heaney, 1984; Nakagiri, 2000; Dick, 2001; Leaño, 2002; Beakes and Sekimoto, 2009; Hulvey *et al*., 2010; Lévesque, 2011; Tojo *et al*., 2012; Beakes, Glockling and Sekimoto, 2012; Thines, 2014; Marano *et al*., 2016; Beakes and Thines, 2017; Bennett and Thines, 2017; Thines, 2018; Bennett *et al*., 2018; Bennett and Thines, 2019; Hassett *et al*., 2019; Dickie *et al*., 2019). In aquatic environments, most of the known species were recorded from freshwater, living as

saprotrophs or parasites of algae and animals (Sparrow, 1960; Marano *et al*., 2016). Only a few species are known from the marine realm, mostly living as obligate biotrophic parasites (Sparrow, 1960), and others thrive as facultative anaerobic saprophytes in anoxic water bodies (e.g. stagnant ponds, and heavily polluted waters) (Emerson and Weston, 1967; Emerson and Held, 1969; Alabi, 1972; Emerson and Natvig, 1981). Most of the terrestrial forms are primarily facultative and obligate biotrophic parasites of many vascular plants in natural and managed ecosystem (Thines, 2014). The obligate parasites of plants are known to cause devastating outbreak on several agriculturally important crops. Examples of these includes *Phytophthora infestans*, the cause of late blight in potatoes (Haas *et al*., 2009; Yoshida *et al*., 2013; Goss *et al*., 2014), *Pythium debaryanum*, causing damping-off diseases on several agricultural seedlings (Drechsler, 1953; Sansome, 1963; van der Plaats-Niterink, 1981), *Plasmopara viticola*, the cause of grape downy mildews, as well as several other species (Burruano, 2000; Gobbin *et al*., 2005; Gessler, Pertot and Perazzolli, 2011).

The phylum *Oomycota* is made up of two major classes, *Saprolegniomycetes* (Thines *et al*., 2015) and *Peronosporomycetes* (Dick, 2001), as well as several early-diverging lineages with unclear relationships (Beakes and Thines, 2017). The early-diverging lineages (*Eurychasmatales*, *Haliphthorales*, *Haptoglossales*, *Olpidiopsidales*) that are mostly holocarpic marine obligate biotrophic parasites (Sparrow, 1960; Dick, 2001; Sekimoto, Hatai and Honda, 2007; Beakes and Thines, 2017). To date, the *Oomycota* comprise about 1,500 species or more grouped into 100 genera (Beakes and Thines, 2017). The present taxonomic arrangement of the oomycetes is largely based on the works of Karling (1942), Sparrow (1960) and Dick (2001), but with larger taxonomic

revisions over the past 15 years (Thines and Spring, 2005; Thines *et al*., 2015; Beakes and Thines, 2017). *Peronosporomycetes* is the largest oomycetes class containing three orders (*Albuginales*, *Peronosporales*, *Rhipidiales*) that are saprophytes (e.g. *Sapromyces*, *Rhipidium*, *Salispina*, *Halophytophthora*, and *Phytopythium*) and parasites of various plants (e.g. *Phytophthora*, *Pythium*, *Pustula*, *Peronospora*, *Hyaloperonospora*, *Peronosclerospora*, *Pseudoperonospora*, *Plasmopara*, *Bremia*, and *Albugo*), vertebrate and invertebrate animals (e.g. *Lagenidium*, and *Myzocytiopsis*) (Dick, 2001; Kamoun, 2003; Lamour and Kamoun, 2009; Thines, 2014; Fawke, Doumane and Schornack, 2015; Kamoun *et al*., 2015; Beakes and Thines, 2017; Thines and Choi, 2016; Derevnina *et al*., 2016). Members of the *Peronosporomycetes* are mostly eucarpic, the majority are terrestrial and several species are specialised obligate hemibiotrophic and biotrophic parasites of plants in agriculture (e.g. *Phytophthora infestans*, *Plasmopara viticola*, *Pseudoperonospora cubensis*, *Peronospora destructor*, *Hyaloperonospora brassicae*, *Bremia lactucae*, and *Albugo candida*) and natural environments (e.g. *Phytophthora ramorum*, *Phytophthora cinnamomi*, *Phytophthora palmivora*, *Hyaloperonospora arabidopsidis*, and *Plasmopara obducens*) (Thines, 2014; Thines and Choi, 2016; Beakes and Thines, 2017; Derevnina *et al*., 2016). Several species are also amphibious and can inhabit both aquatic and terrestrial environments, such as several saprotrophic or facultative parasitic species belonging to genera *Pythium*, *Halophytophthora*¸ *Phytopythium*, and *Phytophthora* (Nakagiri, 2000; Dick, 2001; Nakagiri *et al*., 2001; Hansen, Reeser and Sutton, 2012; Jung *et al*., 2012; Bennett *et al*., 2018). The only known oomycetes species to infect mammals, *Lagenidium giganteum* and *Pythium insidiosum* also belongs to this class, causing a necrotrophic disease called “pythiosis” in

dogs, horses, cattle and humans in the tropics (De Cock, 1987; Gaastra *et al*., 2010; Presser and Goss, 2015). The hyphae of several hemibiotrophic plant parasites grow into healthy or dying cells of the host plant, which will eventually kill the host plant or certain parts after colonization (Thines, 2014; Fawke, Doumane and Schornack, 2015; Thines and Choi, 2016; Whisson *et al*., 2016). The obligate biotrophic pathogens produces specialized hyphal structures termed haustoria that are able to penetrate into host cell wall, expand intracellular, invaginated by the plasma membrane (Dick, 2001; Szabo and Bushnell, 2001; Meng *et al*., 2009; Whisson *et al*., 2016). While most saprotrophic and non-obligate pathogenic species produce zoospores upon germination of the conidiosporangia, many obligate parasites (e.g. *Bremia, Hyaloperonospora*, and *Peronospora*) produce conidia that germinate directly by forming germ tubes, instead of producing zoospores (Voglmayr *et al*., 2004; Thines and Kamoun, 2010; Runge Choi and Thines, 2011; Savory *et al*., 2011; Thines 2014; Yin *et al*., 2017).

The *Saprolegniomycetes* (Thines *et al*., 2015) contain three recognised orders (*Saprolegniales*, *Leptomitales*, *Lagenismatales*) mainly saprobes and parasites of plants/algae (e.g. *Aphanomyces*, *Lagenisma*, and *Ectrogella*), vertebrate (e.g. *Saprolegnia*, and *Achlya*) and invertebrate animals (e.g. *Atkinsiella*, *Chlamydomyzium*, *Aquastella*, *Sommerstorffia*, and *Leptolegnia*) (Tiffney and Wolf, 1937; Karling, 1942; Karling, 1952; Prowse, 1954; Sparrow, 1960; Drebes, 1966; Srivastava and Srivastava, 1978; Zattau and McInnis, 1987; Nakamura and Hatai, 1995; Dick, 2001; van West, 2006; Gaulin *et al*., 2007; Beakes and Sekimoto, 2009; Beakes, Glockling and Sekimoto, 2012; Molloy *et al*., 2014; Beakes, Glockling and James, 2014; Beakes and Thines, 2017; Rocha *et al*., 2018; Buaya, Kraberg and Thines, 2019c). The eucarpic members of the

*Saprolegniomycetes* are mostly placed in the *Saprolegniales*, while the holocarpic ones are in the clade comprised of *Leptomitales* and *Lagenismatales*. The relationships in that clade are largely unclear and several holocarpic genera are assumed to belong to this group. The eucarpic members of the *Saprolegniomycetes* are ubiquitous and abundant in the aquatic environment (freshwater, brackish waters) and moist soil, and are important contributors to organic decomposition and nutrient recycling (Karling, 1942; Sparrow, 1960; Alexopoulos, Mims and Blackwell, 1996; Dick, 2001; Beakes and Thines, 2017). Several species from this class are also facultative parasites and are known to infest several economically important plants (e.g*. Aphanomyces euteiches*) and animals (e.g. *Aphanomyces invadans*, *Aphanomyces astaci*, *Saprolegnia* spp., and *Achlya flagellata*) (Sparrow, 1960; Dick, 2001; van West, 2006; Gaulin *et al*., 2007; Gozlan *et al*., 2014; Rezinciuc *et al*., 2015; James *et al*., 2017; Iberahim, Trusch and van West, 2018). Among the most notable parasites from this class, causing significant problems to aquaculture, are *Saprolegnia parasitica*, the cause of an often fatal fish disease known as "saprolegniosis", and *Aphanomyces astaci*, the cause "crayfish plague" which almost wiped out the wild population of freshwater crayfish in Europe (van West, 2006; Rezinciuc *et al*., 2015; James *et al*., 2017). *Saprolegniomycetes* commonly grow an extensive network of coenocytic mycelium, and undergo both asexual and sexual reproduction (Sparrow, 1960; Dick, 2001). Asexual reproduction is by means of biflagellate zoospores that are dimorphic and diplanetic or polyplanetic (Sparrow, 1960). Sexual reproduction is oogamous, occurring by fusion of a haploid male (antheridium) and a female (oogonium) gametangium, producing one to several diploid oospores (Dick, 2001).

The early-diverging orders that branch before the main split between *Peronosporomycetes* and *Saprolegniomycetes* (*Eurychasmatales*, *Haptoglossales*, *Olpidiopsisdales*, *Haliphthorales*) and a few unresolved families, e.g. *Rozellopsidaceae*, show a great diversity of lifestyles and cytological adaptations and mostly strive in limnic and marine environments (Beakes and Sekimoto, 2009; Beakes, Glockling and Sekimoto, 2012; Beakes and Thines, 2017). Most species are holocarpic, and most seem to be, biotrophic parasites and either parasitoids of phytoplankton and algae, such as *Anisolpidium, Eurychasma*, *Olpidiopsis*, *Petersenia*, *Pontisma*, and *Sirolpidium*, or of invertebrate animals, such as *Haptoglossa*, *Haliphthoros*, *Halioticida*, and *Halodaphnea* (Karling, 1942; Sparrow, 1960; Dick, 2001; Hakariya, Hirose and Tokumasu, 2007; Sekimoto, Hatai and Honda, 2007; Sekimoto *et al*., 2008a; Sekimoto *et al*., 2008b; Gachon *et al*., 2017). While there is accumulating evidence that these holocarpic pathogens are ubiquitous, especially in aquatic environments, their ecological roles are widely unknown (Strittmatter, Gachon and Küpper, 2009; Beakes and Sekimoto, 2009; Skovgaard, 2014; Scholz *et al*., 2015; Beakes and Thines, 2017; Hassett *et al*., 2019). Only asexual reproduction is known to occur from organisms in this order by means of biflagellate zoospores that are monomorphic and monoplanetic (Karling, 1942; Sparrow, 1960; Dick, 2001). The type of oospores observed for the *Peronosporomycetes* and *Saprolegniomycetes* is an apomorphy of this crown group and absent from the early-diverging lineages. However, some species of *Olpidiopsis* (e.g. *Olpidiopsis saprolegniae*, *Olpidiopsis achlyae*, and *Olpidiopsis varians*) produce oospore-like structures, but their formation is not well understood (Cornu, 1872; Barrett, 1912; Shanor, 1939; McLarty, 1941; Sparrow, 1960). Classification of early-diverging oomycetes is mainly based on few

morphological characters and the development of the holocarpic thallus, zoosporangia and zoospores, as well as the mode of zoospores release and encystment (Sparrow, 1960; Dick, 2001; Beakes, Glockling and Sekimoto, 2012; Beakes and Thines, 2017). However, the taxonomy and phylogenetic relationships of the early-diverging oomycetes are poorly resolved as compared to the *Saprolegniales* and *Peronosporales* (Beakes and Thines, 2017). Only recently, interest on the taxonomy and systematics of this group has resurged (Fletcher *et al*., 2015; Klochkova *et al.,* 2016; Buaya *et al*., 2017; Kwak *et al*., 2017; Klochkova, Kwak and Kim, 2017; Garvetto *et al*., 2018; Badis *et al*., 2019; Garvetto *et al*., 2019). However, investigations of these organisms are challenging due to the obligate biotrophic nature of the parasitoids, and huge effort is needed for isolating and establishing a stable dual culture in a defined chemical medium.

## 1.2 General Biology and Characteristics of the Basal Oomycetes

### *1.2.1 Morphology and Life-cycle*

Early-diverging lineages of the oomycetes have rather simple morphological characters unlike the morphological more complex species from the two crown classes. All known species of the early-diverging oomycetes produce endobiotic holocarpic thalli that subsequently matures into sporangia (Karling, 1942; Sparrow, 1960; Dick, 2001; Beakes and Thines, 2017). The typical life-cycle of the early-diverging oomycetes starts as soon an encysted zoospore attached to its host and undergoes germination. After subsequent penetration, growth and elongation, the thallus undergoes rapid differentiation. At early stages of development, the colorless plasmodial thallus is un-walled or very thin-walled, and for most early-diverging oomycetes, growth usually starts close to the host nucleus (Karling, 1942; Sparrow, 1960; Schnepf, Deichgräber and Drebes, 1978b; Dick, 2001;

Beakes, Glockling and Sekimoto, 2012), probably to enable a more efficient deployment of pathogenicity effectors. This is evident on various early-diverging species especially those that parasitise algae (e.g. *Olpidiopsis schekiana*, *Olpidiopsis oedogoniarum*, and *Eurychasma dicksonii*) and diatoms (e.g. *Diatomophthora gillii*, and *Ectrogella perforans*), including also the two early-diverging members of the *Saprolegniomycetes*, *Ectrogella bacillariacearum*, and *Lagenisma coscinodisci* (Zopf, 1884; Scherffel, 1925; Sparrow and Ellison, 1949; Friedmann, 1952; Sparrow, 1960; Drebes, 1966; Johnson, 1966; Schnepf and Drebes, 1977; Raghu Kumar, 1980; Schnepf, Deichgräber and Drebes, 1978a). The majority of the early-diverging oomycetes such as the marine parasitoids of algae (e.g. *Eurychasma*, *Anisolpidium*, *Pontisma*, *Sirolpidium*, and *Petersenia*), diatoms (e.g. *Aphanomycopsis*, and *Ectrogella*) and aquatic oomycetes (e.g. *Olpidiopsis*) produces unbranched or little-branched thalli that are either tubular or spherical (Zopf, 1884; Cornu, 1872; Magnus, 1905; Petersen, 1905; Scherffel, 1925; Karling, 1943; Feldmann and Feldmann, 1955; Sparrow, 1960). Others early-diverging lineages produce branched thalli, such as some species that are parasites of invertebrates (e.g. *Haliphthoros*, *Halocrusticida*, *Halodaphnea*, and *Atkinsiella*), but also *Lagenisma*, a parasite of centric diatoms, produces branched thalli (Vishniac, 1958; Sparrow, 1960; Dick, 1988; Drebes, 1966). The principal chemical composition of the thallus wall of early-diverging oomycetes is not fully known, but it is likely to contain significant amounts of cellulose derivatives, since most species across several genera (e.g. *Ectrogella*, and *Olpidiopsis*) exhibits positive reaction when tested with Melzer's reagent (chloride, zinc, and iodine solution) (Zopf, 1884; Friedmann, 1952; Sparrow, 1960; Dick, 2001).

In several species of *Olpidiopsis* (e.g. *Olpidiopsis saprolegniae*, *Olpidiopsis achlyae*, *Olpidiopsis vexans*, *Olpidiopsis luxurians*, and *Olpidiosis varians*), a number of spherical vacuoles is prominent during the mid-stage of sporangium development (Shanor, 1939; Sparrow, 1960; Barrett, 1912; McLarty, 1941). After subsequent differentiation, these vacuoles disappear before roundish zoospores initials and the discharge tube begin to form. The number and length of discharge tube varies and it is unclear, if this character can be used. While most possess single discharge tubes per thallus or thallus segment (e.g. *Ectrogella monostoma*, *Diatomophthora gillii, Olpidiopsis saprolegniae*, and *Pontisma lagenidioides*), there are several species that have multiple exit tubes (e.g. *Ectrogella bacillariacearum, Ectrogella perforans*, and *Ectrogella licmophorae*) (Zopf, 1884; Cornu, 1872; Petersen, 1905; Scherffel, 1925; Friedmann, 1952). Unlike other early-diverging species, several diatom-infecting oomycetes (e.g. *Ectrogella bacilariacearum*, *E. perforans*, and *E. monostoma*) show a thickening of the base of the discharge tube (Zopf, 1884; Scherffel, 1925; Sparrow, 1960; Johnson, 1966). It is likely that these thickenings are formed as a “forcing apparatus” (Scherffel, 1925) for pushing apart the frustule of the diatoms host during the development of the thallus into a mature sporangium (Johnson, 1966). However, it is unclear and remains to be known if these “thickenings” have significant taxonomic importance (Gavetto *et al*., 2018). From unpublished observations (Buaya and Thines, unpublished) it seems that this character is variable and depends on the actual force needed to enable sporulation.

In addition to the characters mentioned above, the mode of zoospores release also differs among species in the early-diverging lineage (Sparrow, 1960). Most holocarpic species of the early diverging *Saprolegniomycetes* and of the species diverging before the main

*Peronosporomycetes*/*Saprolegniomycetes* split have a zoospore discharge pattern either like *Olpidiopsis* (e.g. *Olpidiopsis saprolegniae*, and *Pontisma lagenidioides*), *Saprolegnia* (e.g. *Ectrogella bacillariacearum*, and *Lagenisma coscinodisci*), or *Achlya* (e.g. *Aphanomycopsis bacillariacearum*, *Ectrogella monostoma*, and *Ectrogella licmophorae*) (Cornu, 1872; Magnus, 1905; Scherffel, 1925; Petersen, 1905). In species with olpidiopsis-like and saprolegnia-like zoospores discharge, zoospores immediately swims away in dissolution after its release (Sparrow, 1960). While the olpidiopsis-like behavior is characterised by spores that swim for some time (sometimes several minutes), in species with saprolegnia-like discharge the spores quickly come to a rest (often within a minute) and form cysts from which a second generation of more vigorously-swimming zoospores emerges. Species with achlya-like discharge first release spores that are non-flagellated (aplanospores), which encyst at the orifice of the discharge tube, and undergo further development before release from the cysts (Scherffel, 1925; Canter, 1949). However, it is still unclear whether those species with achlya-like zoospores discharge pattern are *bona fide* member of the early-diverging oomycetes since none of them has sequence data available (Beakes and Thines, 2017).

All confirmed early-diverging oomycetes only produce pyriform shaped primary zoospores that are monomorphic and might change shape, but without forming cysts. Thus, they are considered as monoplanetic (Dick, 2001; Beakes and Sekimoto, 2009; Beakes, Glockling and Sekimoto, 2012; Beakes and Thines, 2017). Except for *Anisolpidium*, the zoospores of early-diverging oomycetes contain two anteriorly to sub-lateral inserted flagella, with a forwardly directed tinsel flagellum ornamented with mastigoneme hairs, and a whiplash flagellum trailing behind (Beakes, Glockling and

Sekimoto, 2012; Beakes and Thines, 2017). However, the former mastigoneme ornamentation appears to be variable among some lineages such as *Haptoglossa* which entirely lacks mastigonemes (Beakes and Glockling, 1998; Beakes, Glockling and Sekimoto, 2012).

Resting spores of early-diverging oomycetes are diverse and derive from various pathogen stages (Sparrow, 1960; Dick, 2001). In some early-diverging species zoospore cysts are converted into resting spores, e.g. in *Lagenisma coscinodisci* (Schnepf and Drebes, 1977). The encysted zoospores can stay dormant for a longer period of time and only germinate under proper environmental conditions. However, in species of the genus *Haptoglossa* the encysted spores germinate into “gun cells” (Barron, 1990; Barron, 1987; Barron, 1989; Beakes and Glockling, 1998; Beakes, Glockling and Sekimoto, 2012). It functions like a miniature cannon containing a needle-like harpoon projectile that is capable of rupturing the cuticle of its host (usually Adineta rotifers or rhabditid nematodes) establishing new infection site (Beakes, Glockling and Sekimoto, 2012). The ultrastructure and firing mechanism of gun cells has been described in detail by Beakes and Glockling (1998, 2000, 2002) and Barron (1980, 1987). In *Olpidiopsis*, resting spores can either be formed in a manner similar to oospore formation in the crown oomycetes (see below) or from non-discharged thalli that instead of discharge tubes develop a thick, usually ornamented wall (Cornu, 1872; Barrett, 1912; Shanor, 1939; Karling, 1942; Sparrow, 1960).

### *1.2.2 Sexual Reproduction*

Early-diverging oomycete genera are not forming oospores in the way present in the crown groups (*Peronosporomycetes*, and *Saprolegniomycetes*). The absence of

canonical sexual reproduction is likely a key diagnostic feature for all early-diverging species in addition to producing monomorphic and monoplanetic zoospores. However, an obscure form of sexual reproduction is apparently occurring in several species of the genus *Olpidiopsis* sensu Sparrow (1960), *Eurychasma dicksonii*, and *Anisolpidium ectocarpii* (Barrett, 1912; Sparrow, 1960; Beakes and Thines, 2017). This non-canonical reproduction has been reported many times (Cornu, 1872; Barrett, 1912; Coker, 1923; Tokunaga, 1933; Shanor, 1939; McLarty, 1941; Whiffen, 1942; Karling, 1942; Sparrow, 1960), but is still not fully understood. In *Olpidiopsis*, sexual reproduction is by the fusion of two resting thalli of unequal size, in which the smaller thallus (often referred to as companion cells or antheridium) passes its protoplasmic contents into the larger thallus (often referred to as oogonium) (Barrett, 1912; Sparrow, 1960). Karyogamy is assumed to occur after the protoplasmic fusion between two different thalli (Barrett, 1912; McLarty, 1941). Subsequently, a thick layer of exospore material is deposited, resulting in a variety of ornamentations, which can be spiny, smooth, tuberculate, fibrillose, or irregular (Sparrow, 1960). However, it remains to be demonstrated if sexual reproduction in *Olpidiopsis* is homologous of that of the crown groups or has evolved independently in the genus. So far, sexual reproduction in early-diverging oomycetes has been reported or assumed for only few species (Barrett, 1912; Coker, 1923; Scherffel, 1925; McLarty, 1941). The most well-documented case of non-oogamous sexual reproduction among holocarpic oomycetes was documented in the diatom parasite, *Lagenisma coscinodisci*, which is a member of the early-diverging *Saprolegniomycetes* (Drebes, 1966; Thines *et al*., 2015). In this species, encysted zoospores (zoomeiospores) conjugate forming a diploid zygote (with spiny wall ornamentation) that will eventually undergo meiosis,

forming several haploid spores (Schnepf, Deichgraber and Drebes, 1978a; Schnepf, Deichgraber and Drebes, 1978b; Schnepf, Deichgraber and Drebes, 1978c). For other species, e.g. *Eurychasma dicksonii*, a similar mode of reproduction has been assumed, but still needs to be ascertained by detailed cytological studies (Magnus, 1905; Sparrow, 1934; Sparrow, 1960; Sekimoto *et al*., 2008a).

# 2 OBJECTIVES OF THE STUDY

The early-diverging oomycetes, at the start of the work on this thesis, comprised of 4 genera and less than 100 species. These organisms are widely distributed and are all biotrophic parasites. However, little is known on the biology of these organisms and, until now, their phylogeny remained largely incomplete and unresolved. Many species that were previously assigned to the early-diverging lineage of oomycetes remain unsequenced, including several of the type species (e.g. *Aphanomycopsis bacillariacearum*, *Ectrogella bacillariacearum*, *Olpidiopsis saprolegniae*, *Pontisma lagenidioides*, and *Sirolpidium bryopsis*). Indeed, the classification of this group is still mainly based on classical methods (e.g. morphology and development of the thallus, zoosporangia, zoospores, zoospores release, and encystment) and many species have not been isolated since their original description. It is becoming apparent that many of the traditional systematic circumscriptions of the early-diverging oomycetes presented by Karling (1942), Sparrow (1960) and Dick (2001) are not supported by molecular phylogeny and necessitate significant revision. So far, only a few species within the early-diverging lineage have been sequenced and the taxonomic placement of most species still remains provisional because phylogenetic affinities of several type species remain uncertain. Thus, it was the aim of this study to infer the phylogenetic placement of several unsequenced species, especially the type species of several early-diverging oomycetes genera (e.g. *Aphanomycopsis*, *Ectrogella*, *Olpidiopsis*, *Pontisma*, and *Sirolpidium*) to finally settle their taxonomic placement and improve phylogenetic resolution of this lineage. Resolving the taxonomy and phylogeny of the early-diverging oomycetes is important in understanding the evolution of the phylum *Oomycota* which has been

hypothesised to originate from the marine environment. Moreover, this study also aims to search for novel species of early-diverging oomycetes in various aquatic environments, in both marine (e.g. sea, fjord, and littoral) and freshwater (e.g. river, lake, and pond) habitats. Early-diverging oomycetes are ubiquitous and are known to occur in a wide array of aquatic environments, however, there are still many places around the world that have not been sampled for these organisms, especially in the tropics and the arctic. In aquatic environments, multiple molecular studies have reported a great number of environmental sequences clustering to the early-diverging lineage of oomycetes, hinting to a huge, undiscovered diversity. Undoubtedly, there are still many novel, early-diverging oomycetes that remain to be discovered.

# 3 DISCUSSION AND SIDE RESULTS

## 3.1 Classification and Systematics

### *3.1.1 Phylogenetic Relationships of the Early-diverging Oomycetes Orders*

The early-diverging oomycetes previously comprised of 4 orders and families (Dick, 2001; Beakes and Thines, 2017). These include the *Olpidiopsidales*, *Eurychasmatales*, *Haptoglossales*, and *Haliphthorales*. Most of these orders have not been formally published and are mostly monogeneric, containing only a single genus (Beakes and Thines, 2017). Molecular phylogeny of these lineages is mostly based on sequences of the nuclear-encoded small ribosomal subunit (18S) (Lara and Belbahri, 2001; Sekimoto, Hatai and Honda, 2007; Hakariya, Hirose and Tokumasu, 2007) and the mitochondrial-encoded cytochrome c-oxidase subunit II (*cox*2) (Hudspeth, Nadler and Hudspeth, 2000; Hakariya, Hirose and Tokumasu, 2007; Sekimoto *et al*., 2008a; Choi *et al*., 2015), as well as the cytochrome c oxidase subunit I (*cox*1) (Gachon *et al*., 2017; Garvetto *et al*., 2018). However, the systematics of the early-diverging lineages is still in a state of flux (Beakes and Thines, 2017); this is because there are still many species and genera that are supposedly early-divergent, but for which no sequence data are available, and some species have not been isolated since their original descriptions. It is also becoming apparent that the classical systematic accounts by Karling (1942), Sparrow (1960) and Dick (2001) are, in many aspects, not supported by molecular phylogeny and require significant reappraisal. In this study, taxonomic placement of the type species of the genera *Ectrogella* (*E. bacillariacearum*), *Olpidiopsis* (*O. saprolegniae*) and *Pontisma* (*P. lagenidioides*) were resolved, including the introduction of two new early-diverging oomycetes genera, *Miracula* and *Diatomophthora* (Buaya *et al*., 2017; Buaya, Ploch and

Thines, 2019a; Buaya and Thines, 2019b; Buaya *et al*., 2019d; Buaya and Thines, 2020). The recent placement of these key species has resulted in multiple reappraisals and the concluding of a long-standing taxonomic confusion in the early-diverging oomycetes lineages. For instance, the decade-old phylogenetic confusion regarding the diatom-infecting parasitoids and marine red-algae *Olpidiopsis*, has been resolved (Buaya *et al*., 2019d; Buaya and Thines, 2020). Thus, the phylogeny of the early-diverging oomycetes has further expanded, resulting in the establishment of three new orders (*Miraculales*, *Diatomophthorales*, *Pontismatales*) and one order (*Anisolpidiales*) being reinstated (Buaya *et al*., 2017; Buaya, Ploch and Thines, 2019a; Buaya and Thines, 2019b; Buaya *et al*., 2019d; Buaya and Thines, 2020; Buaya and Thines, under review). Three novel diatom parasitoids (*Miracula helgolandica*, *Miracula moenusica*, *Diatomophthora drebesii*) and a single rediscovered species (*Diatomophthora gillii*) are also introduced herein (Buaya *et al*., 2017; Buaya, Ploch and Thines, 2019a; Buaya and Thines, 2019b; Buaya and Thines, 2020). The newly introduced *Miraculales* probably represents the earliest lineage whilst the order *Haliphthorales* is the upper-most lineage, branching just before the main *Saprolegniomycetes/Peronosporomycetes* split (Buaya *et al*., 2017; Sekimoto, Hatai and Honda, 2007).

*3.1.1.1 Parasites of Macro-Algae*

*Eurychasmatales: Eurychasmaceae (Eurychasma)*

The genus *Eurychasma* has often been placed in the *Saprolegniales*, but phylogenetic investigations have shown that it forms a very early-diverging lineage of the oomycetes (Sekimoto *et al*., 2008a; Beakes and Thines, 2017) and often groups with moderate support with the genus *Haptoglossa* (Sekimoto *et al*., 2008a; Beakes and Sekimoto, 2009;

Strittmatter *et al*., 2013; Buaya and Thines, 2020). The type species *Eurychasma dicksonii* is the only confirmed member of the genus and is a biotrophic parasite of various brown algae (Magnus, 1905; Sparrow, 1960, Küpper *et al*., 2006; Gachon *et al*., 2009). Only recently has this order been formally described (Buaya and Thines, under review). This parasite is known to be widely distributed in temperate regions and has been extensively studied with respect to its physiology and cellular ultrastructure (Müller, Küpper and Küpper, 1999; Gachon *et al*., 2009; Tsirigoti *et al*., 2013; Grenville-Briggs *et al*., 2011; Tsirigoti *et al*., 2015; Strittmatter *et al*., 2016). Aside from *E. dicksonii*, *E. succulus* has also been described which is parasitic to the *Rhodophyte* algae *Halosaccion ramentaceum* and *Rhodymenia palmata* (Petersen, 1905). However, the taxonomic status of this species is difficult to assess. Petersen, who described the species, did not later consider it as different from *E. dicksonii* (conversation cited in Sparrow, 1960), but as the host is unrelated to the host of *E. dicksonii* (red algae vs. brown algae), it would be expected that the species are distinct. However, sequence data for *E. succulus* is needed to clarify this situation. It is likely that all marine brown algae basal holocarpic parasites may belong to this genus.

*Anisolpidiales: Anisolpidiaceae (Anisolpidium)*

*Anisolpidiales* (Beakes and Thines, 2017) is a monogeneric order which frequently groups with *Diatomophthora* (Gachon *et al*., 2017; Buaya *et al*., 2019d). If the grouping reflects a host-jump of a common ancestor to members of the Straminipila or if is coincidental, because of the limited knowledge on holocarpic oomycetes, this needs to be clarified by future studies. So far, only two species of this family have been included in molecular phylogenetic investigations, *Anisolpidium ectocarpii* and *Anisolpidium rosenvingei*,

while the phylogenetic placement of the type species (*A. sphacellarum*) remains unknown (Gachon *et al*., 2017). The genus contains nine species (*Anisolpidium sphaecellarum*, *A. ectocarpii*, *A. rosenvingei*, *A. elongatum*, *A. saprobium*, *A. joklianum*, *A. minutum*, *A. olpidium*, and *A. stigeoclonii*), which are all parasites of filamentous marine algae (e.g. *Sphacelaria*, *Ectocarpus*, and *Pylaiella*) (Karling, 1943; Canter, 1950; Karling ,1968; Karling, 1977; Dick, 2001). However, with the recent discovery of a single diatom-infecting species (Buaya and Thines, unpublished), it is likely that there are more *Anisolpidiales* species awaiting discovery, especially in freshwater environments.

*Pontismatales: Pontismataceae (Pontisma)*

The monogeneric order *Pontismatales* (Buaya *et al*., 2019d) has only been recently introduced. Due to its host-range and "olpidiopsis-like" thallus, many parasites of red algae have been assigned to the *Olpidiopsidales*, together with the genera *Olpidiopsis*, *Pontisma*, *Sirolpidium*, and *Petersenia* (Petersen, 1905; Karling, 1942; Sparrow, 1960). However, a recent phylogenetic investigation of the type species of *Pontisma*, *P. lagenidioides*, show that this genus is unrelated to *Olpidiopsidales*; and are probably restricted to oomycete hosts and form a monophyletic grouping together with other parasites of marine red algae (*Pontisma heterosiphoniae*, *P. feldmanii*, *P. pyropiae*, *P. porphyrae*, *P. porphyrae* var*. koreanae*, *P. bostrychiae*, *P. muelleri*, and *P. palmariae*), previously assigned to the genus *Olpidiopsis* (Aleem, 1952; Sekimoto *et al*., 2008b; Sekimoto *et al*., 2009; Fletcher *et al*., 2015; Klochkova *et al*., 2016; Klochkova, Kwak and Kim, 2017; Badis *et al*., 2019; Buaya *et al*., 2019d). Thus, all holocarpic oomycete parasites of red algae were transferred to the genus *Pontisma* (Buaya *et al*., 2019d). Presently, only four *Pontisma* species remain unsequenced: *P. antithamnionis*, *P.*

*dangeardii*, *P. inhabile*, and *P. magnusii* (Petersen, 1905; Feldmann and Feldmann-Mazoyer, 1955; Feldmann and Feldmann-Mazoyer, 1967; Whittick and South, 1972). Interestingly, the type species of the *Sirolpidium* (*S. bryopsidis*) also shows a close phylogenetic relationship to this genus (Buaya and Thines, unpublished). To date, only one type species of marine algae remains unsequenced, *Petersenia lobata* (Sparrow, 1934). It is likely that all *Petersenia* species that are parasites of algae (*P. lobata*, *P. pollagaster*, and *P. palmariae*) may also be related to *Pontisma* and *Sirolpidium* because of their life-cycle and morphological similarities (Sparrow, 1934; Sparrow, 1960). It remains to be known if other *Petersenia* (*Petersenia irregularis*, *P. utriculoba*, *P. panicicola*, *P. catenophlyctidis*, and *P. andreei*), that are not parasites of marine algae, are related to this genus (Sparrow, 1936; Sparrow, 1943; Thirumalachar and Lacy, 1951; Miller, 1962; Sundaram, 1968).

*3.1.1.2 Diatom-Parasitoids and Lagenismatales*

*Miraculales: Miraculaceae (Miracula)*

*Miraculales* (Buaya *et al*., 2017) is a monogeneric order and seems to represent the earliest-diverging lineage of the oomycetes (Buaya *et al*., 2017). The two known members of *Miracula* are obligate biotrophic parasites of diatoms (Hanic, Sekimoto and Bates, 2009; Buaya *et al*., 2017; Buaya and Thines, 2019b) in freshwater (*M. moenusica*, Buaya and Thines, 2019b) and marine (*M. helgolandica*, Buaya *et al*., 2017) environments. The cellular ultrastructure of its type species, *M. helgolandica*, on its host diatom (*Pseudo-nitzschia pungens*), a known producer of the toxin domoic acid (Bates *et al*., 2018), has revealed structures typical for early-diverging lineages (Hanic, Sekimoto and Bates, 2009). The second species, *M. moenusica*, has been studied less well, but it is noteworthy

that its host diatom, *Pleurosira laevis*, is an invasive species in freshwater aquatic environments (Gherardi, 2007; Buaya and Thines, 2019b). It is not known if *M. moenusica* has been introduced together with its host or if the parasitoid has jumped hosts to the alien species, a pattern frequently found in obligate parasites (Thines, 2019); this needs to be determined by future studies. In addition, recent molecular studies have highlighted and revealed multiple environmental sequences of unknown *Miracula* parasitising on different *Pseudo-nitzschia* species (*P. australis*, *P. fraudulenta*, and *P. plurisecta*) and other marine diatoms (*Melosira nummuloides*, and *Thalassiosira pseudonana*) (Garvetto *et al*., 2018; Hassett *et al*., 2019; Buaya and Thines, unpublished). Certainly, there are still numerous *Miracula* species that are left to be discovered and it is likely that this genus could contain one of the largest assemblages of oomycetes diatom-parasitoids.

*Anisolpidiales: Diatomophthoraceae (Diatomophthora)*

The *Diatomophthoraceae* is a monogeneric family (Buaya and Thines, 2020). The genus *Diatomophthora* forms a monophyletic group with *Anisolpidium* and contains a variety of relatively little studied parasitoids of diatoms (Buaya and Thines, 2020). *Diatomophthora* has been previously assigned to *Olpdiopsis*, together with several species of marine red-algal parasites, an assemblage which was long thought to be polyphyletic or paraphyletic (Beakes and Thines, 2017; Buaya *et al*., 2019d; Buaya and Thines, 2020). However, recent phylogenetic placement of the type species of *Olpidiopsis* (*O. saprolegniae*) shows that the olpidiopsis-like diatom parasitoid is largely unrelated to *Olpidiopsis* (Buaya and Thines, 2020). It was speculated that the lineage would be related to *Ectrogella* due to the host-range similarities and thallus morphology (Garvetto *et al*., 2018; Garvetto *et al*., 2019). However, recent phylogenetic investigations of the type species of *Ectrogella*,

*Ectrogella bacillariacearum*, revealed that this genus actually belongs to the early-diverging *Saprolegniomycetes* and does not diverge earlier (Buaya and Thines, 2020). Thus, the genus *Diatomophthora* was introduced to accommodate the monophyletic clade containing olpidiopsis-like diatom parasites (Buaya and Thines, 2020). So far, only six species of this genus have been sequenced: the type species *D. drebesii*, *D. gillii*, *D. perforans*, *D. perforans* subsp. *oslofjordensis*, *D. perforans* subsp. *destruens*, and *D. perforans* subsp. *pleurosigmae* (Buaya *et al*., 2017; Buaya, Ploch and Thines, 2019a; Buaya *et al*., under review). There are still several diatom-infecting oomycetes parasitoids that have not yet been sequenced and, because of close life-cycle similarities, it is likely that some of these may be members of *Diatomophthora*. Examples of these include several *Ectrogella* species (*E. monostoma*, *E. gomphonematis*, *E. licmophorae*, and *E. eunotiae*). However, it is also highly probable that some of these parasitoids are unrelated to each other or any of the early-diverging oomycetes lineages; for instance, *Aphanomycopsis bacillariacearum* and *Lagenidium enecans*, which were formerly assumed to be primitive, turnout to be members of the *Peronosporales* (Buaya and Thines, unpublished). It remains to be seen if the remaining unsequenced *Lagenidium* diatom parasitoids (*L. brachystomum*, and *L. cyclotellae*) are related and if they form monophyletic grouping with the latter species (Zopf, 1884; Petersen, 1905; Scherffel, 1925; Friedmann, 1952).

*Lagenismatales: Ectrogellaceae (Ectrogella)*

*Ectrogellaceae* is a monogeneric family, initially speculated to be a member of the early-diverging oomycetes (Garvetto *et al*., 2018; Garvetto *et al*., 2019). However, recent phylogenetic investigations of the type species, *E. bacillariacearum*, show that this genus

belongs to the early-diverging lineages of the *Saprolegniomycetes* (Buaya and Thines, 2020), in line with earlier taxonomic accounts of Karling (1942), Sparrow (1960) and Dick (2001). It remains to be investigated if other diatom-infecting species (*E. monostoma*, *E. gomphonematis*, *E. eunotiae*, *E. brachystoma*, *E. cyclotellae*, *E. licmophorae*, and *E. eurychasmoides*) and algal parasites (*E. marina*, *E. lauderia*, *E. dicksonii*, and *E. besseyi*), that are traditionally associated with this group, are *bona fide* members of this genus since sequence data are not yet available (Petersen, 1905; Scherffel, 1925; Sparrow and Ellison, 1949; Friedmann, 1952; Feldmann and Feldmann, 1955; Sparrow, 1960; Dick, 2001).

*Lagenismatales: Lagenismataceae (Lagenisma)*

The family *Lagenismataceae* (Dick, 2001) is monotypic, containing a single genus and species which is an obligate parasite of the marine diatom *Coscinodiscus* (Drebes, 1966; Thines *et al*., 2015; Beakes and Thines, 2017). The parasite is known to inhabit temperate and tropical regions, especially during blooms of its diatom host *Coscinodiscus* (*C. granii*, *C. concinnus*, *C. wailesii*, and *C. radiatus*), and *Palmeria* (*P. hardmaniana*) (Parson, 1962; Johnson, 1966; Gotelli, 1971; Grahame, 1976; Wetsteyn and Peperzak, 1991; Thines *et al*., 2015; Buaya, Kraberg and Thines, 2019c). It was initially speculated to belong to the early-diverging oomycetes because it produces a holocarpic thallus and its sexual reproduction is by zoomeiospores. However, Thines *et al*., (2015) have shown that it is embedded within the early-diverging lineage of the *Saprolegniomycetes*, close to *Atkinsiella* (Thines *et al*., 2015). Later, Buaya and Thines (2020) have shown that *Ectrogella* belongs to the same group. This parasite produces diplanetic zoospores which are distinct characteristics for species belonging to the *Saprolegniomycetes* (Schnepf and

Drebes, 1977; Schnepf, Deichgräber and Drebes, 1978a; Schnepf, Deichgräber and Drebes, 1978b; Schnepf, Deichgräber and Drebes, 1978c; Schnepf and Heinzmann, 1980). A few other unsequenced holocarpic species (e.g. *Ectrogella licmophorae*, and *Pythiella vernalis*) are also known to produce diplanetic zoospores (Scherffel, 1925; Couch, 1935; Sparrow, 1960). It seems plausible that these species are also members of the *Saprolegniomycetes*, even though this needs to be ascertained in future studies.

*3.1.1.3 Parasites of Aquatic Oomycetes*

*Olpidiopsidales: Olpidiopsidaceae (Olpidiopsis)*

*Olpidiopsidales* (Dick, 2001; Buaya *et al*., 2019d) is also a monogeneric order and is one of the earliest-diverging lineages of the oomycetes. This order has been previously used as a catch-all for simple holocarpic oomycetes, but has been shown to be largely polyphyletic, thus leading to substantial revision (Dick, 2001; Beakes and Thines, 2017; Buaya *et al*., 2019d). The phylogenetic placement of the type species, *O. saprolegniae* from *Saprolegnia parasitica*, had revealed that *Olpidiopsidales* are largely unrelated to the holocarpic parasites of marine red algae and diatoms (Buaya *et al*., 2019d). This led to a resurrection of *Pontismatales* and a transfer of all holocarpic parasites of *Rhodophyte* algae to the genus *Pontisma* (Buaya *et al*., 2019d), as previously suggested by Dick (2001). Up to now, only four species of *Olpisiopsidales s.s.tr*. have been sequenced: the type species, one newly described species *O. parthenogenetica* and three other rediscovered species, *O. achlyae*, *O. vexans*, and *O. aphanomycis* (Buaya *et al*., 2019d; Buaya and Thines, unpublished). The type species and other *Olpidiopsis* parasites of aquatic oomycetes are known to widely occur in freshwater habitats and moist soil (Karling, 1942; Sparrow, 1960). The remaining unsequenced *Olpidiopsis* that are obligate

endobiotic parasites of aquatic oomycetes and fungi include *O. saprolegniae* var. *levis*, *O. fusiformis*, *O. braziliensis*, *O. index*, *O. varians*, *O. spinosa*, *O. incrassata*, *O. major*, *O. luxurians*, *O. gracile*, *O. pythii*, *O. curvispinosa*, *O. brevispinosa*, *O. verrucose*, and *O. karlingiae* (Cornu, 1872; Maurizio, 1895; Barrett, 1912; Coker, 1923; Tokunaga, 1933; Shanor, 1939; McLarty, 1941; Whiffen, 1942; Karling, 1942; Karling, 1949; Sparrow 1960). For some of these species, placements in other genera have been suggested, but without phylogenetic investigations that may help to pin-point synapomorphies, no decisions can be made on these. The taxonomic placement of several *Olpidiopsis* species parasitising marine and freshwater algae (*O. schenkiana*, *O. oedogoniarum*, *O. fibrillosa*, *O. appendiculata*, *O. zopfii*, *O. andreei*, *O. magnusii*, and *O. myzocytia*) remains uncertain as none were studied for their molecular phylogeny (Zopf, 1884; de Wildeman, 1896; Scherffel, 1925; Sparrow, 1936; Rieth, 1954; Feldmann and Feldmann, 1955; Johnson, 1955).

*3.1.1.4 Parasites of Invertebrates Animals*

*Haptoglossales: Haptoglossaceae (Haptoglossa)*

*Haptoglossales* (Dick, 2001; Beakes and Thines, 2017) is a monogeneric order, often grouping with *Eurychasmatales* (Hakariya, Hirose and Tokumasu, 2007; Sekimoto *et al*., 2008a; Buaya *et al*., 2019d). The genus *Haptoglossa* contains 12 species (*H. beakesii*, *H. dickii*, *H. elegans*, *H. erumpens*, *H. heteromorpha*, *H. heterospora*, *H. humicola*, *H. intermedia*, *H. mirabilis*, *H. northumbrica*, *H. polymorpha*, and *H. zoospora*); all are obligate endobiotic parasites of various rotifers and nematodes (Davidson and Barron, 1973; Barron, 1981; Barron, 1980; Barron, 1989; Barron, 1990; Glockling and Beakes, 2000b; Glockling and Beakes, 2001; Glockling and Serpell, 2010). All species, except *H.*

*heterospora* (Drechsler, 1940), are terrestrial (Beakes and Thines, 2017). As an apomorphy, *Haptoglossa* species produce “gun cells” which are specialised infection structures that enable the parasite to penetrate its host animal (Beakes, Glockling and Sekimoto, 2012). Several species of the genus *Haptoglossa* have been studied for electron microscopy, especially on the “gun cell” ultrastructure and mechanistic function (Barron, 1987; Lee, Vaughan and Durschner-Pelz, 1992; Glockling and Beakes, 2000b; Glockling and Beakes, 2002; Beakes, Glockling and Sekimoto, 2012). However, there are, as yet, no clear-cut synapomorphies that could be used to differentiate subgroups in the genus. In addition, the phylogeny of the genus still remains incomplete since there remain several unsequenced species. Furthermore, knowledge regarding its occurrence in the marine environments is still very scarce and, so far, only one species (*H. heterospora*) is known (Newell, Cefalu and Fell, 1977).

*Haliphthorales: Haliphthoraceae (Haliphthoros, Halocrusticida)*

The order *Haliphthorales* contains one to three genera with, in total, four species that are unanimously assigned to the type genus, *Haliphthoros* (*H. milfordensis*, *H. philippinensis*, *H. zoophthorum*, and *H. sabahensis*), all of which are parasites of crustaceans (Vishniac, 1958; Hatai *et al*., 1980; Dick, 2001; Beakes and Thines, 2017). This order is probably monophyletic and may represent the sister group of the crown oomycetes (Sekimoto, Hatai and Honda, 2007; Beakes and Sekimoto, 2009; Buaya and Thines, 2020). The genera *Halioticida* (*H. noduliformans*), *Halocrusticida* (*H. awabi*, *H. baliensis*, *H. entomophaga*, *H. hamanaensis*, *H. okinawaensis*, and *H. parasitica*), and *Halodaphnea* (*H. pinulirata*) are also members of this group (Kitancharoen and Hatai, 1995; Nakamura and Hatai, 1995; Hatai, Roza and Nakayama, 2000; Muroasa *et al*., 2009). However, as

there are little or no clear-cut morphological differences between the groups and all have a similar lifestyle, they are probably better considered to be synonymous with *Haliphthoros*. Several species in the genus are also likely to be synonymous (Beakes and Thines, 2017). All the species belonging to *Haliphthoros* s.l. forms segmented branched thalli and these are the only known members of the early-diverging oomycetes that can be cultured separate from their host (Vishniac 1958; Sekimoto, Hatai and Honda, 2007; Hatai, 2012).

## 3.2 Likely Phylogenetic Placement of Other Unsequenced Holocarpic Oomycetes

Aside from the previously discussed species, the taxonomic placement of several other holocarpic oomycetes remains largely unresolved (Beakes and Sekimoto, 2009; Beakes and Thines, 2017). These include several species in the genera *Pleotrachelus*, *Rozellopsis*, *Eurychasmidium*, *Pseudolpidium*, *Pseudosphaerita*, and *Blastulidiopsis* (Zopf, 1884; Pfitzer, 1872; Fischer, 1892; Dangeard, 1894; de Wildeman, 1896; Sigot, 1931; Sparrow, 1936; Karling, 1942). These genera were traditionally included in the early-diverging lineage of oomycetes because of their close morphological similarities (thallus, discharge tube, zoospores), stages in life-cycle development (infection, sporangium differentiation, biflagellate zoospores, zoospores release, resting spores) and host-range (Sparrow, 1960). However, most of the species under these genera were incompletely described and none were previously investigated for molecular phylogeny (Karling, 1942; Sparrow, 1960; Dick, 2001). For the past decades, several of these genera (e.g. *Pleotrachelus*, *Pseudolpidium*, and *Rozellopsis*) were subjected to multiple taxonomic revisions (Sparrow, 1960). This resulted in multiple reclassifications and re-

assignments of several species to the early-diverging lineage of oomycetes (e.g. *Olpidiopsis*, and *Eurychasma*) and basal fungal clades (e.g. *Olpidium*) (Sparrow, 1960). However, it is becoming apparent that the classical systematics implemented on these genera are doubtful and require reclassification, although this remains difficult without molecular data and reexamination of the type species. It is likely that some of these organisms will prove to be a member of the early-diverging lineage of oomycetes, once their sequence has become available.

*Pleotrachelus* Zopf

This genus contains 24 species: *Pleotrachelus andreei*, *P. askaulos*, *P. bornovanus*, *P. brassicae*, *P. ectocarpi*, *P. fulgens*, *P. inhabilis*, *P. itersoniliae*, *P. lobatus*, *P. minutus*, *P. olpidium*, *P. paradoxus*, *P. petersenii*, *P. pollagaster*, *P. radicis*, *P. rosenvingii*, *P. rotatoriorum*, *P. sphacelarum*, *P. tumefaciens*, *P. virulentus*, *P. vuilleminiae*, *P. vuilleminii*, *P. wildemanii*, and *P. zopfianus* (Zopf, 1884; de Wildeman, 1893; Lagerheim, 1899; Magnus, 1905; Petersen, 1905; Petersen, 1910; Morini, 1914; Jokl, 1916; Lund, 1930; Scherffel, 1931; Arnaud, 1952; Sahtiyanci, 1962; Bradley, 1967; Barr and Bandoni, 1980; Dick, 2001). *Pleotrachelus* is biotrophic and the majority of the species form olpidioid endobiotic thalli (Zopf, 1884; Karling, 1942; Sparrow, 1960). However, most species in this genus have a similar life-cycle and host-range to most of the basal holocarpic oomycetes. Due to these close similarities (e.g. thallus, biflagellate zoospores, and pattern of zoospores discharge), several of the species formerly assigned to *Pleotrachelus* were reclassified or synonymised into multiple species (e.g. *Anisolpidium*, *Olpidiopsis*, *Pontisma*, *Petersenia*, and *Sirolpidium*) of the early-diverging lineage of the oomycetes (Karling, 1942; Sparrow, 1960; Dick, 2001). These include *Pleotrachelus*

*andreei* (*Petersenia andreei*, *Olpidiopsis andreei*, *Sirolpidium andreei*), *P. ectocarpi* (*Anisolpidium joklianum*), *P. inhabilis* (*Pontisma inhabile*), *P. lobatus* (*Petersenia lobata*), *P. minutus* (*Anisolpidium minutum*), *P. olpidium* (*Anisolpidium olpidium*), *P. paradoxus* (*Olpidiopsis paradoxa*, *Sirolpidium paradoxum*), *P. pollagaster* (*Petersenia pollagaster), P. rosenvingii* (*Anisolpidium rosenvingii*), *P. sphacelarum* (*Anisolpidium sphacelarum*, *Olpidiopsis sphacellarum*), *P. tumefaciens* (*Olpidiopsis tumefaciens*, *Eurychasmidium tumefaciens*), *P. vuilleminiae* (*Olpidiopsis vuilleminiae*) and *P. vuilleminii* (*Olpidiopsis vuilleminiae*) (Zopf, 1884; Lagerheim, 1899; Magnus, 1905; Petersen, 1905; Jokl, 1916; Arnaud, 1952; Dick, 2001). Others were reclassified to the basal clade of the *Mycota* because of the production of posteriorly uniflagellated zoospores (Sparrow, 1960), which include *Pleotrachelus brassicae* (*Chytridium brassicae*, *Olpidium brassicae*), *P. fulgens* (*Olpidium fulgens*), *P. sphacelarum* (*Chytridium sphacelarum*, *Olpidium sphacelarum*), *P. tumefaciens* (*Chytridium tumefaciens*, *Olpidium tumefaciens*), *P. virulentus* (*Olpidium virulentus*, *Chytridium brassicae*, *Asterocystis radicis*), and *P. wildemanii* (*Olpidium wildemanii*) (Zopf, 1884; Magnus, 1905; Petersen, 1905; Petersen, 1910; Sahtiyanci, 1962; Dick, 2001). In the taxonomic account of Sparrow (1960), only four species were retained under *Pleotrachelus*: *Pleotrachelus fulgens*, *P. zopfianus*, *P. wildemanii*, and *P. petersenii*. It is probable that these remaining members of *Pleotrachelus* may also be reassigned to other clades, once their sequence data becomes available, resulting in the dissolution of this genus. Up to the present day, only a few species (e.g. *Anisolpidium rosenvingii*, and *A. ectocarpii*) formerly placed in this genus, have been assigned to their correct taxonomic placement (Gachon *et al*., 2017). It is most probable that the large bulk

of *Pleotrachelus* will fall into the early-diverging lineage of oomycetes while others will be assigned to the basal zoosporic fungi.

*Rozellopsis* Karling

*Rozellopsis* contains four species that are biotrophic parasites of the aquatic phycomycetes *Saprolegniaceae* (*R. septigena*, *R. simulans*), and *Pythiaceae* (*R. inflata*, *R. waterhouseii*) (Fischer, 1882; Butler, 1907; Karling, 1942). Organisms under this genus form intramatrical holocarpic thalli maturing into basipetal succession and produce biflagellate zoospores that are variable in terms of shape and size (Karling, 1942; Sparrow, 1960). Species in this genus are generally segregated as either with sporangia that are septigenate (*R. septigena*, *R. simulans*) or as monosporangiate (*R. inflata*, *R. waterhouseii*) (Fischer, 1882; Butler, 1907; Karling, 1942). So far, only the septigenous species are known to produce resting spores; resting spores are unknown for the monosporangiate species (Karling, 1942; Sparrow, 1960). This genus was introduced by Karling (1942), originally intended for *Rozzella*-like fungi with biflagellate rather than uniflagellate zoospores. Presently, *Rozellopsis* is tentatively assigned to the early-diverging lineage of oomycetes because of its close life-cycle characteristics, however, this is with weak circumscription (Sparrow, 1960). For the past decades, this genus has been subjected to multiple taxonomic revisions and was even reclassified to *Woroninaceae* because of its plasmodial thallus and unequally biflagellate zoopores (Fischer, 1882; Butler, 1907; Karling, 1942; Sparrow, 1960). In Sparrow's (1960) taxonomic account, *Rozellopsis* was tentatively grouped with the *Olpidiopsidaceae*, however, without molecular sequence, taxonomic placement of this genus remains difficult to assess. It is highly probable that *Rozellopsis* is related to the early-diverging

lineage of oomycetes and likely that septigenate and monosporangiate species would form a distinct genus once their sequence becomes known and their life-cycle completely documented.

*Eurychasmidium* Sparrow

This genus is monotypic and the type species (*Eurychasmidium tumefaciens*) has been initially described by Magnus (1879) as *Olpidium tumefaciens*; this was later reclassified by Petersen (1905) as *Pleotrachelus tumefaciens* (Sparrow, 1960). Sparrow (1936) re-established this genus and tentatively assigned it to the early-diverging *Saprolegniales*. However, the exact taxonomic placement of *Eurychasmidium* still remains uncertain. Due to its similar host-range and life-cycle traits to the marine algae oomycetes, it is likely that this *E. tumefaciens* would be closely related to the genus *Petersenia* or, to a lesser extent, to *Eurychasma* once its molecular data becomes available (Sparrow, 1960). *Eurychasmidium tumefaciens* has similar life-cycle traits to *Eurychasma dicksonii*, except that it produces multiple endobiotic discharge tubes and its zoospores encyst outside the sporangium (Magnus, 1875; Wright, 1879; Petersen, 1905; Sparrow, 1960). There are still several significant details of its life-cycle that remain unknown, such as the zoospore encystment, zoospore release and resting spore (Sparrow, 1960). This organism is a weakly hemibiotrophic parasite of various marine *Rhodophyceae*, especially *Ceramium* (*C. flabelligerum*, *C. acanthonotum*, *C. spiniferum*, *C. rubrum*, *C. diaphanum*) and commonly occurs in temperate oceans (Magnus, 1875; Wright, 1879; de Wildeman, 1900; Petersen, 1905; Sparrow, 1936).

*Pseudolpidium* Fischer

*Pseudolpidium* is a monotypic genus and has been established by Fischer (1882), originally containing large assemblages of aquatic phycomycetes and filamentous green algae parasitoids that were later reclassified, mostly to the genus *Olpidiopsis* (Cornu, 1872; Karling, 1942; Sparrow, 1960; Dick, 2001; Buaya *et al*., 2019d). Presently, this genus is tentatively maintained, comprising three incompletely known parasites, *Pseudolpidium deformans*, *P. glenodinianum*, and *P. sphaeritae* (Dangeard, 1888; Dangeard, 1889; Fischer, 1892; Serbinow, 1907; Sparrow, 1960). *Pseudolpidium* species have similar morphological characters (e.g. thallus, and biflagellate zoospores) to *Olpidiopsis*, except that their life-cycles are not completely known (Karling, 1942; Sparrow, 1960). It is highly probable that *Pseudolpidium deformans*, *P. glenodinianum*, and *P. sphaeritae* will be reclassified into different taxons once their molecular data becomes available, resulting in the dissolution of this genus. Karling (1942) suggested earlier that *P. deformans* would likely be related to *Rozellopsis* or *Woronina* because of its similar life-cycle and host-range, although McLarty (1941) and Shanor (1939) believed that *P. glenodinianum*, and *P. sphaeritae* would eventually be reassigned to the genus *Olpidiopsis*. However, *P. glenodinianum* and *P. sphaeritae* have different host-ranges and it is probable that these two parasitoids would fall into separate genera (Karling, 1942; Sparrow, 1960), although without molecular sequence, the taxonomic placements of these three parasitoids are still difficult to assess.

*Pseudosphaerita* Dangeard

This genus was founded by Dangeard in 1933 for his isolates of *Sphaerita*-like forms with biflagellate zoospores, infecting *Euglena* and *Cryptomonas*. This genus originally

contained two species, *Pseudosphaerita euglenae* and *P.* radiata, with five more species being subsequently added to this genera, *Pseudosphaerita dinobryi*, *P. dinobryonis*, *P. dryli*, *P. drylii*, and *P. phaci* (Dangeard, 1895; Dangeard, 1933; Karling, 1942; Sparrow, 1960; Canter, 1968; Reyes *et al*., 1985; Dick, 2001). All *Pseudosphaerita* species have a high degree of morphological similarities to *Olpidiopsis*, except for thallus differentiation, zoospore formation, discharge tube, zoospores release and resting spore (Dangeard, 1895; Karling, 1942; Sparrow, 1960). *Pseudosphaerita* have an unusual thallus division and zoospores formation; organisms within this genus are generally diagnosed in terms of these two characteristics of either not being radiately arranged (e.g. *P. euglenae*) or the zoospores globules being radiately arranged in the sporangium (e.g. *P. radiata*) (Karling, 1942; Sparrow, 1960). However, the exact taxonomic placement of this taxon still remains unknown and its assignment to the early-diverging lineage of oomycetes remains provisional since its type species has not yet been sequenced. It is probable that several species of this genus are unrelated to the *Oomycota* because of their unusual thallus development and they are likely to be related to other clades within Straminipila or Alveolata (e.g. *Developayella*, and *Pirsonia*).

*Blastulidiopsis* Sigot

*Blastulidiopsis*is a monotypic genus containing only a single species (*Blastulidiopsis chattoni*) that is parasitic on the eggs of *Cyclops* crustaceans (Sigot, 1931; Karling, 1942; Sparrow, 1960). Its morphology and life-cycle is closely similar to *Olpidiopsis*, while its lobed thallus resembles that of *Petersenia* and, to a lesser extent, *Pontisma* (Karling, 1942). Details on the life-cycle and development of *Blastulidiopsis* are still obscure and this parasitoid has only been isolated once. So far, it is only known to infect the eggs of

*Cyclops* crustaceans from which it was originally isolated (Sigot, 1931). Its placement in the early-diverging lineage of oomycetes, as suggested by Karling (1942) and Sparrow (1960), is still provisional because its molecular sequence is, as yet, unavailable. However, based on its morphology and life-cycle traits, it is likely that this parasitoid is an oomycete and could either be related to early-diverging *Saprolegniales* or closer to other invertebrate pathogens.

## 3.3 Host-range and Ecology of the Early-Diverging Oomycetes

### *3.3.1 Host-range and Occurence*

The early-diverging oomycetes are ubiquitous and widely distributed, especially in the aquatic environment (Karling, 1942; Sparrow, 1960; Dick, 2001; Beakes and Sekimoto, 2009; Strittmatter, Gachon and Küpper, 2009; Beakes and Thines, 2017). In marine environments, they are known to infect diatoms (Petersen, 1905; Johnson, 1966; Johnson, 1967; Hanic, Sekimoto and Bates, 2009; Garvetto *et al*., 2018; Garvetto *et al*., 2019), algae (Magnus, 1905; Petersen, 1905; Sparrow, 1934; Sparrow, 1936; Karling, 1943; Aleem, 1952; Feldmann and Feldmann, 1955; Kupper *et al*., 2006; Sekimoto *et al*., 2008a; Sekimoto *et al*., 2008b; Sekimoto *et al*., 2009; Gachon *et al*., 2017; Grenville-Briggs *et al*., 2011; Tsirigoti *et al*., 2013; Klochkova *et al*., 2016; Klochkova, Kwak and Kim, 2017; Badis *et al*., 2019) and invertebrate animals (Drechsler, 1940; Vishniac, 1958; Davidson and Barron, 1973; Fisher, Nilson and Shleser, 1975; Tharp and Bland, 1977; Hatai *et al*., 1980; Nakamura and Hatai, 1995; Hatai, Roza and Nakayama, 2000; Dick, 2001; Leaño, 2002; Sekimoto, Hatai and Honda, 2007). Likewise, these parasites are also widely occurring in freshwater, parasitizing diatoms (Zopf, 1884; Gill, 1893; Scherffel, 1925; Friedmann, 1952), filamentous algae (de Wildeman, 1895; de Wildeman, 1896;

Canter, 1949) and invertebrate animals (Barron, 1981; Barron, 1989; Barron, 1990; Glockling and Beakes, 2000a; Glockling and Beakes, 2000b; Glockling and Beakes, 2001; Glockling and Serpell, 2010). However, despite their widespread occurrence, little is known regarding the ecology of these organisms especially on how they interact with other organisms, occurrence and roles in nature (Skovgaard, 2014; Scholz *et al*., 2015). To date, almost all of the known early-diverging oomycetes were recorded from temperate regions, and knowledge about the existence of this group in the tropics is scarce (Grahame, 1976; Hatai *et al*., 1980; Raghu Kumar, 1987; Leaño, 2002; Chukanhom *et al*., 2003; Strittmatter, Gachon and Küpper, 2009; Raghu Kumar, 2009). In terms of host-range, little is also known, and for the past decades there were only few studies have been conducted on this aspect (Drebes, 1966; Müller, Küpper and Küpper, 1999; Strittmatter, Gachon and Küpper, 2009; Gachon *et al*., 2009). Perhaps the last extensive host-range study was conducted almost eighty years ago on the aquatic, oomycete-parasitic genus *Olpidiopsis* (*O. varians*, *O. fusiformis*, *O. saprolegniae*, *O. luxurians*, *O. incrassata*, *O. aphanomycsis*) (Shanor, 1940). At present, there were only few host-range studies were conducted on two pathogens of marine algae, *Eurychasma dicksonii*, and diatoms, *Lagenisma coscinodisci* (Drebes, 1966; Müller, Küpper and Küpper, 1999; Gachon *et al*., 2009; Strittmatter *et al*., 2009). However, it is becoming clearer that a remarkable diversity in terms of host ranges exists. While *Eurychasma dicksonii* seems to have a rather broad host range, at least under laboratory conditions, species of *Olpidopsis* seem to be more specialised, often even below the genus level (Shanor, 1940). Application of molecular techniques in ecological studies of oomycetes have recently expanded the present understanding on the diversity and distribution of these

seemingly intractable organisms (Beakes and Sekimoto, 2009). In the marine environment, a few molecular studies have been conducted and revealed environmental sequences that are thought to correspond to many unknown species and higher-level clades of the early diverging lineages, suggesting that these organisms are indeed widespread, with many species still awaiting discovery (Moon-van der Staay *et al*., 2001; Massana *et al*., 2004; Massana *et al*., 2006; Garvetto *et al*., 2018; Hassett *et al*., 2019).

*3.3.1.1 Parasites of Macro-algae*

Several early-diverging oomycetes species are known to be obligate biotrophic parasites of marine and freshwater algae (Karling, 1942; Sparrow, 1960; Dick, 2001). In marine environments, the majority of these are infecting red-algae (*Pontisma*, *Petersenia*) and a smaller number is parasitic in brown (*Anisolpdium*, *Eurychasma*) or green algae (*Sirolpdium*) (Petersen, 1905; Magnus, 1905; Karling, 1942; Karling, 1943). In freshwater environments, early-diverging oomycetes were only reported from a small number of species of filamentous green algae, usually as *Olpidiopsis* species (Zopf, 1884; de Wildeman, 1896; Scherffel, 1925). The host-range of most algae-infecting parasites are still not well established, except for *E. dicksonii* (Müller, Küpper and Küpper, 1999; Gachon *et al*., 2009). However, based on morphological identifications, several algal parasites have a broad host-range (e.g. *E. dicksonii*, and *P. lagenidioides*), while some are assumed to have rather narrow host-ranges (e.g. *P. bostrychiae*, and *P. porphyrae*) (Sekimoto *et al*., 2008b; Strittmatter, Gachon and Küpper, 2009; Sekimoto *et al*., 2009). The genus *Pontisma* (*P. bostrychiae*, *P. heterosiphoniae*, *P. muelleri*, *P. palmariae*. *P. porphyrae*, *P. pyropiae*) is widespread, which is also reflected by the fact that it has the highest number of species recorded (Petersen, 1905; Sekimoto *et al*., 2008b; Sekimoto

*et al*., 2009; Klochkova *et al*., 2016; Klochkova, Kwak and Kim, 2017; Badis *et al*., 2019). It is noteworthy that the type species, *P. lagenidioides* infects often only old and moribund tissues, which suggests that it has little effect on its host populations. Also, the other species have rarely been reported to cause massive losses in natural ecosystems (Petersen, 1905; Magnus, 1905; Sparrow, 1936; Gachon *et al*., 2009; Tsirigoti *et al*., 2013; Gachon *et al*., 2017; Badis *et al*., 2019), indicating a well-balanced host pathogen relationship. If *Petersenia* (*P. lobata*, *P. palmariae*, *P. pollagaster*) is distinct from *Pontisma* remains to be demonstrated. It is conceivable that its lobed thallus represents a beginning compartmentalisation, which is absent in most species of *Pontisma*, but pronounced in *P. lagenidioides*, to which, in term of infection strategy*, Petersenia* is closely connected. Also, *Sirolpdium* (*S. bryopsidis*) seems to favour old thallus parts and has probably only a limited detrimental effect on its host populations. *Anisolpidium* (*A. sphacellarum*, *A. ectocarpii*, *A. rosenvingii*), and *Eurychasma* (*E. dicksonii*) only contains a few records (Magnus, 1905; Sparrow, 1934; Karling, 1943; Karling, 1942; Sparrow, 1943; Van der Meer and Pueschel, 1985; Dick, 2001). The scarcity of the records does not necessarily mean that the species are indeed rare, it could also be that they are rather attenuated pathogens, as, e.g. it is possible to co-culture *Eurychasma* and some of its host under optimal growth conditions for several months (Ploch an Thines, unpublished experiments).

#### *3.3.1.2 Parasites of Diatoms*

Diatom-infecting basal oomycetes are widely occurring, containing several species from different genera (Karling, 1942; Sparrow, 1960; Dick, 2001). Most of these parasites were recorded from marine environments and almost all were isolated from temperate regions

(Sparrow, 1960). The ecological role and occurrence of these parasitoids remains largely speculative (Scholz *et al*., 2015). In both freshwater and marine habitats, the occurrence of these parasites often seems to coincide with the bloom of their respective host with most species additionally seem to favor cooler temperatures, occurring in high abundance during spring or autumn (Sparrow, 1936; Sparrow, 1960; Hanic, Sekimoto and Bates, 2009; Beakes and Thines, 2017). However, this remains to be proven systematically since reports of diatom parasitoids are rather rare. The bulk of the known species has been assigned to the genus *Ectrogella* (Zopf, 1884). However, the phylogenetic affiliations of most of these remains unresolved since most have not yet been investigated for their molecular phylogeny. The host-range of these parasitoids remains also largely unknown, even though it has been speculated that there is some degree of host specificity (Sparrow, 1936; Drebes, 1966; Gotelli, 1971; Wetsteyn and Peperzak, 1991; Garvetto *et al*., 2018), and to date, only one species has been successfully cultivated together with its host diatom (Schnepf and Drebes, 1977; Buaya *et al*., 2019d).

*3.3.1.3 Parasites of Aquatic Oomycetes*

Early-diverging oomycetes that are obligate parasites of aquatic crown oomycetes are widely distributed in various freshwater environments (Karling, 1942; Sparrow, 1960; Dick, 2001). The majority of these parasitoids are members of the genus *Olpidiopsis* (*O. saprolegniae*, *O. saprolegniae* var. *levis*, *O. braziliensis*, *O. fusiformis*, *O. achlyae*, *O. varians*, *O. index*, *O. incrassata*, *O. major*, *O. vexans*, *O. spinosa*, *O. luxurians*, *O. aphanomycis*, *O. gracile*, *O. pythii*, *O. curvispinosa*, *O. brevispinosa*), but also *Lagenidium destruens*, *Petersenia irregulare*, *Pythiella besseyi*, *Pythiella vernalis*, and *Pythium utriculoba* have been reported as parasitoids of aquatic oomycetes (Cornu, 1872;

Maurizio, 1895; Barrett, 1912; Coker, 1923; Tokunaga, 1933; Shanor, 1939; McLarty, 1941; Karling, 1942; Whiffen, 1942; Sparrow, 1960; Miller, 1962). The ecological role of these organisms with respect to regulating the pathogen pressure on e.g. invertebrates remains largely unknown. In a cross-infection study conducted with five parasite species (*O. saprolegniae*, *O. varians*, *O. fusiformis*, *O. incrassata*, *O. luxurians*, *O. aphanomycis*), results have shown that a few are able to affect a broader host range, while others apparently infect only single host (Shanor, 1940).

*3.3.1.4 Parasites of Invertebrate Animals*

Early-diverging oomycetes that parasitize invertebrate animals are also ubiquitous and widely occurring in aquatic and terrestrial environments (Sparrow, 1960; Beakes and Sekimoto, 2009; Beakes and Thines, 2017). Among the hosts of marine species of the genus *Haliphthoros* s.l. are several crustaceans (e.g. *Homarus americanus*, *Penaeus monodon*, and *Haliotis sieboldii*) cultivated in aquaculture or with economic importance (Fisher, Nilson and Shleser, 1975; Kitancharoen and Hatai, 1995; Chukanhom *et al*., 2003). The genus *Haptoglossa* is a widespread, mostly terrestrial obligate endobiotic parasite of invertebrate animals (Beakes, Glockling and Sekimoto, 2012). Most of the species of *Haptoglossa* parasitize nematodes (e.g. *H. beakesii*, and *H. erumpens*), and few others on rotifers (*H. mirabilis*, *H. elegans*) (Beakes and Sekimoto, 2009). Their regulating effects on their often abundant host populations is poorly understood, but the widespread nature and species-richness of the genus suggests that there is a longstanding evolutionary equilibrium between the parasitoids and their invertebrate hosts.

*3.3.2 Methods of Study and Cultivation*

Most of the early-divergent oomycetes are widely distributed and can be readily isolated from their natural environments after a thorough screening process. Methods for the collection, isolation and culturing these parasites can be found in Karling (1942), Sparrow (1960) and Dick (2001). Fresh aquatic samples (e.g. water, mud, filamentous algae, floating organic debris and insect carcasses) can be directly collected from the field. Field collection of basal oomycetes infecting phytoplankton are usually done using a plankton net, preferably with mesh size of 20 *µm* (Hanic, Sekimoto and Bates, 2009; Thines *et al*., 2015). Screening for parasites and isolation is probably most efficient using an inverted light microscope. Early-diverging oomycetes that are parasites of diatoms (e.g. *Diatomophthora gillii*, and *Ectogella bacillariacerum*) and algae (e.g. *Pontisma lagenidioides*, and *Eurychasma dicksonii*) can be directly isolated using pipettes and scalpels, respectively (Müller, Gachon and Küpper, 2008). Others require some additional techniques for isolation such as baiting (Karling, 1942; Sparrow, 1960; Karling, 1981). Baiting is especially useful for isolating *Olpidiopsis* (e.g. *O. saprolegniae*, and *O. achlyae*) species (Barrett, 1912; Coker, 1923; Shanor, 1939; McLarty, 1941). Samples (e.g. water, mud, soil sediments and organic substance) are baited with various seeds (e.g. sesame, and hemp) diluted with autoclaved pond water, and incubated for several days (Sparrow, 1960; Karling, 1981; Beakes and Thines, 2017). Baiting is also useful for the isolation of invertebrate animal parasites such as *Haptoglossa* (Beakes and Thines, 2017). Removal of the bacterial or fungal contaminants can be achieved by the addition of antibiotics to the medium or through stepwise rinsing using sterile water (Sparrow, 1960). After isolation, specimens can be processed for morphological characterization and also for

molecular investigations. So far, only a handful of holocarpic oomycetes can be cultivated in agar medium. These includes few species that are marine parasites of various crustaceans from the genus *Haliphthoros* (*H. milfordensis*, *H. philippinensis*), which can be cultivated using PYG medium (peptone, yeast extract, and glucose agar) or synthetic sea-water medium containing glucose and sodium aspartate dissolved in sterile sea water (Vishniac, 1958; Hatai *et al*., 1980; Chukanhom *et al*., 2003). A few early-diverging oomycetes species were also cultivated together with their host, such as some species of aquatic oomycetes parasites from *Olpidiopsis* genus (*S. saprolegniae*, *O. incrassata*, *O. varians*), the marine algae parasite *Eurychasma dicksonii* and the diatom-infecting oomycetes *Lagenisma coscinodisci* (Shanor, 1940; Müller, Gachon and Küpper, 2008; Buaya, Kraberg and Thines, 2019c). If (temporal) cultures are aimed for, it is important to first isolate some healthy hosts and to establish their culture, before dividing it in half and inoculating half of the culture, for each asexual cycle of the parasites.

### *3.3.3 Challenges in Studying Early-Diverging Oomycetes*

Understanding of the biology and ecology of the early-diverging oomycetes has gradually increased in the past few decades (Beakes and Sekimoto, 2009; Beakes and Thines, 2017). However, despite these developments, early-diverging oomycetes remain challenging to study as compared to other taxa of *Oomycota* (Beakes and Thines, 2017). Information on the occurrence of most basal parasitoids in their natural environments is not, as yet, well established and baseline knowledge is scarce (Beakes and Thines, 2017). Some basal holocarps occur seasonally in the year, while others do not follow the same pattern of seasonal recurrence (Karling, 1942; Sparrow, 1960; Strittmatter, Gachon and Küpper, 2009; Scholz *et al*., 2016; Hassett *et al*., 2019). Screening for early-diverging

oomycetes directly from their natural environments is often challenging and requires much patience in performing microscopy. Isolation of these parasitoids is usually carried out by direct screening or by a baiting technique, as previously mentioned; these methods are often time consuming and require thorough microscopic work (Karling, 1942; Sparrow, 1960). In most cases, it is usually not so easy to detect the presence of these parasitoids from field or baited samples since most parasitoids produce unpronounced inconspicuous symptoms (e.g. *Diatomophthora gillii*, and *Ectrogella bacillariacearum*), especially at the early and mid-stage of infection (Zopf, 1884; Friedmann, 1952). In addition, isolation of biotrophic holocarps is generally carried out using either a micropipette (usually 10 *µl*) or heated glass needles, as previously mentioned. However, this method is sometimes difficult and requires a steady-hand while pipetting for cell picking as most of the parasitoid thalli are very small and stick to most plastic pipette tips, thus making it difficult to separate them from unwanted contaminants, as well as for subsequent serial transfer. Furthermore, some parasitoid's thalli are very fragile and easily plasmolyse during subsequent transfers. Due to their biotrophic nature, most basal parasitoids do not grow on common mycological medium and no extant cultures for most species can be found in public culture collections or herbariums dedicated to these organisms (Beakes and Thines, 2017). In fact, only a dozen of these early-diverging oomycetes can be cultured on agarised medium (Strittmatter, Gachon and Küpper, 2009; Beakes and Thines, 2017). Most of these early-diverging species are members of *Haliphthorales* which are mostly facultative parasites of different marine invertebrates (Vishniac, 1958; Hatai *et al*., 1980; Kitancharoen and Hatai, 1995; Nakamura and Hatai, 1995; Hatai, Roza and Nakayama, 2000; Muroasa *et al*., 2009), while other parasitoids

can be cultivated in dual-culture, containing the host and parasite (e.g. *Lagnisma coscinodisci*). Only recently, have a few of these basal parasitoids been successfully established on stable host-parasite culture by using enrichment medium (e.g. f/2 medium, and Provasoli´s enriched Seawater Medium) (Guillard and Ryther, 1962; Provasoli, 1968; Guillard, 1975; Müller, Gachon and Küpper, 2008; Buaya, Kraberg and Thines, 2019c). Although there have been successful culture establishment attempts in the past for a few basal species (e.g. *Olpidiopsis saprolegniae*, *O. varians*, *O. fusiformis*, *O. incrassata*, *O. luxurians*, *Haliphthoros milfordensis*, *Ectrogella perforans*, and *Lagenisma coscinodisci*), these cultures were discontinued and lost over time (Barrett, 1912; Shanor, 1939; Shanor, 1940; Vishniac, 1958; Schnepf and Drebes, 1977; Raghu Kumar, 1978; Bortnick, Powell and Bangert, 1985). The dual-culture method has become very useful for obtaining materials that can be utilised for further investigations, especially morphological, life-cycle and phylogenetic studies (Strittmatter *et al*., 2013; Buaya, Kraberg and Thines, 2019c). Presently, only a few early-diverging oomycetes host-parasite cultures exist that can be accessed by the public (on request) and, thus, can be subjected for further studies (Karling, 1942; Sparrow, 1960; Beakes and Thines, 2017; Gachon *et al*., 2017; Buaya, Kraberg and Thines, 2019c). These include three marine parasitoids of diatoms *Lagenisma coscinodisci*, *Diatomophthora perforans* subs. *destruens*, and *D. perforans* subs. *pleurosigmae* (Buaya, Kraberg and Thines, 2019c; Buaya *et al*., under review), the brown algae parasitoid *Eurychasma dicksonii* and two species of *Olpidiopsis*, *Olpidiopsis aclyae* and *O. vexans* (Müller, Küpper and Küpper, 1999; Müller, Gachon and Küpper, 2008; Buaya and Thines, unpublished). However, the establishment and maintenance of host-parasite dual culture is often laborious and requires uninterrupted periodic

subculturing by continuously replenishing the parasite with new, susceptible host strains. Aside from the challenging nature of isolation and cultivation of the basal oomycetes, molecular phylogeny of the group also remains incomplete and largely unresolved (Beakes and Thines, 2017; Beakes and Sekimoto, 2009). Presently, most of the sequenced basal species have only been based on a few genetic markers, in particular the nuclear-encoded small ribosomal subunit (18S) and on the mitochondrial-encoded cytochrome c-oxidase subunit II (*cox*2), mitochondrial-encoded cytochrome c-oxidase subunit I (*cox*1) or the large ribosomal subunit (LSU) (Beakes and Thines, 2017). Because of suboptimal primer fitting due to the high degree of genetic divergence in the basal taxa, multigene phylogenies reconstructions in the early-diverging lineage of the *Oomycota* still remain largely unresolved. Presently, several 18S primers are available for the investigation of early-diverging oomycetes, including a few *cox*2, *cox*1 and LSU primers (Sekimoto, Hatai and Honda, 2007; Strittmatter *et al*., 2013; Beakes, Glockling and James, 2014; Fletcher *et al*., 2015; Gachon *et al*., 2017; Beakes and Thines, 2017; Garvetto *et al*., 2018; Garvetto *et al*., 2019). However, most of these are unspecific and, because all of these parasitoids are endobiotic, often the DNA of the host organism or the DNA of other contaminants are amplified, rather than the target organism's DNA. Although most of the key species (Sekimoto, Hatai and Honda, 2007; Hakariya, Hirose and Tokumasu, 2007; Sekimoto *et al*., 2008a; Gachon *et al*., 2017) have been sequenced, the phylogeny and systematics of the early-diverging oomycetes clade is far from complete; and development of molecular markers are needed for enhancing molecular identification and multigene phylogenies to improve the resolution of the early-diverging oomycetes lineages.

## 3.4 Practical and Economic Importance

Only a few basal oomycetes are known to infect various, economically important, marine algae and crustaceans (Strittmatter, Gachon and Küpper, 2009; Beakes and Thines, 2017). In marine algae, the widely cultivated red-algae, *Porphyra spp.*, is periodically parasitised by two *Pontisma* species (*P. porphyrae*, and *P. bostrychiae*) causing a disease known as “chytrid blight” (Sekimoto *et al*., 2008b; Sekimoto *et al*., 2009; Li *et al*., 2010; Klochkova *et al*., 2012). *Porphyra* spp. are widely cultivated in East Asia (Japan, Korea, China), where they are used for nori production (Pereira and Yarish, 2008; Baweja *et al*., 2016). In aquaculture, *Pontisma* spp. sporadically infect the blades of algae including conchocelis, causing spots, holes and discolorations affecting the quality and yield of the diseased crop (Ding and Ma, 2005; Sekimoto *et al*., 2008b; Sekimoto *et al*., 2009; Strittmatter, Gachon and Küpper, 2009). The eucarpic oomycete, *Pythium porphyrae*, is also known to infect *Porphyra*, occurring simultaneously with *Ponstima porphyrae* and *P. bostrychiae*, causing a “red-rot” disease (Kawamura *et al*., 2005; Park, Kakinuma and Amano, 2006; Park and Hwang, 2015). Aside from marine algae, as mentioned before, a few species of the genus *Haliphthoros* s.l. (*H. milfordensis*, *H. philippinensis*, *H. sabahensis*, and *H. okinawaensis*) are also known to have an economic impact on important marine crustaceans (shrimp, lobster and mud crab) (Vishniac, 1958; Hatai *et al*., 1980; Nakamura and Hatai, 1995; Strittmatter, Gachon and Küpper, 2009; Lee, Hatai and Kurata, 2017). These parasites usually attack the larvae of crustaceans, causing mycotic infections and, subsequently, killing the larvae (Fisher, Nilson and Shleser, 1975; Tharp and Bland, 1977; Leaño, 2002). Apart from these negative impacts, it can be assumed that some holocarpic oomycetes affecting toxic diatoms can also have

a positive economic impact by controlling harmful algal blooms (Hanic, Sekimoto and Bates, 2009; Lelong *et al*., 2012; Trainer *et al*., 2012; Garvetto *et al*., 2018; Bates *et al*., 2018).

## 3.5 Future Research Directions

The phylogeny of the oomycetes early-diverging lineage is far from complete and much work is left to understand the taxonomy and systematics of these organisms. The results presented in this thesis have only partially resolved the taxonomy and phylogeny of the early-diverging lineages and, yet, there are many holocarpic oomycetes that remain to be rediscovered and phylogenetic placements to be settled. Thus, it will be the core focus of our future work to complete and expand the phylogeny of the early-diverging oomycetes. So far, the taxonomic placement of most species remains unresolved. It is highly important, at present, to sequence all early-diverging holocarpic oomycetes, most especially the type species, as well as to develop specific primers for multilocus studies, therefore, further improving the resolution and phylogenetic analyses. Likewise, high priority of our future work will also focus on re-isolating and culture establishment of all described early-diverging oomycetes, which can then be further utilised for studies on the pathogen life-cycle, ultrastructure (e.g. SEM, and TEM), genomics (e.g. genes, and effectors), chemistry (e.g. metabolomics, and metabolites) and physiology (host-pathogen interactions, and host susceptibility). Up to now, only a few of these parasitoids (e.g. *Lagenisma coscinodisci*, *Diatomophthora perforans* subs*. destruens*, *Diatomophthora perforans* subs*. pleurosigmae*, and *Eurychasma dicksonii*) have been successfully cultivated, mostly together with their hosts and none in agarised medium (Müller, Gachon and Küpper, 2008; Buaya, Kraberg and Thines, 2019c; Buaya *et al*.,

under review). Little is also known regarding the ecology and occurrence of these organisms. Almost all of the studies on holocarpic oomycetes have been conducted in the temperate regions whilst knowledge about these organisms remains scarce in the tropics and arctic. Our future work will also address these lapses and prioritise the sampling for holocarpic oomycetes in several aquatic environments, especially in habitats (e.g. tropical oceans, benthos, arctic and glacial lakes) and substrates (mud, soil and bottom sediments) that have not been previously explored for these organisms.

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# APPENDICES AND FULL TEXT

1. Phylogeny of *Miracula helgolandica* gen. et sp. nov. and *Olpidiopsis drebesii* sp. nov., two basal oomycete parasitoids of marine diatoms, with notes on the taxonomy of *Ectrogella*-like species. *Mycological Progress*, 16:1041-1050

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2. Sampling and experimentation

   Doctoral Student: AB (70%)- field collection (German isolates), parasite screening, isolation, light microscopy, PCR assays, characterization, herbarium voucher & deposit

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ORIGINAL ARTICLE

# Phylogeny of *Miracula helgolandica* gen. et sp. nov. and *Olpidiopsis drebesii* sp. nov., two basal oomycete parasitoids of marine diatoms, with notes on the taxonomy of *Ectrogella*-like species

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**Abstract** Despite their widespread nature and economic impact, little is known regarding the diversity and phylogeny of diatom-infecting oomycetes. While the phylogenetic affinities of *Lagenisma*, affecting large centric diatoms, has recently been resolved, no member of the widespread genus *Ectrogella* has, so far, been investigated using molecular phylogenetics. The genus *Ectrogella* contains about a dozen species, which are all holocarpic. The species in the genus are diverse in terms of morphology and development, and primarily set apart from other holocarpic oomycete genera on the basis of their occurrence in unicellular or colonial algae, predominantly licmophoroid and bacillarioid diatoms. Here, we report the phylogenetic placement of two oomycete parasitoids one parasitic to *Pseudo-nitzschia pungens* and the other parasitic to *Rhizosolenia imbricata*. While both parasitoids were placed outside the crown oomycete groups represented by Saprolegniomycetes and Peronosporomycetes, they did not form a monophyletic assemblage. The *Rhizosolenia* parasitoid was embedded amongst marine *Olpidiopsis* species, while the *Pseudo-nitzschia* parasitoid was placed as the sister clade to all remaining oomycetes. The taxonomy of *Ectrogella*-like organisms and *Olpidiopsis* is discussed and, as a consequence of morphological differences and phylogenetic placement, two new species, *Miracula helgolandica* and *Olpidiopsis drebesii*, are introduced.

**Keywords** Diatom · Oomycetes · Parasitoids · Pathogens · Phylogeny · Taxonomy

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## Introduction

Diatom parasitoids have been reported in various hosts from both freshwater and marine ecosystems (Karling 1942; Sparrow 1960; Dick 2001). The majority of these parasitoids have been assigned to the oomycete genus *Ectrogella*, which contains almost exclusively pathogens of diatoms. Only a few oomycete pathogens of diatoms have been classified in other genera, e.g. *Aphanomycopsis* (Scherffel 1925) and *Lagenisma* (Drebes 1968), while a few others are of unclear affinity (Braun 1856; Sparrow 1936, 1960; Dick 2001). Since its description by Zopf (1884), the genus *Ectrogella* has been used rather indiscriminately for holocarpic oomycete pathogens of diatoms with similar thallus morphology. Initially, it contained parasitoids exhibiting a clear diplanetism (Zopf 1884). This was also given as the key diagnostic feature separating the genus *Ectrogella* from *Olpidiopsis*, with *Ectrogella* placed in the diplanetic Saprolegniales and *Olpidiopsis* placed in the monoplanetic Lagenidiales by Sparrow (1960). However, after the description of the type species, several species were added to the genus with rather olpidioid spore development or

unclear diplanetism, on the basis of their diatom hosts (Scherffel 1925; Karling 1942; Sparrow 1960; Dick 2001). However, Sparrow (1960) retained species in *Olpidiopsis* in which no cyst formation but only a resting phase, in which the morphology of the zoospores changed, was observed, e.g. *O. gillii*. It remains unclear if the distinction between primary and secondary spores formed after encystment in most species of *Ectrogella* and the dimorphism without encystment reported for some species in *Olpidiopsis* (Sparrow 1960) has phylogenetic significance. Currently available phylogenies (Hakariya et al. 2007; Sekimoto et al. 2009; Thines et al. 2015) suggest that diplanetism might be an ancestral stage for oomycetes that has been variously modified or lost. Oomycete parasitoids of diatoms have gained considerable attention from the end of the nineteenth century to the mid-twentieth century (Zopf 1884; Petersen 1905; Sparrow 1934, 1936, 1960; Karling 1942; Friedmann 1952; Aleem 1953; Feldmann and Feldmann 1955; Johnson 1965, 1967), but have been studied relatively little in more recent times (e.g. Drebes 1968; Schnepf et al. 1978; Raghukumar 1978, 1980).

Pathogens of marine diatoms are probably known even less than their freshwater counterparts, with only a few species described (Sparrow 1960; Dick 2001). This is surprising, given their potential role in the collapsing of diatom blooms and, thus, their ecological importance, which has only recently been reappraised (Strittmatter et al. 2009; Scholz et al. 2016a, b; Raghukumar 2017). A newly recognised pathogen of *Pseudo-nitzschia pungens* has been characterised in detail (Hanic et al. 2009), but its taxonomic affinities remained obscure. *Pseudo-nitzschia* species are a major part of the marine phytoplankton and some can cause poisonous blooms (Bates et al. 1998). They are, thus, closely monitored (Bates and Trainer 2006), on which occasion also the parasitoid had been encountered (Pauley et al. 1994). It has been speculated that it belongs to the basal oomycetes, as there are some similarities to *Eurychasma* and *Haptoglossa* (Hanic et al. 2009), such as the lack of mastigoneme hairs on the anterior flagellum. But as *Lagenisma*, which has also been thought to represent a basal lineage (Beakes et al. 2014), was found to be a member of the Saprolegniomycetes (Thines et al. 2015), this assessment has to be regarded as provisional. There are currently no sequence data available for the parasitoid affecting *Pseudo-nitzschia* or any other oomycete diatom parasitoids, apart from *Lagenisma*. However, the presumably basal oomycete lineages are crucial for understanding oomycete macro-evolution, as the phylum Oomycota is hypothesised to have its ancestors in the marine environment (Beakes and Sekimoto 2009; Thines 2014; Beakes et al. 2014; Beakes and Thines 2017). Thus, it was the aim of the current study to infer the phylogenetic placement of the oomycete parasitoids affecting *Pseudo-nitzschia pungens* and a second diatom pathogen, which was found to affect *Rhizosolenia imbricata*.

## Materials and methods

### Sample collection and microscopy

Diatom samples were obtained from Prince Edward Island, Canada and Helgoland, Germany, as described previously by Hanic et al. (2009) and Wiltshire et al. (2010), respectively. Concentrated phytoplankton samples containing parasitised filaments of *Pseudo-nitzschia pungens* were deep-frozen in a freezer for shipment, in case of the samples from Canada, or directly processed, in case of the samples from Germany. Individuals of *P. pungens* and *Rhizosolenia imbricata* infected with oomycetes from Germany were collected from July to August 2016 and in July 2017, from Helgoland Roads, located in between the rocky island of Helgoland and the sandy island of Düne. Around 10 mL of raw net samples was poured onto a Petri dish and screened for infected diatoms by using a compound microscope (Zeiss Axioskop 2, Zeiss, Germany) or a dissecting microscope (Leica MZ16, Leica, Germany). Infected cells were mounted on glass slides using sterile marine water or 0.1% Lugol's iodine solution (Kraberg et al. 2012). Photographs were taken using a Zeiss Axiocam HRc digital camera (Zeiss, Germany). Oomycete-infected diatoms occurring singly or in chains were picked using a 10-μL micropipette (Braun, Germany and Eppendorf, Germany) from raw net collections, transferred up to three times through droplets of sterile seawater until no non-target cells were visible and then immersed in 1 mL Ambion RNAlater™ solution (Sigma-Aldrich, Germany) or 70% ethanol (VWR, Germany). Approximately 50 infected cells were collected per tube for subsequent DNA extraction. In the diatom sample from Canada, infected filaments of *P. pungens* were observed in samples from 2013, while the sample from Helgoland contained infected filaments of both *P. pungens* in 2015 and 2016, and *R. imbricata* in 2016 and 2017.

### DNA extraction, PCR and sequencing

For DNA extraction, the samples were centrifuged at maximum speed for 2 min to pellet the cells. Ethanol (70% aq.) or RNAlater were carefully removed by pipetting and, subsequently, the SLS buffer of the innuPREP Plant DNA Kit (Analytik jena, Germany) was added. About 10–15 tungsten carbide beads (1 mm) were added to 2-mL tubes before homogenisation in a Retsch Mixer Mill MM 200 (Retsch GmbH, Germany) at 25 Hz for 5 min. Subsequently, the DNA extraction was conducted following the manufacturer's instructions of the innuPREP Plant DNA Kit. Polymerase chain reaction (PCR) for the *Pseudo-nitzschia* pathogen was carried out using Ranger DNA Polymerase (Bioline, UK) with each 12.5-μL reaction mix containing 1× Ranger Reaction buffer, 0.4 μM of the primers Euk573 and Euk1422 (Wang et al. 2014), 1 U of Ranger DNA Polymerase and 1 μL of

DNA extract. The amplification was conducted on an Eppendorf Mastercycler pro S system equipped with a vapo.protect lid (Eppendorf, Germany) by a two-step PCR with an initial denaturation at 95 °C for 3 min and 40 cycles at 98 °C for 10 s followed by 55 °C for 60 s. Two positive PCR reactions (one from RNAlater, one from ethanol-stored samples) were equally mixed and diluted by a factor of ten. Subsequently, the mixture was cloned into *Escherichia coli* using a StrataClone PCR Cloning Kit (Agilent Technologies, USA), following the manufacturer's instructions. Single colonies were picked into 20 μL of distilled molecular biology grade water and a colony PCR was carried out with the Mango DNA Polymerase (Bioline, UK). Reaction mixes of 12.5 μL contained 1× Mango Reaction buffer, 200 μM dNTPs, 2 mM $MgCl_2$, 0.8 μg BSA (bovine serum albumin, Carl Roth GmbH, Germany) 0.4 μM of T3 and T7 plasmid primers, 0.5 U of Mango DNA Polymerase and 0.5 μL of the colony suspension as template. PCR was carried out on an Eppendorf Mastercycler pro S system equipped with a vapo.protect lid with an initial denaturation at 96 °C for 10 min, 36 cycles at 96 °C for 20 s, 54 °C for 20 s and 72 °C for 60 s, 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 (BiK-F, Frankfurt, Germany) using the plasmid primer T7. The 18S loci of the *R. imbricata* pathogen was amplified with Mango DNA Polymerase using the same conditions as described above, except that 5 μL of the DNA extract was used as the template. The amplification conditions were set to an initial denaturation at 96 °C for 10 min, 40 cycles at 96 °C for 20 s, 65 °C for 40 s and 72 °C for 120 s, concluding with a final elongation at 72 °C for 10 min. In addition, single-cell PCRs were carried out as described for the PCRs on extracted DNA, except that, instead of DNA, the same amount of molecular biology grade water and a single infected cell was added. Positive PCRs were sent for sequencing to the laboratory centre of the Senckenberg Biodiversity and Climate Research Centre (BiK-F, Frankfurt, Germany) using the PCR primers Euk422 and Euk1422, as well as the internal forward primer Euk573 (Wang et al. 2014).

The final sequences were prepared using Geneious 5.6 with forward and reverse sequences merged. Reference sequences of holocarpic oomycetes, in particular *Olpidiopsis* spp., and representative additional species were extracted from GenBank (https://www.ncbi.nlm.nih.gov/) and added to the dataset. Partial 18S sequences obtained in this study were deposited in GenBank as MF926412 for the *P. pungens* parasitoid and MF926411 for the *R. imbricata* parasitoid.

### Alignments and phylogenetic analyses

Alignments were done using MAFFT (Katoh and Standley 2013) on the TrEase webserver (http://www.thines-lab.senckenberg.de/trease), applying the G-INS-i algorithm. Alignments were subjected to phylogenetic analysis using MEGA v5 (Tamura et al. 2011). Three different methods were used for phylogenetic inference using the Tamura–Nei substitution model (the most comprehensive classical model offered by MEGA): minimum evolution, with 1000 bootstrap replicates and all other parameters set to default; maximum parsimony, with 100 bootstrap replicates and all other parameters set to default; and maximum likelihood, with 100 bootstrap replicates and all other parameters set to default. This rather simplistic approach was taken as it was not the aim to resolve the higher-level relationships within oomycetes in detail, which would also have been impossible, given the rather short SSU sequence stretches available.

## Results

### Morphology

In the current study, oomycete parasitoids were found in *Rhizosolenia imbricata* and *Pseudo-nitzschia pungens*. Our own observations of the latter from the frozen sample from Canada are fully in line with those of Hanic et al. (2009), even though zoospore release could not be studied. In the samples from Helgoland, which contained living parasitoids in *Pseudo-nitzschia pungens* (Figs. 1a and 2a–c), thallus development and zoospore release were followed. Thalli of this parasitoid were initially inconspicuous and became easily visible as slightly granular, colourless structures, when they began to fill the breadth of the diatom hosts. Upon further growth, the phaeoplasts of the diatom were forced outwards, finally disintegrating and aggregating into orange to chestnut-coloured relicts near the outer tips of the diatom. During this stage, the granular structure of the thallus became more pronounced but was then lost for some time, before zoospores began to form. The cleavage of the zoospores appeared to occur in the cytoplasm around a central vacuole, which was not always regular in its shape. However, due to the slender nature of the *Pseudo-nitzschia* cells investigated, this pattern was difficult to observe. With the formation of zoospores, a single discharge tube with a thickened base formed per parasitoid thallus and usually emerged laterally within the central third of the diatom cell. After the apical part of the discharge tube ruptured, broadly pyriform to reniform zoospores of 3–5 μm in length and 2–4 μm wide were released and swam away, often in a spiral movement. Flagella were almost equally long, usually about two times the length of the spore, inserted in a lateral grove, usually sub-apical but also sub-central (Fig. 2c). Hypertrophy of infected cells was sometimes apparent, especially when diatoms at the ends of the filaments were affected.

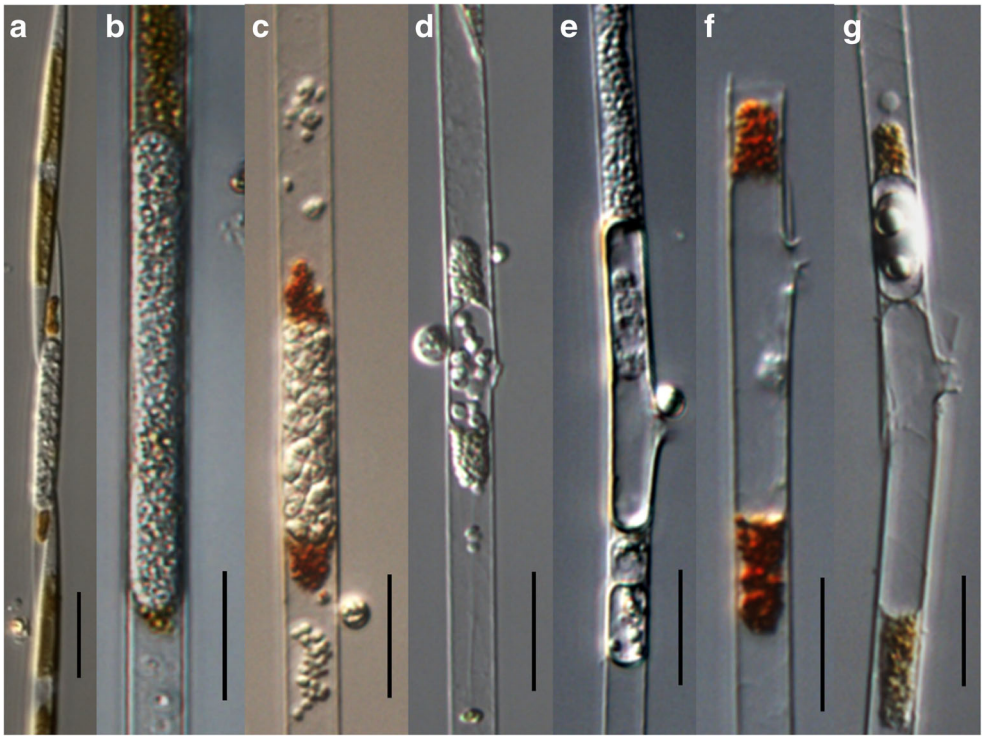



**Fig. 1** Light micrographs of oomycete parasitoids. **a** Developing thallus of the oomycete parasitoid affecting *Pseudo-nitzschia pungens*; **b–g** differentiation of the thallus of the oomycete parasitoid affecting *Rhizosolenia imbricata*; **b** mature thallus; **c** zoospore differentiation; **d** zoospore discharge; **e** adjacent thalli at different developmental stages; **f** empty sporangium with discharge tube; **g** empty sporangium with exit tube and adjacent resting-spore-like structures. Scale bar = 20 μm in all images

The pathogen of *R. imbricata* developed in a similar manner, but never caused hypertrophy (Figs. 1b–g and 2d–f). The single discharge tube mostly developed in the central half, breaking through the overlapping girdle bands, did not have a pronounced thickening at the base, was longer (up to 10 μm) than in the parasitoid affecting *Pseudo-nitzschia* spp., did not taper towards the end and was 3–7 μm wide. Upon the rupturing of the apical portion of the discharge tube, the broadly pyriform zoospores that were about 3–4 μm in length and about 3 μm wide immediately swam away from the host (Figs. 1d and 2f). The two flagella were sub-apically inserted and about two times longer than the zoospore body, but

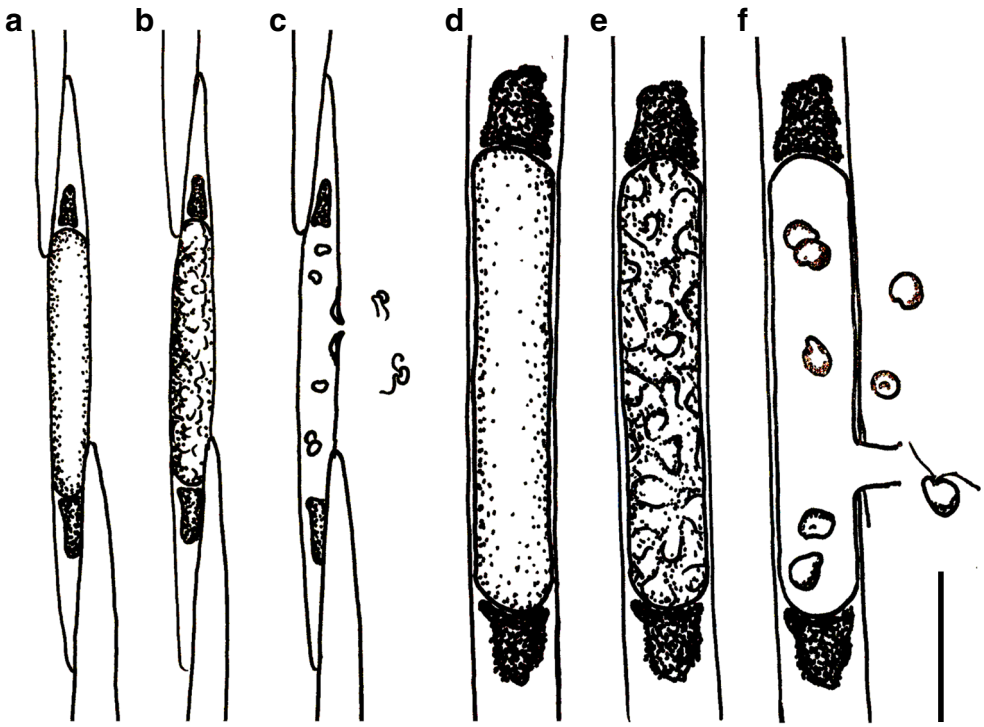



**Fig. 2** Line drawings for the illustration of thallus development and zoospore release. **a–c** Parasitoid affecting *Pseudo-nitzschia pungens*; **a** mature thallus; **b** beginning zoospore differentiation; **c** zoospore release; **d–f** parasitoid affecting *Rhizosolenia imbricata*; **d** mature thallus; **e** beginning zoospore differentiation; **f** zoospore release. Scale bar = 20 μm in **a–c** and 10 μm in **d–f**

sometimes seemed to be much shorter (Fig. 2f). After some time, zoospores came to rest. It was not observed if motility was assumed again. Occasionally, resting-spore-like structures with large refractive droplets formed upon infection in discrete thallus sections. However, no antheridial structures were observed. Within the plankton samples, *R. styliformis* and *R. setigera* were present as well, but were not observed to becoming infected with the pathogen of *R. imbricata* when plankton samples were kept for several days, while cells of *R. imbricata* quickly declined.

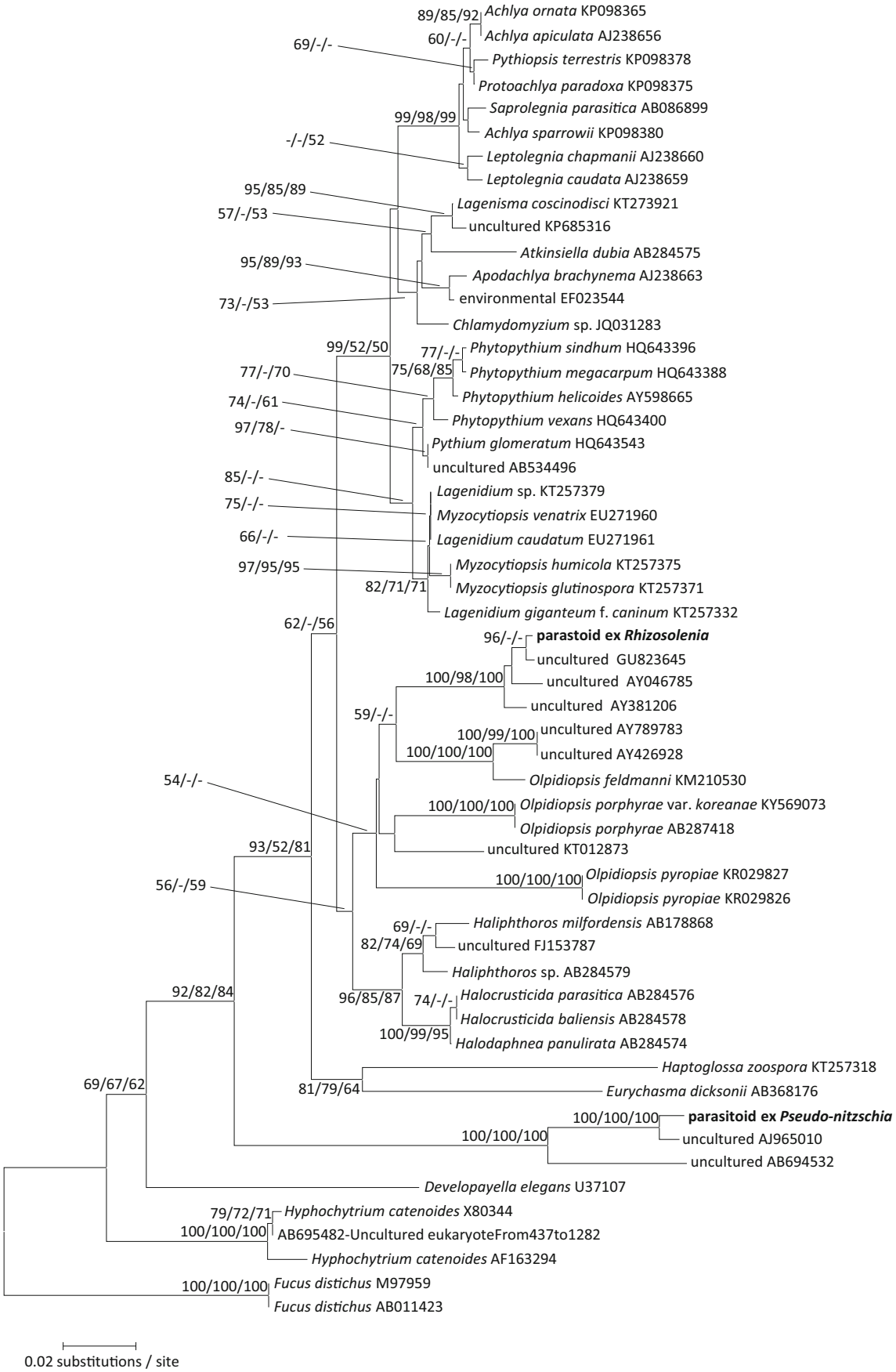
### Phylogeny

In the phylogenetic reconstructions based on partial nrSSU sequences (Fig. 3), the pathogen of *R. imbricata* is placed within a weakly supported *Olpidiopsis* clade, and, with some environmental sequences, inferred as the sister group to a clade containing the rhodophyte pathogen, *Olpidiopsis feldmannii*, again with weak support. The three environmental sequences included in the same clade as the parasitoid from *R. imbricata* are grouping with it with maximum support in all analyses. The *Olpidiopsis* clade was resolved as the sister to a well-supported clade containing the genera *Haliphthoros* and *Halocrusticia*, with weak support. This larger clade was inferred as the sister group of the crown oomycete groups, the Peronosporomycetes and Saprolegniomycetes, with weak support. A moderately supported clade containing the genera *Eurychasma* and *Haptoglossa* was in a basal position to the previously mentioned groups, resembling the core oomycetes, which were grouped together with moderate to high support. The parasitoid of *P. pungens* was placed in the sister group to the core oomycetes together with two environmental sequences. The Oomycota as whole were resolved as monophyletic with high to maximum support as the sister group to the marine protist *Developayella elegans*. Sequences from single-cell PCRs were rather short and, thus, were not added to the phylogenetic tree. However, good quality sequence stretches matched those obtained from extracted genomic DNA.

## Discussion

With the single exception of *Lagenisma* infecting *Coscinodiscus* (Thines et al. 2015), holocarpic oomycete pathogens of diatoms, despite their widespread nature and high abundance (Zopf 1884; Sparrow 1960; Johnson 1965, 1967), have not been investigated using molecular phylogenetic investigations. The majority of oomycetes with unbranched thalli affecting diatoms are currently placed in the genus *Ectrogella* (Dick 2001). The genus contains species with both a clear diplanetism, e.g. the type species, *E. bacillariacearum*, and others in which diplanetism has not been fully established, e.g. *E. perforans* (Sparrow 1960) or *O. gillii*, which was synonymised with *E. bacillariacearum* by Karling (1942) and Dick (2001). In this study, the first two members of unbranched oomycete parasitoids of diatoms were investigated. The oomycete parasitoid affecting *Pseudo-nitzschia* spp. has been known since the 1990s (Pauley et al. 1994; Hanic et al. 2009) and its morphogenesis and ultrastructure have been studied by Hanic et al. (2009). Hanic et al. (2009) concluded that the pathogen could not be assigned to any known oomycete species, while its ultrastructure suggested that it was a basal oomycete and its thallus development, zoospore structure and host spectrum were similar to some species in the genera *Ectrogella* and *Olpidiopsis*. The genus *Ectrogella* has been described by Zopf (1884) and was further refined in its circumscription by Scherffel (1925). The genus contains five to about a dozen species, depending on how narrow species are delineated (Karling 1942; Sparrow 1960; Dick 2001). Within the genus, different modes of zoospore formation have been reported. The two extremes are represented by *Ectrogella perforans* and *E. monostoma*. Zoospores are swarming within the sporangia and directly swim away after discharge in *E. perforans* (Sparrow 1960), with no diplanetism reported, similar to species in *Olpidiopsis*. In *E. monostoma*, spores are non-flagellate when discharged and form a cluster at the mouth of the exit tube (achlyoid), where they encyst and differentiate into secondary zoospores (Scherffel 1925), similar to members of the oomycete crown group Saprolegniomycetes (Beakes et al. 2014; Thines et al. 2015). The type species of Ectrogella, *E. bacillariacearum*, shows a saprolegnioid zoospore differentiation, where primary zoospores encyst at some distance from the sporangium to form secondary zoospores (Zopf 1884; Sparrow 1960). The size of the zoospores is also variable in the diatom parasites assigned to *Ectrogella* sensu Dick (2001), ranging from 3 μm in *E. eurychasmoides* and *E. perforans* to 8 μm in *E. monostoma*, while most species have zoospores that are 4–5 μm long (Sparrow 1960). In addition, the amount of exit tubes varies from usually many, as in *E. bacillariacearum* (Zopf 1884), to strictly one, as in *E. monostoma* (Scherffel 1925; Sparrow 1933).

The species affecting *Pseudo-nitzschia* cannot be assigned to any of the known species of *Ectrogella* or *Olpidiopsis*, or even either genus, as it displays a unique combination of characters. It has only a single exit tube, as in *E. monostoma*, and several *Olpidiopsis* species, but differs from the former species in having much smaller spores that directly swim away after escape and from most other *Ectrogella* species (except *E. gomphonematis* and *E. licmophorae*) and *Olpidiopsis* species in forming a thickened basal plate from which the short exit tube arises. Thus, in line with Hanic et al. (2009), it is concluded that the species has not been named previously. In addition, the lack of mastigonemes observed by Hanic et al. (2009), a feature shared with species of the early-branching, nematode-parasitic genus *Haptoglossa*, hints at a basal position of the *Pseudo-nitzschia* parasitoid.


89/85/92
Achlya ornata KP098365
60/-/-
Achlya apiculata AJ238656
69/-/-
Pythiopsis terrestris KP098378
Protoachlya paradoxa KP098375
99/98/99
Saprolegnia parasitica AB086899
Achlya sparrowii KP098380
-/-/52
Leptolegnia chapmanii AJ238660
Leptolegnia caudata AJ238659
95/85/89
Lagenisma coscinodisci KT273921
57/-/53
uncultured KP685316
Atkinsiella dubia AB284575
95/89/93
Apodachlya brachynema AJ238663
73/-/53
environmental EF023544
Chlamydomyzium sp. JQ031283
99/52/50
77/-/-
Phytopythium sindhum HQ643396
75/68/85
Phytopythium megacarpum HQ643388
77/-/70
Phytopythium helicoides AY598665
74/-/61
Phytopythium vexans HQ643400
97/78/-
Pythium glomeratum HQ643543
uncultured AB534496
85/-/-
Lagenidium sp. KT257379
75/-/-
Myzocytiopsis venatrix EU271960
66/-/-
Lagenidium caudatum EU271961
97/95/95
Myzocytiopsis humicola KT257375
82/71/71
Myzocytiopsis glutinospora KT257371
Lagenidium giganteum f. caninum KT257332
62/-/56
96/-/-
parastoid ex Rhizosolenia
uncultured GU823645
100/98/100
uncultured AY046785
uncultured AY381206
59/-/-
100/99/100
uncultured AY789783
100/100/100
uncultured AY426928
Olpidiopsis feldmanni KM210530
54/-/-
100/100/100
Olpidiopsis porphyrae var. koreanae KY569073
Olpidiopsis porphyrae AB287418
uncultured KT012873
93/52/81
100/100/100
Olpidiopsis pyropiae KR029827
56/-/59
Olpidiopsis pyropiae KR029826
69/-/-
Haliphthoros milfordensis AB178868
82/74/69
uncultured FJ153787
Haliphthoros sp. AB284579
92/82/84
96/85/87
74/-/-
Halocrusticida parasitica AB284576
Halocrusticida baliensis AB284578
100/99/95
Halodaphnea panulirata AB284574
Haptoglossa zoospora KT257318
69/67/62
81/79/64
Eurychasma dicksonii AB368176
100/100/100
parasitoid ex Pseudo-nitzschia
100/100/100
uncultured AJ965010
uncultured AB694532
Developayella elegans U37107
79/72/71
Hyphochytrium catenoides X80344
100/100/100
AB695482-Uncultured eukaryoteFrom437to1282
Hyphochytrium catenoides AF163294
100/100/100
Fucus distichus M97959
Fucus distichus AB011423
0.02 substitutions / site

◀ **Fig. 3** Phylogenetic reconstruction using minimum evolution inference on partial nrSSU sequences. Support values in minimum evolution, maximum parsimony and maximum likelihood inference are given at the branches, in the respective order. – Indicates lack of support

The second parasitoid investigated in this study is pathogenic to *Rhizosolenia imbricata*. When plankton samples were kept for several days at 16 °C (14/10 h light/darkness), a decline of the cells of *R. imbricata* was seen. But while in 2016 a few Lugol-fixed cells of *R. styliformis* were seen parasitised, this species and *R. setigera*, which was more common in the plankton samples investigated than *R. imbricata*, were not observed to be affected. This suggests either a high degree of host specificity or that non-resistant cells had already been eliminated previously (Friedmann 1952). The former assumption seems to be more likely, as attempts to infect pure cultures of several lines of *R. setigera* failed. Even though these supporting findings will need to be followed up by infection studies involving a broad spectrum of potential hosts to test the degree of host specificity, this casts doubts regarding the validity of the broad host range assumed for *E. bacillariacearum* and *E. perforans* (Karling 1942; Sparrow 1960), and suggests that these species might, rather, be species complexes. To our knowledge, the only oomycete pathogen observed in *Rhizosolenia* was a species determined as *Petersenia* sp. that formed two exit tubes and did not fill the entire cell (Johnson 1967). However, this designation was tentative, and as spore release has not been followed and the report remained anecdotal, a determination, even at the genus level, does not seem feasible at present. As the parasitoid observed in this study always formed just a single exit tube and filled the inner width of the diatom host completely, it seems unlikely that it is conspecific with the pathogen reported by Johnson (1967). The mode of thallus development and zoospore release is similar to other unbranched holocarpic diatom parasitoids. When the thallus enlarges within the cells, the cellular content is pushed outward and shrivels into small, dark orange to chestnut-brown structures, similar to the ones already observed by Braun (1856) in infected cells of *Eunotia*. As with the pathogen on *Pseudo-nitzschia*, the parasitoid of *R. imbricata* cannot be assigned to any of the known species of oomycete diatom parasitoids. In always forming a single exit tube, it is similar to *E. monostoma*, some marine *Olpidiopsis* species and the vesiculate holocarpic diatom parasitoids often classified as *Lagenidium* (Scherffel 1925; Sparrow 1960), but its zoospores scatter and usually swim away from the sporangium, while in *E. monostoma* and *Lagenidium* spp., the zoospores cluster at the mouth of the exit tube. In addition, the zoospores of the parasitoid of *R. imbricata* are significantly smaller, measuring less than half (3–4 μm) of the length of *E. monostoma* zoospores (8 μm) and the zoospores reported for the *Lagenidium* species affecting diatoms. They are also smaller than those reported for the diatom-infecting *Olpidiopsis gillii*, from which it can also be distinguished by forming a shorter and more cylindrical exit tube. The taxonomic affinity of the diatom parasitoids currently placed in *Lagenidium* is uncertain. The vesiculate appearance of zoospore release might probably be because of the production of mucous, in which the zoospores are trapped until it dissolves, similar to the situation in *O. glenodinianum* (Sparrow 1960).

In the phylogenetic analyses, the two investigated diatom parasitoids occupied different positions. While the species affecting *P. pungens* was placed as the sister group to all remaining oomycetes with weak to strong support, the pathogen of *R. imbricata* grouped within a weakly supported group represented by *Olpidiopsis* species. However, as only partial nrSSU sequences could be obtained in the current study, the exact placement of both species cannot be inferred, similar to previous studies on basal oomycetes species (Beakes and Sekimoto 2009; Sekimoto et al. 2009). Resolving the exact phylogenetic placement, in which shifts can be expected, will require the investigation of the species included in this study and, also, additional ones not yet covered, using multigene phylogenies. However, as the pathogen of *R. imbricata* shares several characteristics typical for the genus *Olpidiopsis* in which it is placed based in the phylogenetic analyses, we assign it to that genus, even though the placement of this species and the other species parasitic on marine algae in *Olpidiopsis* should be considered provisional, until sequence data become available for its type species, *O. saprolegniae*. The pathogen of *Pseudo-nitzschia* needs to be assigned to a new genus, based on its phylogenetic placement. It seems possible that also other *Ectrogella* species with short exit tubes with a thickened base, such as *E. monostoma*, will turn out to belong to that genus once corresponding sequence data become available.

It is noteworthy that several environmental sequences clustered with the sequences obtained in this study, hinting to a high, undiscovered diversity, in line with the host specificity deduced in this study. The pathogen of *R. imbricata* clustered with three environmental sequences obtained from various regions and habitats, including the Caribbean Sea (Edgcomb et al. 2011), a hydrothermal vent in the Guaymas Basin (Edgcomb et al. 2002) and the Northwestern Mediterranean Sea (Massana et al. 2004), highlighting the widespread occurrence of similar organisms. The pathogen of *P. pungens* clustered together with two environmental sequences, one originating from surface water of the Japanese Sagami Bay (Kok et al. 2012), the other, which was very similar to the sequence in this study, from the North Sea near Helgoland (Medlin et al. 2006), again demonstrating the widespread occurrence of similar eukaryotes. Given the potential host specificity of the diatom parasitoids and the sequence diversity observed, it seems likely that several new oomycete pathogens of marine diatoms await discovery.

## Taxonomy

Based on phylogenetic investigations, paired with unique morphological and physiological characteristics, two new species are introduced here. The morphology and development of the parasitoid of *Rhizosolenia imbricata* is similar to *Olpidiopsis*, in which it is embedded in terms of phylogeny and, thus, described in this genus, while the parasitoid of *Pseudo-nitzschia pungens* is described in a new oomycete genus, based on its phylogenetic distinctiveness.

***Olpidiopsis drebesii*** A. Buaya et Thines, **sp. nov.**, MycoBank MB 822746.

**Etymology** – In honour of Dr. Gerhard Drebes, in recognition of his contribution to the knowledge of pathogens of marine plankton.

**Type** – Germany, Helgoland Roads, 29th of June 2017, isolated by Anthony T. Buaya, FR-0247058. Ex-paratype partial nrSSU sequence MF926410.

**Description** – Thallus endobiotic, holocarpic, at early development naked, later surrounded by a thin wall, increasing in size, with an undifferentiated amorphous cytoplasm, finally disintegrating the host cytoplasm and phaeoplasts, which are pushed outward until they become an aggregated, dark orange to chestnut-brown mass at the periphery of the developing thallus; upon zoospore development, the thallus is oblong, greatly varying in size, but always covering the full breadth of the host cell, colourless, occurring usually singly in the centre of the host cell, smooth-walled; with one short cylindrical discharge tube, which is 3–7 μm wide and 3–5 μm long, protruding through the cell wall in the central half of the host cell; the broadly pyriform zoospores are about 3 μm in length and almost equally wide, forming within the sporangium, and escaping into the surrounding medium after the dissolution of the tip of the discharge tube, scattering in all directions in vortical motion; after a short time suddenly coming to rest. Occasionally, resting-spore-like structures form within the host cells, about 8–10 μm in diameter.

**Miraculaceae** A. Buaya, L. Hanic et Thines, **fam. nov.**, MycoBank MB 822742.

**Type** – ***Miracula*** A. Buaya, L. Hanic et Thines, **gen. nov.**, MycoBank MB 822743.

**Description** – Thallus endobiotic, holocarpic, non-walled, discharge tube with a thickened base, zoospores swarming into the surrounding medium after discharge.

***Miracula*** A. Buaya, L. Hanic et Thines, **gen. nov.**, MycoBank MB 822743.

**Etymology** – *Miracula* refers to the wondrous nature of the genus.

**Type** – ***Miracula helgolandica*** A. Buaya, L. Hanic et Thines, **sp. nov.**, MycoBank MB 822744.

**Description** – Thallus endobiotic, holocarpic, non-walled, increasing in size, with an undifferentiated amorphous cytoplasm, finally disintegrating the host cytoplasm and phaeoplasts, which are pushed outward, colourless, discharge tube single, short and conical with thickened base, zoospores discharging into the surrounding medium subsequent to the dissolution of the tip of the discharge tube. Resting spores not observed.

***Miracula helgolandica*** A. Buaya, L. Hanic et Thines, **sp. nov.**, MycoBank MB 822744.

**Etymology** – Referring to the place where the type of the parasitoid was isolated.

**Type** – Germany, Helgoland Roads, 8th of August 2016, isolated by Anthony T. Buaya, FR-0247059. Ex-paratype partial nrSSU sequence MF926411.

**Description** – Thallus endobiotic, holocarpic, non-walled, increasing in size, with an undifferentiated amorphous cytoplasm, finally disintegrating the host cytoplasm and phaeoplasts, which are pushed outward until they become an aggregated, dark orange to chestnut-brown mass at the periphery of the developing thallus; upon zoospore development, the thallus is oval, varying in size, but always covering the full breadth and usually at least two-thirds of the length of the host cell in the case of single thalli, colourless, occurring usually singly in the centre of the host cell, with one short conical discharge tube with thickened base, which is 2–4 μm wide and 3–5 μm long, protruding through the girdle band region in the central third of the host cell; zoospores initially subglobose when forming within the sporangium, after discharge into the surrounding medium, subsequent to the dissolution of the tip of the discharge tube, becoming pyriform to reniform, 3–4(5) μm in length and 2–3 μm wide, swimming in all directions in a vortical or irregular motion; after a short time coming to rest. Resting-spores not observed.

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**Author contributions** MT, LH and AK conceived the study; AB, BN, LN and SP screened plankton samples, isolated infected diatoms and carried out PCR assays; AB and AK performed light microscopy; AB, AK, SP and MT analysed the data; MT, AB and SP wrote the manuscript, with contributions from the other authors.

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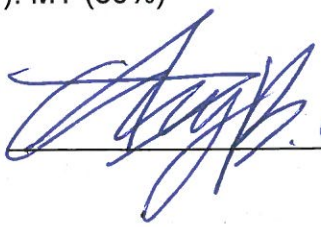
Student signature & date:  04.02.20 Place: Frankfurt am Main

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Full paper

# Rediscovery and phylogenetic placement of *Olpidiopsis gillii* (de Wildeman) Friedmann, a holocarpic oomycete parasitoid of freshwater diatoms

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ABSTRACT

The genus *Olpidiopsis* of the Oomycota includes several species that are aquatic parasites and hyperparasites. Despite their widespread occurrence and potential ecological importance, only a handful of these species has been subjected to phylogenetic investigations, so far. Most species have not been observed and reported for several decades. In the current study, the freshwater diatom parasite *Olpidiopsis gillii* (de Wild.) Friedmann was rediscovered from the river Main in Germany and investigated for its phylogenetic placement using nuclear small ribosomal subunit (SSU) sequences. The absence of a zoospore diplanetism is a characteristic of the genus *Olpidiopsis*, which is in contrast to the diplanetism observed in species of *Ectrogella*. The phylogenetic reconstruction revealed that *Olpidiopsis gillii* is a basal lineage within the oomycetes, grouping together with the recently-described marine diatom parasite *Olpidiopsis drebesii* with high support, and loosely associated with *Olpidiopsis* species parasitising red algae. However, as there are no sequence data available for the type species of both *Olpidiopsis* and *Ectrogella* the taxonomic assignment of these simple holocarpic parasites of algae and diatoms remains fraught with uncertainty.



## 1. Introduction

The oomycetes are fungus-like organisms of the eukaryotic kingdom Straminipila. They include eucarpic and holocarpic forms inhabiting a myriad of terrestrial and aquatic habitats (Dick, 2001; Thines, 2014; Beakes & Thines, 2016; Derevnina et al., 2016). The majority of species belong to Peronosporomycetes and Saprolegniomycetes, which include well-known pathogens of agricultural plants (Thines, 2014), fish (van West, 2006; Derevnina et al., 2016), crustaceans (Hatai et al., 1980, 2000; Chukanhom, Borisutpeth, Khoa, & Hatai, 2003) and algae (Takahashi, Ichitani, & Sasaki, 1977; Li, Zhang, Tang, & Wang, 2010; Fletcher et al., 2015; Klochkova, Shin, Moon, Motomura, & Kim, 2015), which cause significant economic losses yearly. However, the early diverging holocarpic forms, i.e. those that branch below the Peronosporomycetes/Saprolegniomycetes split, are still understudied. This concerns especially the parasites of diatoms, despite their potential ecological role during the breakdown of phytoplankton blooms (Raven & Waite, 2004) and their importance in the understanding of the evolution of oomycetes in general (Beakes, Glockling, & Sekimoto, 2012; Beakes & Thines, 2016). Oomycete parasites of diatoms are well-documented from pennate and centric forms predominantly from freshwater habitats in temperate countries, mainly from articles published during the second half of the 19th until the 60ies of the 20th century (Karling, 1942; Sparrow, 1960). The oomycete pathogens of diatoms were placed in the genera *Lagenidium* (Schenk, 1859), *Aphanomycopsis* (Scherffel, 1925), *Ectrogella* (Zopf, 1884) and *Olpidiopsis* (Cornu, 1872), mainly on the basis of thallus development and characteristics of zoospore release. To these four the genus *Lagenisma* was added later (Drebes, 1966). With the exception of *Lagenisma coscinodisci* Drebes, research on diatom pathogens has been rather stagnant throughout the remainder of the 20th century (Drebes, 1966; Schnepf & Drebes,

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1977). Only recently, interest in diatom-infecting oomycetes has resurged (Hanic, Sekimoto, & Bates, 2009; Thines, Nam, Nigrelli, Beakes, & Kraberg, 2015; Scholz et al., 2016a, b; Buaya et al., 2017). But, because isolation and culture of holocarpic pathogens are challenging due to their often obligate biotrophic nature, it will probably take some time, until all known species of diatom-infecting oomycetes have been rediscovered and added to molecular phylogenetic analyses. Most pathogens of terrestrial diatoms have been classified in the genus *Ectrogella*, and only one species of *Olpidiopsis*, *O. gillii* (de Wild.) Friedmann is known from terrestrial diatoms, predominantly parasitic to species of genus *Gyrosigma* (*G. attenuatum* (Kützing) Rabenh., *G. acuminatum* (Kützing) Rabenh.) and *Pleurosigma* (*P. attenuatum* (Kützing) W. Smith) (Sparrow, 1960). The parasite was first described by de Wildeman in 1896 as *Olpidium gillii* de Wildeman. Friedmann (1952) reclassified the organism as *Olpidiopsis gillii*. The species has been synonymised sometimes with *Ectrogella bacillariacearum* Zopf due to its similar life cycle, except that its zoospores do not undergo diplanetism (Friedmann, 1952; Karling, 1942; Sparrow, 1960). In the light of the recent description of a new species of *Olpidiopsis*, *O. drebesii* A. Buaya & Thines (Buaya et al., 2017) from a marine plankton sample, the question arose as to the closeness of the phylogenetic relationship between *O. gillii* and the *Rhizosolenia* parasitoid *O. drebesii*, as they share some morphological similarities despite their largely divergent hosts, e.g. the formation of a single, tubular discharge tube. A literature research revealed that *O. gillii* was often reported abundantly from summer to early autumn, e.g. from New River near London (1892) and from ponds in Floridsdörfer (Vienna) and Gaaden in Austria (Friedmann, 1952; Gill, 1893). Thus a series of sampling efforts was conducted in the Main River in Frankfurt am Main, Germany from summer to autumn in 2017 and in summer 2018. The parasite, which occurred sporadically, infecting *Gyrosigma* and *Pleurosigma*, was successfully isolated, enabling investigation of its phylogenetic affinities and life cycle, which are reported in the current study.

## 2. Materials and methods

### 2.1. Collection, isolation and microscopy

Phytoplankton samples were taken from the river Main in Frankfurt am Main, Germany (GPS: N50°06.195′, E008°40.323′) as described previously by Huang, Hattermann, Krysanova, and Bronstert (2013) and Lange-Bertalot (1979). Diatoms were collected using a phytoplankton net (Hydrobios, Kiel) with a net mesh size of 20 μm and algal concentrates were poured into plastic collection bottles. Approximately 10 mL diatom concentrate from the net tows was poured into each of several 15 mL Petri dishes, and screened for infected diatoms using a compound inverted light microscope (Type AE31, Motic, Xiamen). Oomycete-infected diatoms were individually picked using a 10 μL micropipette (Brandt, Wertheim), rinsed by transferring them through a series of droplets of sterile distilled water to remove attached debris from the frustules, and subsequently immersed in 250 μL of RNA*later* (Life Technologies, Carlsbad), or in 70% ethanol (VWR, Radnor). Approximately 100 infected cells were collected per 2 mL tube (Sarstedt, Nümbrecht) for DNA extraction. The phytoplankton containing *O. gillii* infecting *G. acuminatum* was collected between Aug and Dec 2017 and in Jun and Jul 2018. Infected cells were also mounted onto glass slides using sterile distilled water. For morphological characterisation and DIC micrographs of life cycle stages, a compound light microscope (Imager2, Carl Zeiss, Göttingen) mounted with a Zeiss Axiocam MRc5 (Carl Zeiss) was used. Then, isolates were stained with Schutze's reagent (chlorzinc-iodine solution, Carl Roth GmbH, Karlsruhe) to detect the presence of cellulose in the parasitoid thallus. Samples preserved in 70% ethanol were deposited in the herbarium collection of the Senckenberg Museum of Natural History (FR), Frankfurt am Main, Germany, under the accessions FR0046005 (collection from 2017) and FR0046006 (collection from 2018).

### 2.2. DNA extraction, PCR and phylogenetic analyses

Isolated cells were centrifuged at 19,000 *g* for 2 min at 22 °C to pellet the cells. Subsequently, RNA*later* or 70% (v/v) ethanol were carefully removed by pipetting and 400 μL SLS buffer of the innuPREP Plant DNA Kit (Analytik Jena AG, Jena) was added. Approximately 100 mg of sterile 0.1 mm silica glass beads (Carl Roth GmbH) were added to each 2 mL tube (Sarstedt) of cell suspension and homogenisation was done at 25 Hz for 25 min in a Retsch Mixer Mill MM 200 (Retsch GmbH, Haan). Extraction of DNA was carried using the innuPREP Plant DNA Kit following the protocol provided by the manufacturer. The PCR reaction mixes of 20 μL contained 1x Mango Reaction buffer (Meridian Bioscience, Cincinnati), dNTP (200 μM), $MgCl_2$ (2 mM), bovine serum albumin (0.8 μg/mL) (Carl Roth GmbH), EUK422-445 (0.4 μM) (5′-GGCAGCAGGCRCGAAMTTRCCCA-3′) forward primer, EUK1422-1440_R (0.4 μM) (5′-GGCATCACAGACCTGTTAT-3′) reverse primer (both from Wang, Tian, Gao, Bougouffa, and Qian (2014)), 0.5 U Mango-Taq DNA Polymerase (Bioline) and 5 μL DNA extract. PCR cycling was carried out on an Eppendorf Mastercycler proS (Eppendorf AG, Eppendorf) 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 as described previously (Buaya et al., 2017). In addition, single cell PCRs were also done as described for extracted DNA, except that single infected cells were each directly added to 5 μL of molecular grade water (Life Technologies), to which reaction mixes were added. The resulting sequences were edited and assembled using Geneious (version 5.6). Sequences obtained from *Olpidiopsis gillii* were added to the dataset of Buaya et al. (2017). The partial 18S (nrSSU) sequences obtained in this study were deposited in GenBank (accession numbers: MH971238 for 2017 isolate; MH971239 for 2018 isolate). Alignments were done using the Q−INS−i algorithm of MAFFT (Katoh & Standley, 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 Minimum Evolution inference were done using MEGA v. 6.0 (Tamura et al., 2011) as described in Buaya et al. (2017).

## 3. Results

In a series of sampling conducted between summer to late autumn of 2017 and in summer of 2018 from the river Main in Frankfurt, Germany, sometimes the majority of *G. acuminatum* individuals found were infected by *O. gillii*, while on other occasions infection frequency was much lower. Infections were also rarely noted for *P. attenuatum*, but as no high-quality nrSSU sequences could be obtained from the sampled diatoms, they were excluded from the phylogenetic analyses. *Cocconema lanceolatum* Ehrenberg and *Nitzschia sigmoidea* (Nitzsch) W. Smith, which are also reported as a host for the parasite (Sparrow, 1960) were also co-occurring with infected individuals of *G. acuminatum*, but none were seen to be parasitised during the entire sampling period. The pathogen isolated in the current study agrees well with previous descriptions of *O. gillii* of Friedmann (1952). The development of the parasitoid

thallus until zoospore release was followed in several infected diatoms (Fig. 1A–F). The host normally contains one or two broadly tubular thalli, rarely more than 10 but even up to 20 thalli were found in a single host when concentrated samples were allowed to develop over the course of several days. Thalli of *O. gillii* were of 4–20 μm in diameter and 5–120 μm long. When thalli were single in the cells, they were usually slightly constricted in the centre and pushed apart the host valves at later stages of infection. Thallus development starts close to the host nucleus, the cytoplasm of the pathogen being colourless to slightly whitish (Fig. 1A). Gradually, tiny light-refracting bodies appear and become highly granular at the mid-stage of thallus development. At this stage, the cytoplasm of the host is almost fully disintegrated and reduced to an orange-brown to chestnut coloured residue at the opposite apices of the host frustules. At the end of this stage, the thallus enlarges, pushing the frustules apart (Fig. 1B and C). The granulose appearance of the sporangium gets less coarse for some time, then irregular zoospore initials become visible. After further maturation, when the zoospore initials are assuming a roundish shape, a single elongated discharge tube develops (Fig. 1E), which is usually subapically inserted, and is variable in length, but can reach more than 40 μm with a diameter of about 5 μm at the apex, and 8–20 μm at the base. Zoospores become motile inside the sporangium, before the majority of the sporangium content is driven out within a few seconds. Zoospores exhibit no or an imperfect diplanetism. The discharged pyriform zoospores have two apically inserted flagella during a first swarming period. Zoospores are 4.5–5 μm long and 2–3 μm broad, and after a period of rest and halting motion, zoospores assume a second swarming period and are slightly broader than before resting. No oospores or other resting spores were observed. The entire thallus wall and the discharge tube tested positive for the presence of cellulose, as evidenced by a strong bluish to violet colour after staining with Schultze's reagent.

In thehe phylogenetic tree (Fig. 2) inferred from partial nrSSU sequences of *O. gillii* parasitising *G. acuminatum* from both sampling years formed a monophyletic clade with maximum support. *Olpidiopsis gillii* was grouped with the marine diatom pathogen *O. drebesii* infecting *Rhizosolenia* spp. and three environmental sequences with moderate support. The clade with the diatom parasites was embedded within the *Olpidiopsis* species infecting rhodophyte algae without support. All *Olpidiopsis* species included formed a monophyletic clade, sister to Haliphthorales, again without support. *Miracula helgolandica*, a parasite of the marine pennate diatom *Pseudo-nitzschia pungens*, formed the earliest-diverging lineage of oomycetes with two sequences derived from environmental sequencing.

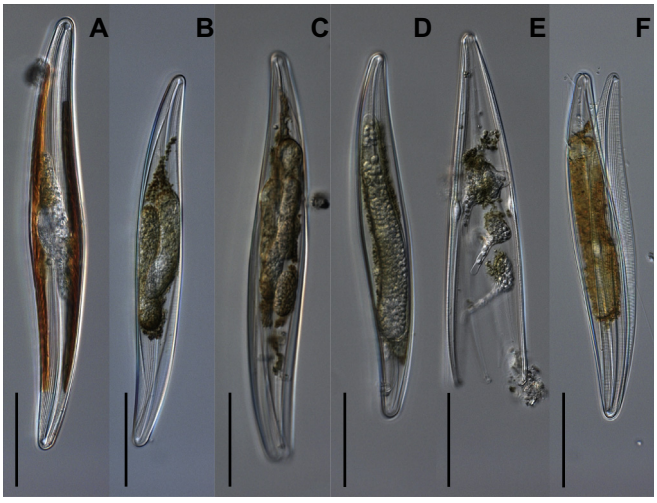


**Fig. 1.** Light microscopy (400×) of *Olpidiopsis gillii* in *Gyrosigma acuminatum*. **A:** Developing endobiotic thallus at an early stage. **B, C:** Elongation of the mature thallus with multiple infection in a single host. **D:** Zoospore differentiation. **E:** Multiple thalli with a subapically inserted discharge tube containing mature zoospores. **F:** Empty parasitoid thallus with a single discharge tube. *Bars*: 50 μm.

## 4. Discussion

Recent studies on oomycetes were focused much on parasites of plants and animals, especially those associated with economic importance (Raffaele & Kamoun, 2012; Thines, 2014; Voigt, Marano, & Gleason, 2013). In contrast, little is known about oomycetes that are forming holocarpic thalli and are predominantly aquatic, for example parasites of invertebrates (e.g. crustaceans, nematodes, mollusks, rotifers), algae (e.g. *Porphyra*, *Ectocarpus*, *Ceramium*), and parasites of aquatic oomycetes (e.g. *Saprolegnia*, *Achlya*, *Pythium*) (Atami, Muroasa & Hatai, 2009; Czeczuga, Kozłowska, & Godlewska, 2002; Czeczuga & Proba, 1980; Glockling & Beakes, 2000; Hakariya, Masuyama, & Saikawa, 2002; Kitancharoen & Hatai, 1995; Molloy et al., 2014; Nakamura & Hatai, 1994; Sekimoto et al., 2008; Sparrow, 1960; Strittmatter et al., 2009, 2016; Vishniac, 1958). The few of these understudied species that have been investigated phylogenetically are mostly included in the early-diverging lineages of oomycetes, but the majority of known species has not been subjected to phylogenetic investigations, even though they are widely occurring (Sparrow, 1960). Among these are oomycete pathogens of diatoms, which have been placed in the genera *Lagenidium*, *Aphanomycopsis*, *Olpidiopsis*, *Ectrogella*, *Lagenisma*, and *Miracula* (Buaya et al., 2017; Cornu, 1872; Drebes, 1966; Schenk, 1859; Scherffel, 1925; Sparrow, 1960; Zopf, 1884). Little is known regarding their ecological importance in nature, but as they have sometimes been observed at high abundance (Zopf, 1884), it can be assumed that they are key parasites of limnic and marine diatoms. Several species have only been reported a few times since their original description. With the exception of *Lagenisma coscinodisci,* a virulent parasite of species of the centric diatom genus *Coscinodiscus* (e.g. *C. granii* L.F. Gough, *C. wailesii* Gran & Angst), for which the phylogenetic placement has recently been resolved (Drebes, 1966; Schnepf & Drebes, 1977; Thines et al., 2015), parasites of diatoms are not well understood. For example, recent metagenomic studies revealed an unexpected diversity of marine oomycetes with several basal lineages (Lara & Belbahri, 2011; Massana et al., 2004, 2006). Two of these oomycetes were recently isolated off the coast of Helgoland in the North Sea and described as new species (Buaya et al., 2017). One of them was placed in the genus *Olpidiopsis*, as *O. drebesii*. Aside from the latter species, which infects *Rhizosolenia* spp. (Buaya et al., 2017), *O. gillii* is the sole diatom parasitoid placed in the genus *Olpidiopsis* (Sparrow, 1960).

*Olpidiopsis gillii* has been known since 1892 (Gill 1893) as a parasite infecting *P. attenuatum*. As Gill (1893) was unsure of the taxonomic identity of the species, he did not describe or assign it to any genus. The parasite was formally described in 1896 by de Wildeman (de Wildeman, 1896) in the fungal genus *Olpidium* as *Olpidium gillii*, based on life-cycle similarities to aquatic chytrids. Friedmann (1952) recognised that the parasite was an oomycete and regrouped the parasite into *Olpidiopsis* based on morphology and the absence of a clear-cut diplanetism (Friedmann, 1952). Subsequently, the pathogen has been synonymised with *Ectrogella bacillariacearum* due to similarities in thallus development, zoospores and host spectrum, but controversy over its correct generic placement persisted (Dick, 2001; Karling, 1942; Sparrow, 1960). The recent finding that the marine diatom genus *Rhizosolenia* is infected by a species that groups with *Olpidiopsis* pathogens infecting red

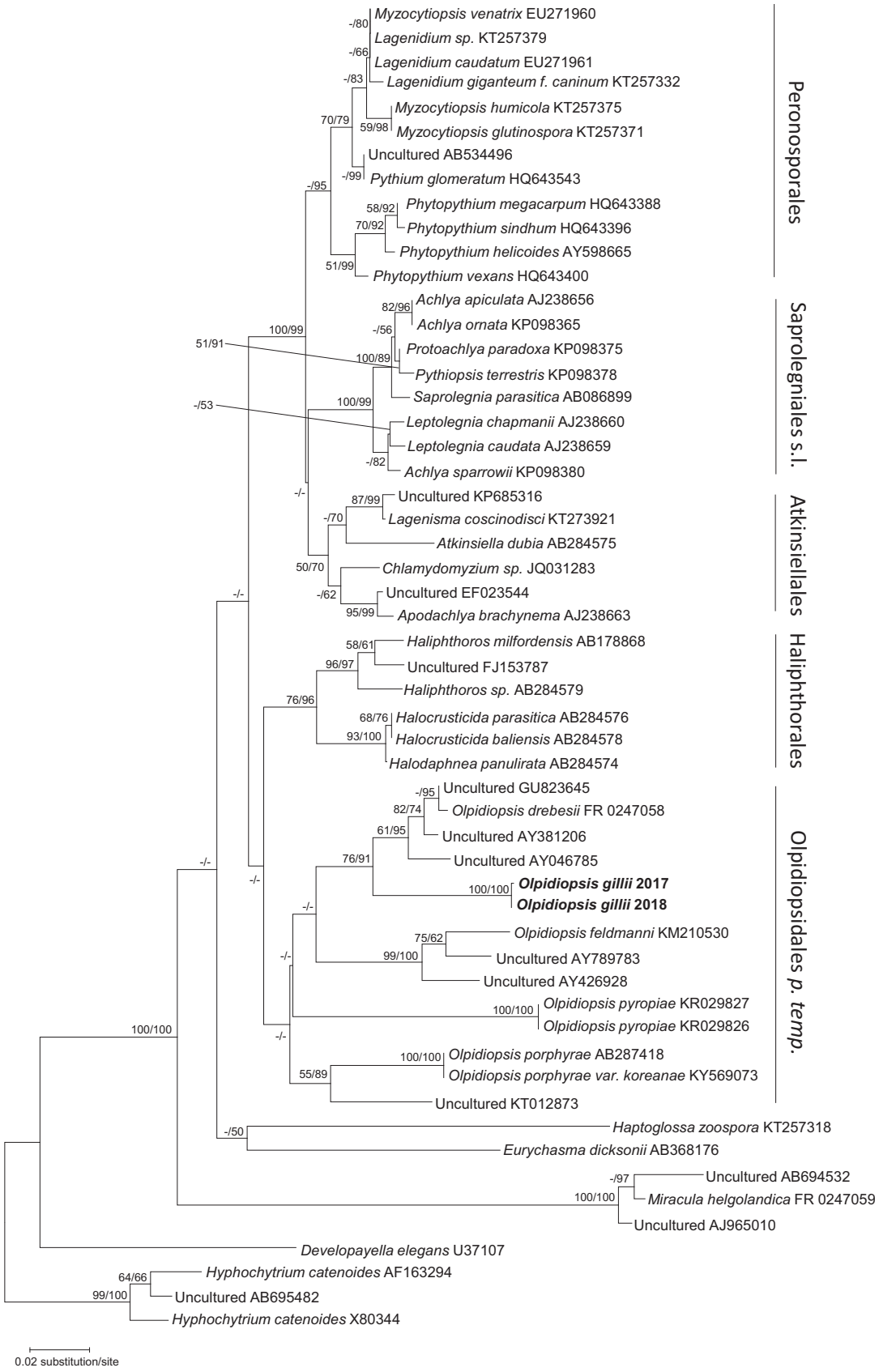


**Fig. 2.** Molecular phylogenetic tree from Minimum Evolution analyses based on nrSSU sequences. Numbers on branches denote bootstrap values from Maximum Likelihood and Minimum Evolution analyses, in the respective order. A dash indicates bootstrap support lower than 50 %.

algae, had fuelled interest in the limnic species, *O. gillii*, leading to the present study, in which *O. gillii* infecting *G. accuminatum* grouped with *O. drebesii* with moderate support. As the same clade also included sequences from environmental sequencing, we hypothesise, that these might also represent diatom-parasitic oomycetes, prompting to an overlooked diversity of *Olpidiopsis* species infecting diatoms in the marine environment.

Should the most recent placement of *O. gillii* in the genus *Ectrogella* be correct (Dick, 2001) the genus *Ectrogella* would be embedded or closely related to *Olpidiopsis* infecting macro-algae (Sekimoto et al., 2008; Sekimoto, Kochkova, West, Beakes, & Honda, 2009; Fletcher et al., 2015; Klochkova, Kwak, & Kim, 2017; Buaya et al., 2017, this study). Dick (2001) suggested the reclassification of *Olpidiopsis* species into three genera based on host-range, retaining the genus *Olpidiopsis* for parasites of aquatic *Saprolegniales* and *Pythiales*. Concomitantly, he assigned the parasites of marine rhodophyte algae to the genus *Pontisma* and the species infecting marine chlorophyte algae to the genus *Sirolpidium*. Even though it might be possible that future phylogenetic investigations encompassing a larger set of genes will resolve that *Olpidiopsis*-like diatom parasites are monophyletic and embedded rhodophyte-infecting pathogens, a classification of these parasites into *Pontisma* or *Ectrogella* would be premature at present, given the low support for the grouping of diatom and rhodophyte pathogens.

In addition, even though phylogenetic investigations of the past two decades have revealed a variety of early-diverging oomycete lineages, e.g. infecting marine crustaceans parasites such as *Haliphthoros* (*H. milfordensis* Vishniac, *H. philippinensis* Hatai, Bian, Batic. & Egusa), *Halocrusticida* (*H. okinawaensis* (K. Nakam. & Hatai) K. Nakam. & Hatai), *Halodaphnea* (*H. panuliri* (Kitanch. & Hatai) M.W. Dick) and *Halioticida* (*H. noduliformans* Muraosa & Hatai), the brown-algae-infecting *Eurychasma dicksonii* (E.P. Wright) Magnus (Magnus, 1905), and the nematode and rotifer parasitic genus *Haptoglossa* (*H. heterospora* Drechsler) (Drechsler, 1940; Hakariya et al., 2002), the type species of *Olpidiopsis*, *O. saprolegniae* (A. Braun) Cornu or other members of *Olpidiopsis* infecting oomycetes could not be included in molecular phylogenetic investigations, so far. In contrast to marine species of *Olpidiopsis*, the oomycete-infecting *O. saprolegniae* and *O. varians* Shanor (Barrett, 1912; Bortnick, Powell, & Bangert, 1985; Cornu, 1872; Martin & Miller, 1986), and *O. schenkiana* Zopf, infecting sexual stages of the limnic green algal genus *Spirogyra*, were reported to form oospore-like resting spores (Barrett, 1912; Zopf, 1884). Thus, until similar stages have been observed in *Olpidiopsis* species infecting marine algae or sequence data become available for *O. saprolegniae*, the genus *Olpidiopsis* s.l. needs to be considered taxonomically unresolved.

## Disclosure

The authors declare no conflict of interest for this study. All of the experiments undertaken in this study comply with the current laws of Germany.

## Author contributions

AB and MT conceived the study; AB collected and screened plankton samples, isolated infected diatoms, performed light microscopy; AB and SP carried out PCR assays; AB, SP and MT analysed the data; AB and MT wrote the manuscript, with contributions from SP.

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# *Miracula moenusica*, a new member of the holocarpic parasitoid genus from the invasive freshwater diatom *Pleurosira laevis*

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**Abstract:** Holocarpic oomycetes are poorly known but widespread parasites in freshwater and marine ecosystems. Most of the holocarpic species seem to belong to clades that diverge before the two crown lineages of the oomycetes, the *Saprolegniomycetes* and the *Peronosporomycetes*. Recently, the genus *Miracula* was described to accommodate *Miracula helgolandica*, a holocarpic parasitoid of *Pseudo-nitzschia* diatoms, which received varying support for its placement as the earliest-diverging oomycete lineage. In the same phylogenetic reconstruction, *Miracula helgolandica* was grouped with some somewhat divergent sequences derived from environmental sequencing, indicating that *Miracula* would not remain monotypic. Here, a second species of *Miracula* is reported, which was found as a parasitoid in the limnic centric diatom *Pleurosira leavis*. Its life-cycle stages are described and depicted in this study and its phylogenetic placement in the genus *Miracula* revealed. As a consequence, the newly discovered species is introduced as *Miracula moenusica*.

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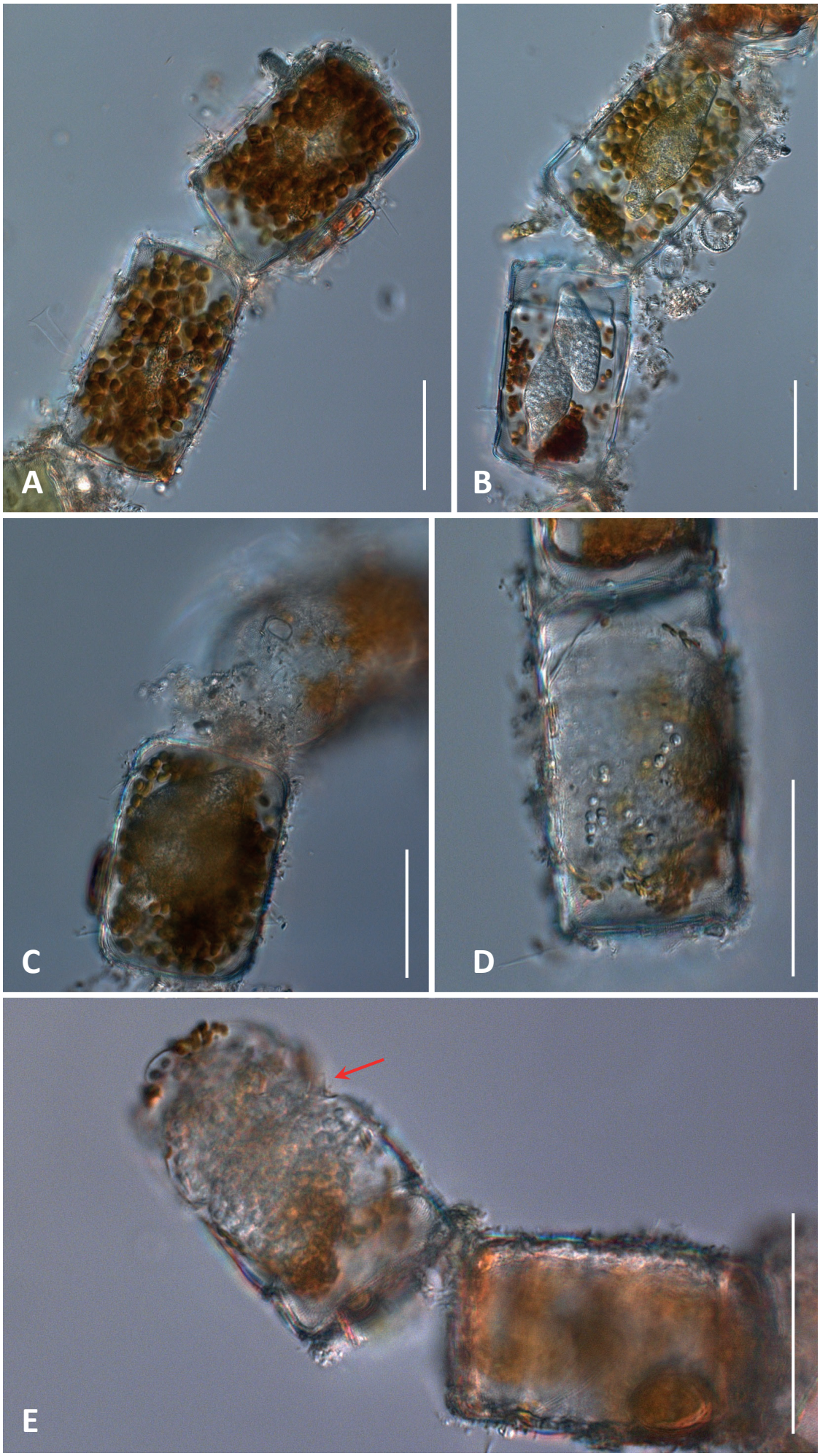
## INTRODUCTION

Despite their global distribution in various habitats, including streams, lakes, and oceans, holocarpic oomycetes are still poorly known (Scholz *et al*. 2016). However, these organisms play a pivotal role in the breakdown of plankton blooms, as parasitoids of multicellular and unicellular algae (Scholz *et al*. 2016, Raghukumar 2017, Buaya *et al*. 2017). Most work on diatom parasitoids has been published in the late 19th and early 20th century, with the monographic treatments of Karling (1942) and Sparrow (1960) pretty much reflecting the current knowledge of this group. Only recently, research interest in oomycete parasitoids of diatoms has increased again, leading to the phylogenetic characterisation of *Lagenisma coscinodisci*, a pathogen of centric diatoms of the genus *Coscinodiscus* (Thines *et al.* 2015a), and the description of two new diatom parasitoids, *Olpidiopsis drebesii* in *Rhizosolenia* spp. and *Miracula helgolandica* in species of the genus *Pseudo-nitzschia* (Buaya *et al.* 2017). While *L. coscinodisci* was found to belong to the early-diverging members of one of the two crown oomycete lineages, the *Saprolegniomycetes*, the other two parasitoids were branching below the *Peronosporomycetes*/*Saprolegniomycetes* split. *Olpidiopsis drebesii* grouped loosely with other *Olpidiopsis* species on red algae, while *Miracula helgolandica* was inferred to likely be the most early-divergent oomycete lineage (Buaya *et al*. 2017). Both new species were grouped with several somewhat divergent environmental sequences, suggesting a widespread nature and the presence of additional, still undiscovered species. While screening for diatom-infecting oomycetes in water and sediment samples from the river Main, a tributary to the central to western European stream Rhine, an unusual parasitoid was found in *Pleurosira leavis*, an invasive species (Litchman 2010) which had not been reported as host for holocarpic oomycetes before. It was the aim of this study to characterise this pathogen in terms of phylogenetic relationships and life cycle and to clarify its taxonomic assignment.

## MATERIALS AND METHODS

### Diatom sampling

In September 2018, sediment surface samples were taken from the banks of the river Main in Frankfurt am Main Germany, by scraping biofilms into 1 L plastic bottles, which were subsequently filled half with water from the river. Samples were brought to the laboratory and screened by pouring sediment suspension into 9-cm-diam Petri dishes and observing them at 50–100 × magnification on an inverted microscope (AE31, Motic, China). Infected diatom cells were transferred to droplets of tap water and observed at 400 × using a Zeiss Imager equipped with DIC and an AxioCam (Zeiss, Oberkochen, Germany). For phylogenetic investigations, around 20 infected filaments were collected in a 2 mL vial containing 1 mL of Ambion RNA Later™ solution (Sigma-Aldrich, Munich, Germany).



### DNA extraction, PCR, and sequencing

For DNA extraction, the tube was centrifuged in a table centrifuge at 19 000 *g* for 2 min and the RNA Later was removed by pipetting. Subsequently, samples were disrupted, and DNA was extracted using the innuprep plant DNA extraction kit (analyticjena, Jena, Germany), as described earlier (Buaya *et al*. 2017). PCR for the amplification of partial small ribosomal subunit (18S nrDNA) and sequencing were performed as described in Buaya *et al*. (2017). Sequencing was done by the Laboratory Centre of the Senckenberg Biodiversity and Climate Research Centre, with the primers Euk573 and Euk1422 (Wang *et al.* 2014), which were also used in PCR. The consensus sequence of the parasite of *Pleurosira laevis* was deposited in GenBank under the accession number MK239934.

### Phylogenetic inference

Sequences were added to the dataset of Buaya *et al.* 2017 and aligned using MUSCLE with standard settings in MEGA v. 5 (Tamura *et al.* 2011), except for using a gap opening penalty of -200 and a gap extension penalty of -4. Phylogenetic inference was done using RAxML v. 8 (Stamatakis 2014) with the GTRGAMMA model and running 1 000 bootstrap replicates for Maximum Likelihood analysis, and using MEGA v. 5 (Tamura *et al*. 2011) with the Tamura-Nei model and running 1 000 bootstrap replicates for Minimum Evolution analysis.

## RESULTS

### Life-cycle observation

Filaments infected with oomycete parasitoids were observed from September 2018 to November 2018, usually at low abundance (less than 5 % of filaments infested). The parasitoid becomes first visible near the central nucleus, rod-shaped, elongating towards the periphery. Subsequently the central part enlarges, giving the thalli a lemon-shaped appearance. Parasitoids remain at this shape for some time, steadily increasing in volume. Towards the end of this stage, chloroplasts degrade into irregular shapes and assume a reddish-brown colouration. Subsequently, a large, central vacuole is forming, the thallus again increasing in size, until almost filling the diatom cells. Within the cytoplasm, the formation of refractive structures can be observed, and zoospores start to mature. When compartmentation is almost concluded, tubular exit tubes with a slightly thickened base develop at or close to the girdle region and push between the valves. Zoospores begin moving within the mature thallus, and then the discharge tube ruptures at the apex, releasing roundish, biflagellate zoospores into the surrounding medium, which swim away from the host cell. After a few minutes, zoospores come to rest. If they assume movement again has not been seen. Frequently, a few zoospores come to rest within the empty thallus and take a globose shape. If they develop further into meioszoospores or if they start moving again has not been observed. The different stages of the life-cycle are illustrated in Fig. 1.

### Phylogenetic inference

In the phylogenetic trees based on partial small ribosomal subunit sequences of the parasitoid of *Pleurosira leavis* grouped together with *Miracula helgolandica* and two sequences derived from environmental sequencing with maximum support in all analyses (Fig. 2). Collectively, they formed the earliest-diverging oomycete lineage, but without strong support. The parasitoid from the river Main was sister to all remaining lineages in *Miracula*, which were grouped together with maximum support in all analyses. Apart from *Lagenisma coscinodisci*, which grouped with other early diverging members of the *Saprolegniomycetes*, all other holocarpic parasitoids of algae and diatoms branched before the split of *Peronosporomycetes* and *Saprolegniomycetes*. These crown oomycete classes were grouped together with moderate to strong support. While the *Peronosporomycetes* and the crown *Saprolegniomycetes* were each grouped together with strong support, the sister-group relationship of the crown *Saprolegniomycetes* and the early-diverging lineages was only weakly supported. The branching order of the early-diverging subclades was not well resolved, but some of the groups received moderate (*Haptoglossa* and *Eurychasma*; *Haliphthoros*, *Halocrusticia*, and *Halodaphnea*), to strong support (phaeophyte parasitoids – *Anisolpidium ectocarpi*, *Olpidiopsis drebesii*, and three sequences derived from environmental sequencing). The parasitoids of red algae did not form a monophyletic assemblage, however, without support.

### Taxonomy

Due to its unique development, diatom host and phylogenetic placement, a new species of *Miracula* is introduced here.

***Miracula moenusica*** A. Buaya & Thines, ***sp. nov*.** MycoBank MB829271. Fig. 1.

*Etymology*: From *moenus*, the Latin name of the river Main.

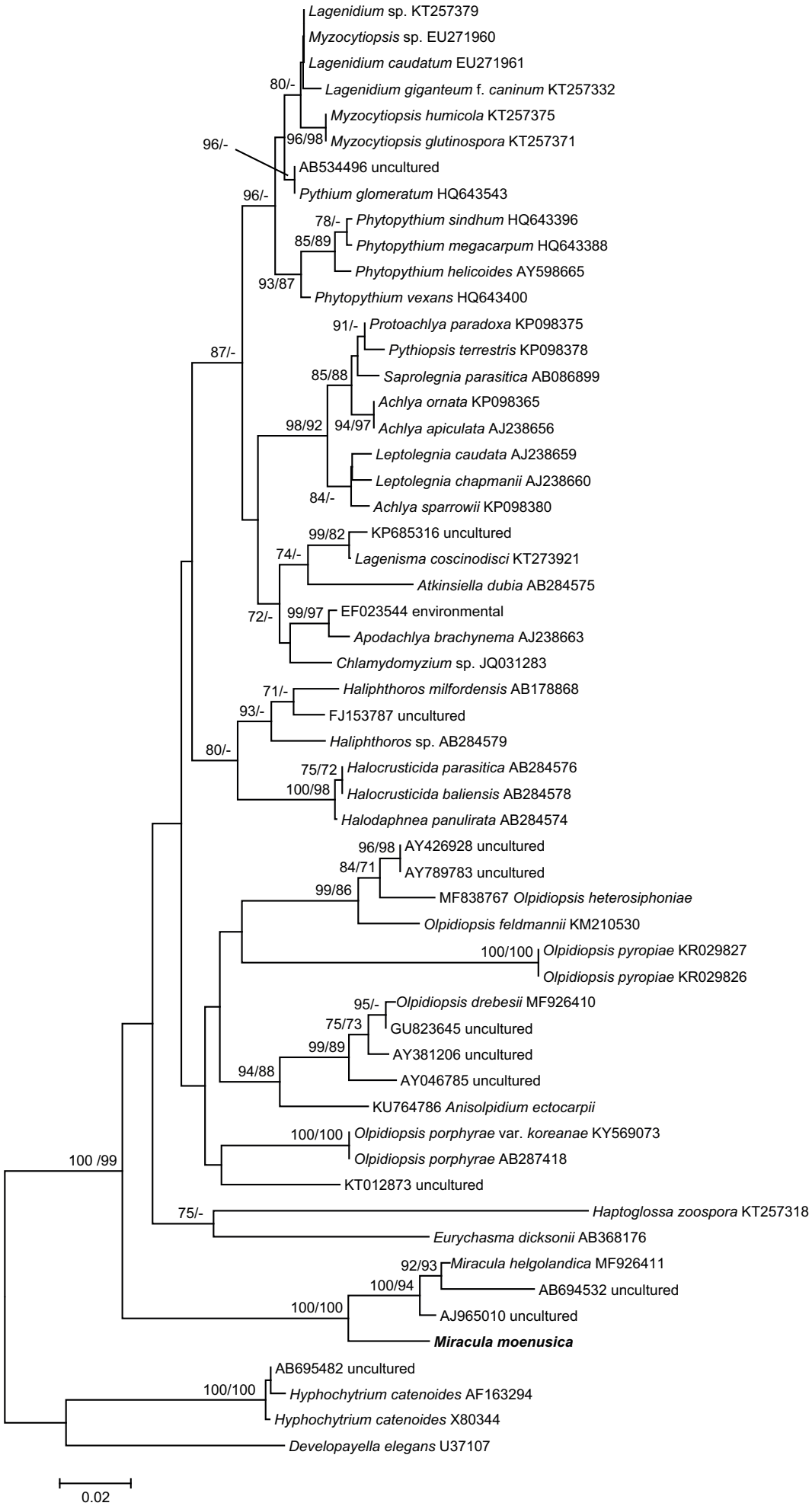
*Diagnosis*: Differs from *Miracula helgolandica* by its lemon-shaped maturing thallus, its more elongated discharge tube, its host in *Coscinodiscophyceae*, and its occurrence in a freshwater habitat.

*Description*: *Thallus* hyaline, normally one, rarely two to three, endobiotic in *Pleurosira leavis*, rod shaped when young, lemon-shaped during maturation, expanding until large parts of the host cell are filled, up to 100 µm long; *wall* thin, smooth, colourless; *zoospore cleavage* from a large central vacuole; *zoospores* roundish to grape-seed-shaped, 2–3 µm in diameter, beginning movement within the thallus; *exit tube* single, with a somewhat thickened base, 4–6 µm wide, 4–8 µm long.

*Typus*: **Germany**, Hessen, Frankfurt, northern bank, in *Coscinodiscophyceae* in freshwater, *leg*. *A. Buaya*, Sep. 2018 (**holotype** specimen in the Herbarium Senckenbergianum under the accession number FR0046007). Ex-type sequence deposited in GenBank under the accession number MK239934.

*Known distribution*: Germany, river Main.

**Fig. 1.** Micrographs (DIC) of various developmental stages of *Miracula moenusica*. **A.** Young, elongate thalli. **B.** Early limoniform stage. **C.** Late limoniform stage with intermediate thallus expansion. **D.** Fully expanded and empty thallus with several encysted zoospores that failed to escape inside. **E.** Discharge tube (arrow) developing from a thallus with maturing zoospores. Scale bars: 50 µm.



## DISCUSSION

While the past two decades have seen huge advances towards a natural system of the crown oomycetes, in particular for both obligate biotrophic plant pathogens (Constantinescu 1998, Constantinescu & Fatehi 2002, Göker *et al*. 2003, Voglmayr *et al.* 2004, Constantinescu *et al.* 2005, Thines & Spring 2005, Thines *et al.* 2006, 2007, 2015b, Voglmayr & Constantinescu 2008, Telle & Thines 2011) and cultivable *Peronosporomycetes* (Bala *et al*. 2010, Hulvey *et al*. 2010, Uzuhashi *et al*. 2010, Li *et al.* 2016, Bennett *et al.* 2017, Jung *et al.* 2017), many genera of the *Saprolegniomycetes* and even more that were assumed to belong to the early-diverging oomycetes have not been revised, so far (Beakes & Sekimoto 2009, Beakes *et al.* 2014, Beakes & Thines 2017). However, the finding that some oomycete lineages diverged before the two major classes (Hudspeth *et al*. 2003), has spurred some interest in holocarpic oomycetes and has revealed the genera *Haptoglossa* and *Eurychasma* as the earliest-diverging lineages (e.g. Sekimoto *et al*. 2008, 2009, Gachon *et al.* 2017), a placement that only recently has been contested by the holocarpic diatom parasitoid *Miracula helgolandica* (Buaya *et al*. 2017). *Miracula helgolandica* parasitises the filamentous diatoms of the genus *Pseudo-nitzschia* (Hanic *et al*. 2009, Buaya *et al.* 2017) and could not be assigned to any of the five holocarpic genera known to parasitize diatoms (*Aphanomycopsis*, *Ectrogella*, *Lagenidium*, *Lagenisma*, and *Olpidiopsis*), which was the reason the new genus *Miracula* had been introduced. Overall, the evolutionary diversity of oomycetes parasitising diatoms seems to be very high, as witnessed by the relatively many descriptions of such organisms in the second half of the 19th and the first half of the 20th century (Zopf 1884, Karling 1942, Sparrow 1960, Drebes 1968, and references therein). So far, sequence data are available only from *Lagenisma*, *Miracula*, and *Olpidiopsis* diatom parasitoids. For all these genera, sequences from environmental sequencing exist, suggesting the presence of additional species that await their discovery. The finding of a second member of the genus *Miracula* in this study supports this notion. That *Miracula moenusica* was found in a freshwater environment is another example of the wide ecological amplitude of water-borne oomycetes, in which the border between marine and freshwater environments has been crossed several times, e.g. in *Haptoglossa* (Beakes & Sekimoto 2009), *Phytopythium* (Thines 2014), and *Halophytophthora* (Yang & Hong 2014). *Miracula moenusica* bears some similarity to *Ectrogella monostoma* (Scherffel 1925, Sparrow 1960), in the central swellings of the thallus. However, the behaviour or the zoospores is rather olpidioid in the former species, as they swim away from the host after emergence, rather than encysting directly at the orifice for the formation of secondary zoospores in the latter species. As in addition to these differences, the host species, *Pleurosira leavis*, has not been reported as a host of oomycete parasitoids, it seems that the species has not been observed previously. The unexpected observation of a second species of *Miracula* in a freshwater diatom, as well as the recent finding of a marine *Olpidiopsis* species in *Rhizosolenia* diatoms highlights that the diversity of holocarpic oomycetes is largely uncharted and promises to hold additional surprises for the future.

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**Fig. 2.** Minimum Evolution (ME) phylogenetic reconstruction. Numbers at branches are bootstrap support values in ME and Maximum Likelihood analyses, respectively. A minus sign denotes support values below 70 % for the presented node or a conflicting topology. The scales bar indicates the number of substitutions per site. The new species introduced in this study is highlighted in bold.

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2. Sampling and experimentation

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3. Data analysis and interpretation

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# Holocarpic oomycete parasitoids of red algae are not *Olpidiopsis*

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**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*.

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## 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).

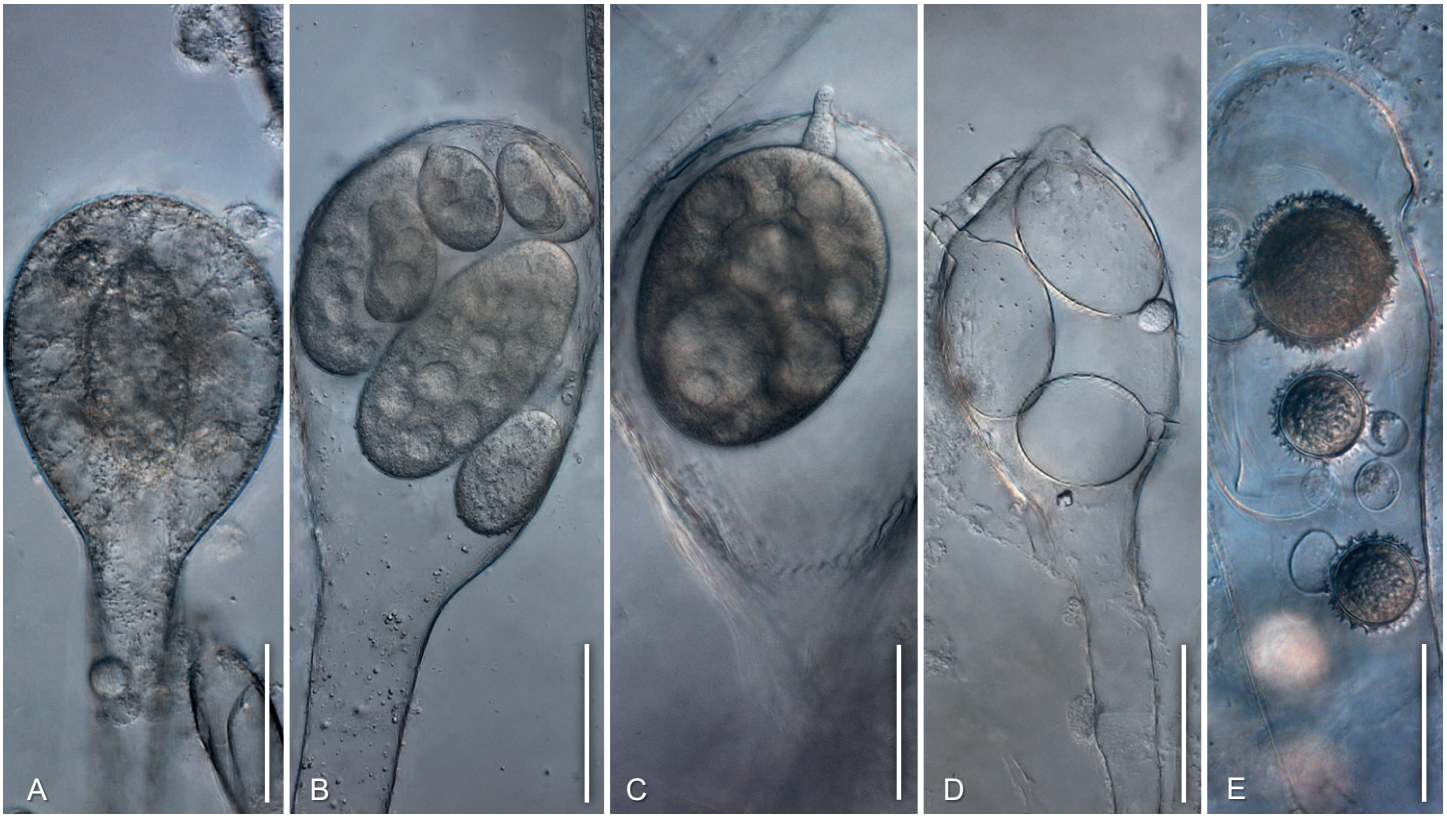


**Fig. 1.** DIC-light microscopy of *Olpidiopsis saprolegniae* at different life cycle stages on hypertrophied terminal hyphae of *Saprolegnia parasitica*. **A.** Single young thallus surrounded by a dense layer and radiating strands of host cytoplasm. **B.** Several asexual thalli, each with numerous 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.

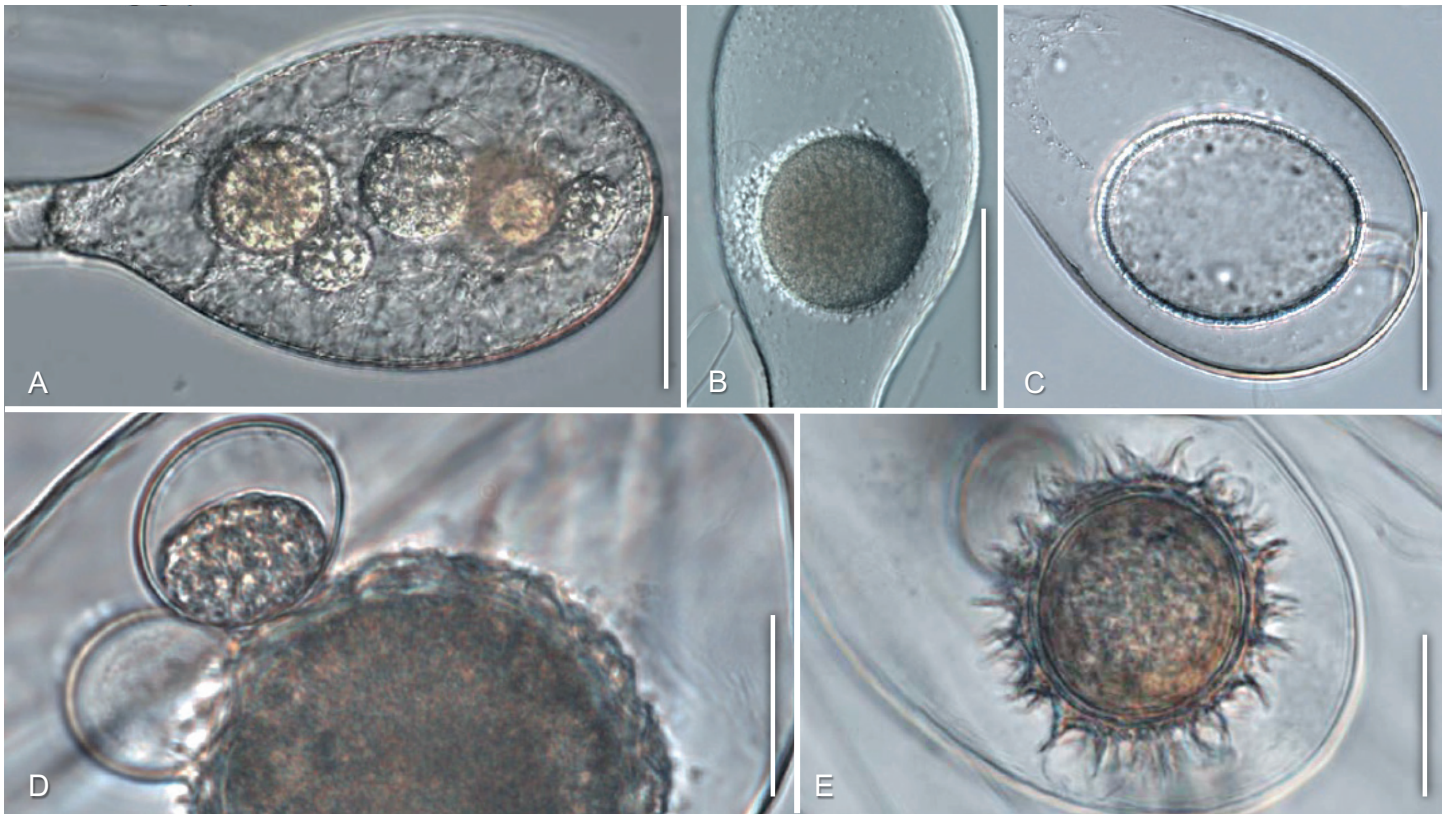


**Fig. 2.** Light microscopy of *Olpidiopsis saprolegniae s.lat*. at different life cycle stages on hypertrophied terminal hyphae of *Saprolegnia ferax*. **A.** Several young thalli with beginning differentiation into asexual and sexual thalli. **B.** Mature asexual thallus. **C.** Empty asexual thallus 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.

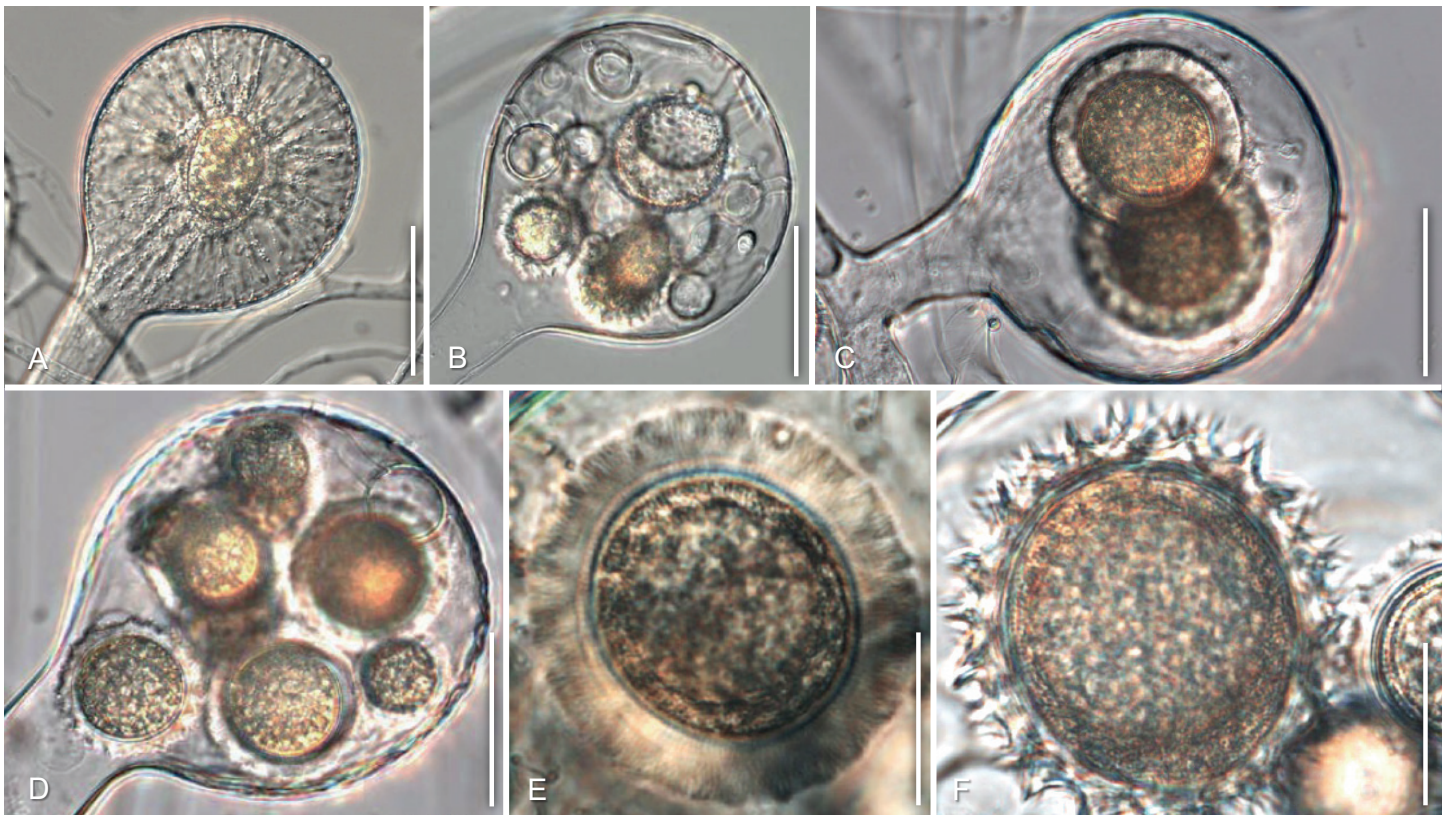


**Fig. 3.** Light microscopy of *Olpidiopsis* sp. at different life cycle stages on hypertrophied terminal hyphae of *Saprolegnia terrestris*. **A.** Single young thallus surrounded by a dense layer and radiating strands of host protoplasm. **B.** Sexual and asexual thalli at different developmental 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.

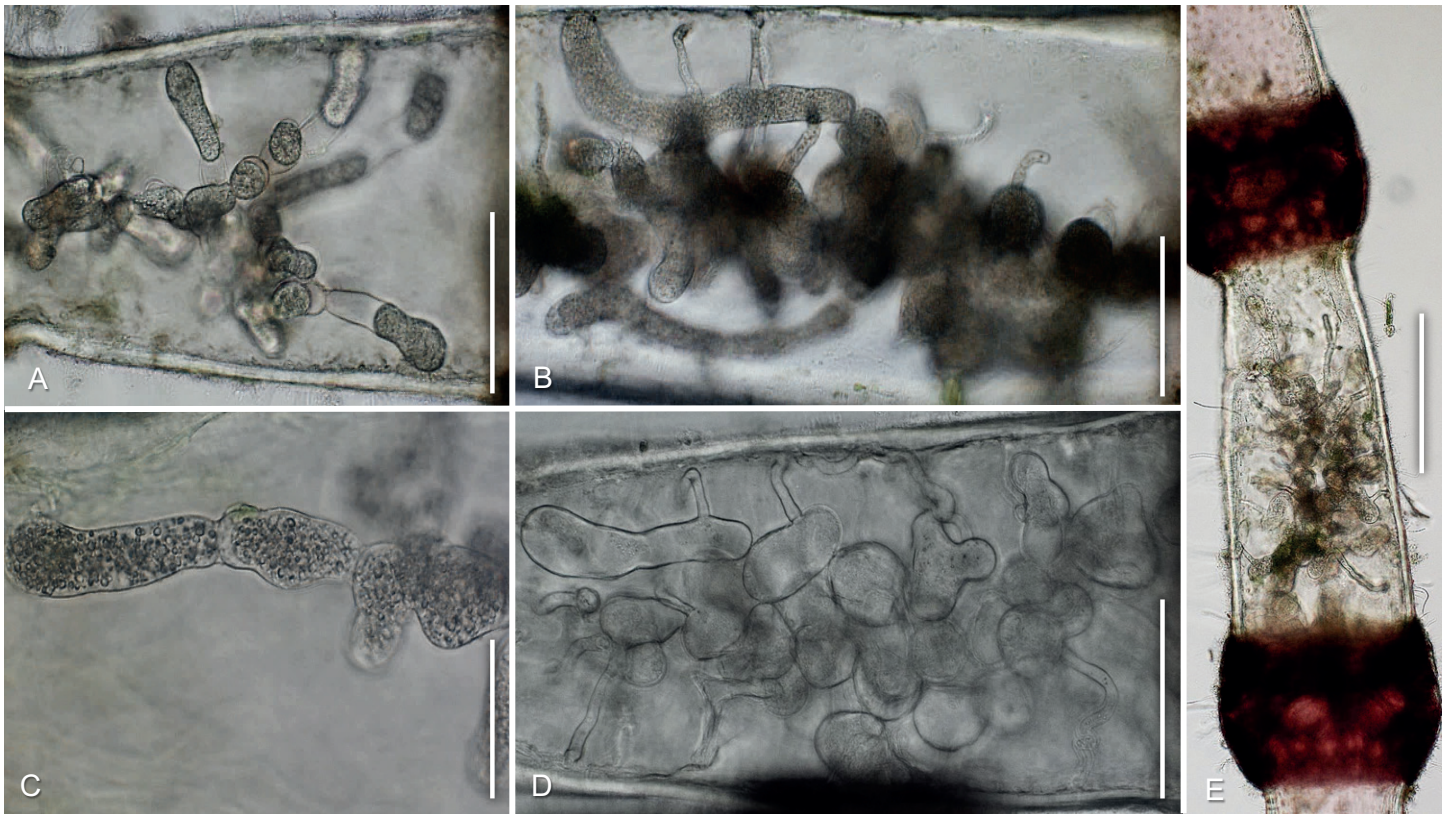


**Fig. 4.** DIC-light microscopy of *Pontisma lagenidioides* at different life cycle stages in *Ceramium rubrum*. **A.** Irregularly shaped, mature parasite thallus with multiple constrictions. **B.** Multiple mature tubular segments each with a developing single discharge tube. **C.** Segment containing 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).

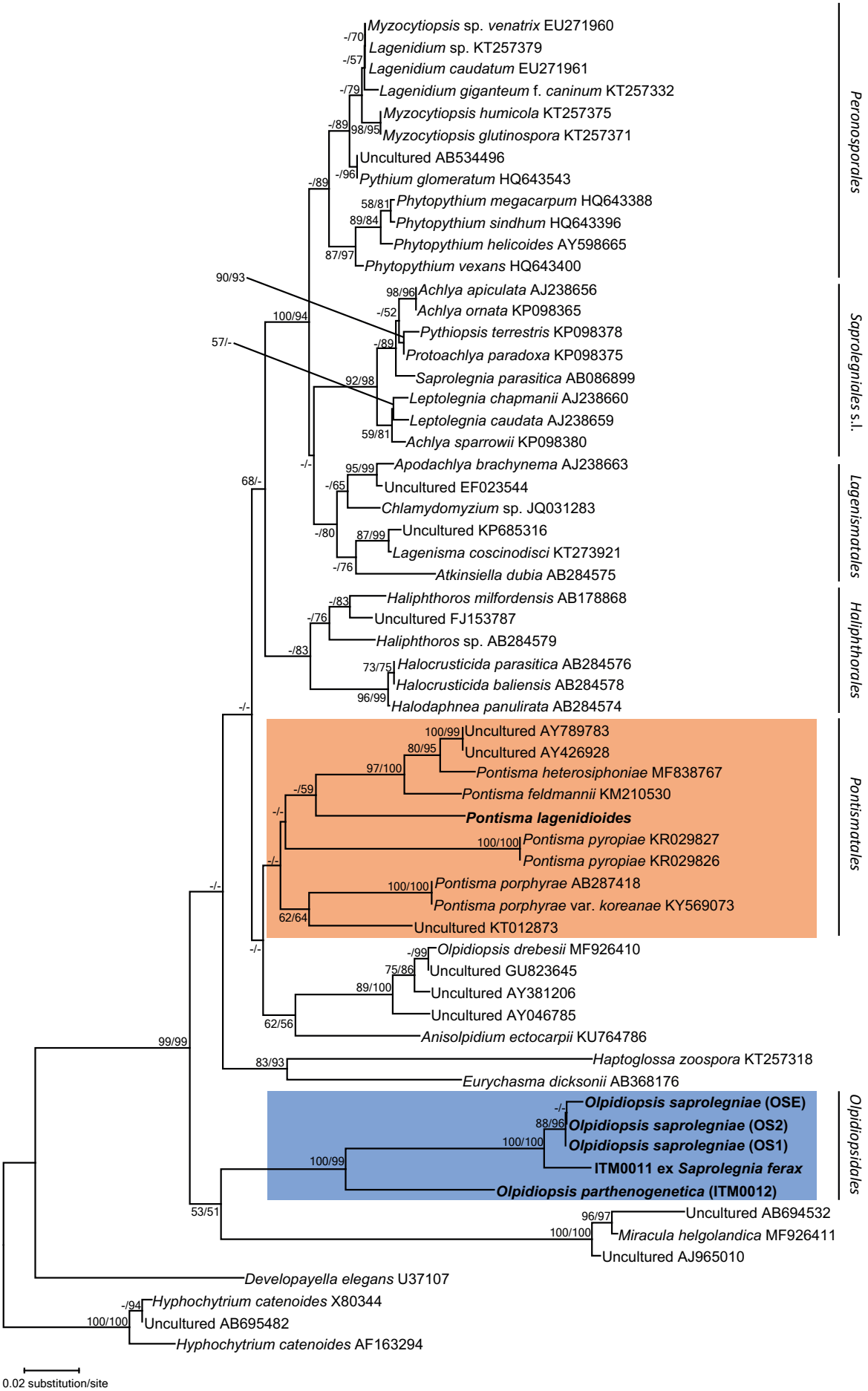


**Fig. 5.** Molecular phylogenetic reconstruction from Minimum Evolution analyses inferred from 18S rDNA sequences. Numbers on branches denote bootstrap values from maximum likelihood and minimum evolution analyses, in the respective order. A minus sign indicates less than 50 % bootstrap support.

***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).

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# *Diatomophthoraceae* – a new family of olpidiopsis-like diatom parasitoids largely unrelated to *Ectrogella*

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**Abstract:** The oomycete genus *Ectrogella* currently comprises a rather heterogeneous group of obligate endoparasitoids, mostly of diatoms and algae. Despite their widespread occurrence, little is known regarding the phylogenetic affinities of these bizarre organisms. Traditionally, the genus was included within the *Saprolegniales*, based on zoospore diplanetism and a saprolegnia/achlya-like zoospore discharge. The genus has undergone multiple re-definitions in the past, and has often been used largely indiscriminately for oomycetes forming sausage-like thalli in diatoms. While the phylogenetic affinity of the polyphyletic genus *Olpidiopsis* has recently been partially resolved, taxonomic placement of the genus *Ectrogella* remained unresolved, as no sequence data were available for species of this genus. In this study, we report the phylogenetic placement of *Ectrogella bacillariacearum* infecting the freshwater diatom *Nitzschia sigmoidea*. The phylogenetic reconstruction shows that *Ectrogella bacillariacearum* is grouped among the early diverging lineages of the *Saprolegniomycetes* with high support, and is unrelated to the monophyletic diatom-infecting olpidiopsis-like species. As these species are neither related to *Ectrogella*, nor to the early diverging lineages of *Olpidiopsis s. str*. and *Miracula*, they are placed in a new genus, *Diatomophthora*, in the present study.

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## INTRODUCTION

*Ectrogella bacillariacearum* (*Oomycetes*, *Saprolegniales*, *Ectrogellaceae*, *Ectrogella*) is an endobiotic, holocarpic, obligate parasite of freshwater pennate diatoms (Sparrow 1960). Described by Zopf in 1884, the parasite is the type species of its genus, and the genus is the type of the family *Ectrogellaceae*. Except for three green algal pathogens (*Ectrogella marina*, *E. lauderia*, and *E. dicksonii*) and one oomycete hyperparasite (*E. besseyi*), all species of the genus are obligate parasites of freshwater diatoms (*E. bacillariacearum*, *E. monostoma*, *E. gomphonematis*, *E. eunotiae*, *E. brachystoma*, *E. cyclotellae*) and marine diatoms (*E. licmophorae*, *E. perforans*, *E. eurychasmoides*) (Zopf 1884, Petersen 1905, Scherffel 1925, Sparrow & Ellison 1949, Friedmann 1952, Feldmann & Feldmann 1955, Dick 2001). The type species, *E. bacillariacearum* and other members of the genus in a strict sense have a saprolegnioid and achlyoid zoospore formation, *i.e*. they produce zoospores which exhibit diplanetism. This can be contrasted to the species with olpidiopsidoid or lagenidioid zoospore formation, *i.e.* members of the genera *Olpidiopsis* and *Lagenidium* (Sparrow 1960). The genus *Ectrogella* has faced different interpretations in the past, and in the latest taxonomic treatment of Dick (2001), it was used as a catch-all for simple holocarpic diatom parasites, irrespective of their mode of zoospore formation. The taxonomic placement of the genus *Ectrogella* in the *Saprolegniales* has been questioned (Beakes & Thines 2017), because of the absence of oospores and the placement of *Eurychasma*, which was assumed to be related to *Ectrogella* (Scherfel 1925, Sparrow 1960), and was found to be a very early-diverging lineage of the oomycetes (Sekimoto *et al*. 2008). To date, only five oomycete diatom parasitoids have been sequenced and included in the phylogeny of the *Oomycota* (Thines *et al.* 2015, Buaya *et al.* 2017, 2019a). Two of these were classified in the genus *Olpidiopsis* (*O. drebesii*, *O. gillii*) because of their placement within a monophyletic, yet unsupported *Olpidiopsidales* (Buaya *et al.* 2017). The parasite of some species of the centric diatom genus *Coscinodiscus*, *Lagenisma coscinodisci*, also previously speculated to represent an early-diverging lineage, was found to belong to the *Saprolegniomycetes*, a placement which is also supported by its diplanetism (Thines *et al.* 2015). In contrast, the olpidiopsidoid parasitoid of *Pseudo-nitzschia* spp. was initially suspected to be a member of either *Ectrogella* or *Olpidiopsis* (Hanic *et al.* 2009), but was found to be the earliest diverging oomycete lineage in Buaya *et al.* (2017) and consequently assigned to the new genus *Miracula* as *Miracula helgolandica*, to which a second, limnic species was recently added (Buaya & Thines 2019). A recent study (Buaya *et al.* 2019b) has shown that the genus *Olpidiopsis*, with its type species, *O. saprolegniae*, is largely unrelated to the diatom parasites currently placed in the genus, necessitating a taxonomic revision. However, the taxonomy of diatom-infecting oomycetes of the genera *Ectrogella*, *Olpidiopsis*, *Lagenidium*, and *Aphanomycopsis* is still uncertain, as no sequence data have been available for the type of *Ectrogella*, *E. bacillariacearum*.

As a consequence, interpretations regarding the relatedness and taxonomy of these species have been largely based on their original descriptions made during the late 18th until the early 19th centuries (*e.g*. Cornu 1872, Zopf 1884, Petersen 1905, Scherffel 1925). Also, the few ultrastructural studies on *E. perforans*, *L. coscinodisci*, and *M. helgolandica* (Schnepf *et al.* 1978a, b, Raghu Kumar 1980, Hanic *et al.* 2009), are rather singular and, thus, while yielding some interesting insights into the cytology of basal oomycetes, they did not provide a basis for taxonomic revision. To clarify the taxonomy of the diatom-infecting parasitoids, which are important for understanding the evolution of holocarpic oomycetes (Beakes & Sekimoto 2009), attempts were made to sample *E. bacillariacearum*. Its presence in the river Main, a tributary to the western European stream Rhine, was monitored in Frankfurt am Main from the autumn of 2017 onward. In autumn of 2018, *E. bacillariacearum* was observed occurring in parallel to the bloom of its pennate diatom host *Nitzschia sigmoidea,* enabling the phylogenetic investigation of the parasitoid, the clarification of its relationship to the diatom-infecting species of *Olpidiopsis*. This also opened the possibility for a taxonomic revision of the diatom-infecting genus *Ectrogella*, which was the aim of the current study.

## MATERIALS AND METHODS

### Sampling, isolation, and microscopy

Diatom samples were collected from the River Main, Frankfurt am Main, Germany (N50°06.195', E008°40.323') as described previously (Buaya & Thines 2019). Approximately 10 mL of biofilm suspension was poured into each of several 15 mL Petri-dishes, and screened for infected diatoms using a compound inverted light microscope (AE31, Motic, USA). *Ectrogella bacillariacearum* infecting *Nitzschia sigmoidea* was observed and collected between Sep. and Nov. 2018. Parasitised diatoms were individually picked using a 10 µL micropipette (Braun, Germany), and rinsed by transfer through a series of droplets of sterile distilled water to remove attached debris from the frustule and subsequent immersion in 250 µL of RNA*later* (Invitrogen, Thermo Fisher, Lithuania) for DNA extraction or into 5 µL molecular grade water (Life Technologies, USA) for direct PCR. Approximately 30 infected cells were collected per 2 mL tube (Sarstedt, Germany) for extraction, and 10 cells per 200 µL PCR tube (Sarstedt, Germany) for direct PCR. For morphological characterisation and DIC micrographs of life cycle stages, infected cells were also mounted onto glass slides using sterile distilled water. Microscopy was done using a compound light microscope (Imager2, Carl Zeiss, Göttingen, Germany) equipped with a Zeiss Axiocam MRc5 (Carl Zeiss, Göttingen, Germany). Infected cells preserved in 70 % ethanol were deposited in the herbarium collection of the Senckenberg Museum of Natural History, Frankfurt am Main (accession number: FR-0046108).

### DNA extraction, PCR and molecular phylogeny

Infected diatom samples were centrifuged at 19 000 *g* for 1 min to pellet the cells. Subsequently, RNA*later* was carefully removed by pipetting and 400 µL SLS buffer of the innuPREP Plant DNA Kit (Analytik Jena AG, Germany) was added. Samples mixed with 100 mg of sterile 0.1 mm silica glass beads (Carl Roth GmbH, Germany) were homogenised at 25 Hz for 25 min in a Retsch Mixer Mill MM 200 (Retsch GmbH, Germany). Extraction of DNA was carried using the innuPREP Plant DNA Kit, as described in the protocol provided in the kit. PCR for the amplification of partial nuclear ribosomal small subunit (18S) and sequencing was performed as described in Buaya *et al.* (2017). PCR amplicons were sent for sequencing to the laboratory centre of the Senckenberg Biodiversity and Climate Research Centre (Frankfurt am Main, Germany), with the primers used in PCR. The partial 18S (nrSSU) sequence obtained in this study was deposited in GenBank (accession number: MK253531). Alignments based on the dataset of Buaya *et al*. (2019b) with the addition of the newly obtained sequence were done using the Q-INS-i algorithm of MAFFT (Katoh & Stadley 2013) on the TrEase webserver (http://thines-lab.senckenberg.de/trease/). Minimum Evolution phylogenetic inference was done using MEGA v. 6.0 (Tamura *et al.* 2013) as described in Buaya *et al.* (2017), and Maximum Likelihood inference using RAxML version 8, (Stamatakis 2014) with the GTRGAMMA model and running 1 000 bootstrap replicates.

## RESULTS

### General results and morphology

During autumn of 2018, biofilm samples containing abundant phytoplankton were collected at the river Main, Frankfurt am Main, Germany. During a careful screening for the presence of diatom-infecting oomycetes, about 5 % of *Nitzschia sigmoidea* agg. were observed to be infected by *Ectrogella bacillariacearum*. Infections were also noted on a few S*ynedra* species at very low incidence, so they could not be included in the phylogenetic analyses. Other species of pennate diatom genera (*e.g. Pinnularia, Meridion, Licmophora, Eunotia*), which are also reported as hosts for *E. bacillariacearum* (Karling 1942, Sparrow 1960), were co-occurring with infected individuals of *N. sigmoidea*, but none were observed to be infected during the entire sampling period. Light microscopic examination of the isolated specimens revealed that, as the thallus matures, the host chloroplasts begin to lose their normal colouration and gradually disintegrate. Usually, one thallus was present per host cell (Fig. 1C, E), but multiple infections, resulting in multiple thalli per host cell were also observed (Fig. 1A, B, D). Upon maturity, thalli normally measured 200 µm or more in length when single, with a smooth, very thin, colourless wall. The unbranched, fusiform to tubular thallus undergoes rapid development and subsequent zoosporogenesis. Mature thalli develop multiple discharge tubes predominantly at the apices of the host cell. Discharge tubes protrude at the girdle band and are short, often with a thickened base (Fig. 1F, G). The pyriform primary zoospores become briefly motile within the sporangium following zoospore cleavage. Zoospores are about 4 µm long and 2 µm broad, with two short, laterally inserted flagella. Zoospore discharge is fast, taking only a few seconds. Zoospores undergo encystment shorty after release, usually near the mouth of the discharge tube, or sometimes a few trapped spores encyst inside the sporangium. After some rest, ovoid secondary zoospores escape from the cysts, which have laterally inserted, unequal flagella, and swim with a dashing motion, frequently changing direction. No resting spores were observed.

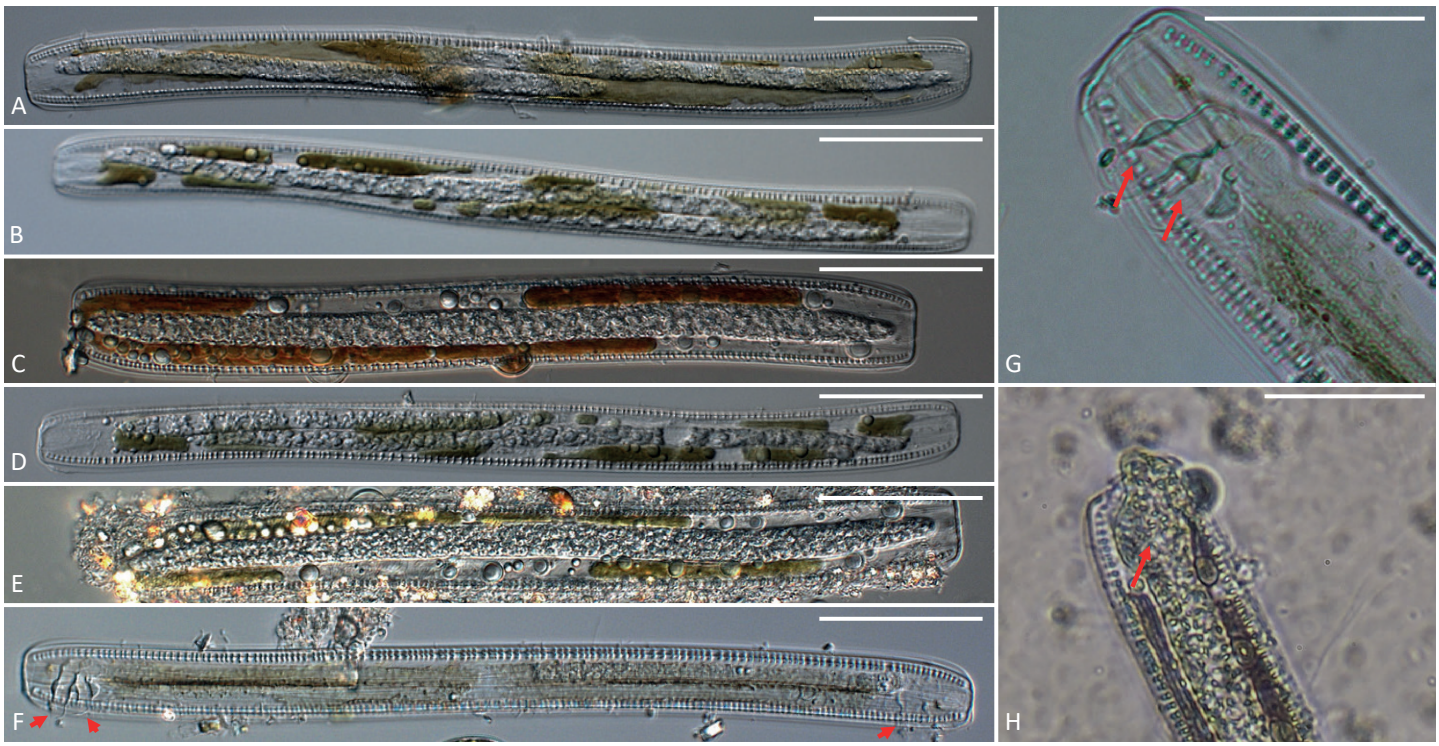


**Fig. 1.** Light micrographs of *Ectrogella bacillariacearum*, at different developmental stages on *Nitzschia sigmoidea*. **A.** Early infection of *N. sigmoidea* cell, with two developing endobiotic thalli. **B.** Two mature sporangia with emerging multiple vacuoles. **C.** Zoospore differentiation following centrifugal cleavage. **D, E.** Zoospore maturation in host with multiple (**D**) and single infection (**E**). **F.** Empty zoosporangium with multiple discharge tubes on the opposite apices of *Nitzschia* girdle bands. **G.** Empty zoosporangium with two discharge tubes (red arrows). **H.** Developing discharge tube (red arrow). Scale bars: A–F = 50 µm; G, H = 20 µm.

## Molecular phylogeny

In phylogenetic reconstructions inferred from partial 18S sequences of *E. bacillariacearum* on *N. sigmoidea* (Fig. 2), the parasitoid clustered in a well-supported clade with two marine parasites, *Atkinsella dubia* (crustacean parasite) and *Lagenisma coscinodisci* (*Coscinodiscus* parasite), the freshwater oomycetes, *Apodachlya brachynema* (saprophyte) and *Chlamydomyzium sp.* (rhabditid nematode parasite), as well as two environmental sequences. Other sequenced oomycete diatom parasitoids, classified in the genus *Olpidiopsis* (*O. drebesii*, *O. gillii*) and genus *Miracula* (*M. helgolandica*, *M. moenusica*) were forming earlier-diverging lineages, diverging before the split of the two major oomycete lineages, the *Peronosporomycetes* and the *Saprolegniomycetes*.

## Taxonomy

***Diatomophthoraceae*** A.T. Buaya & Thines, ***fam. nov.*** MycoBank MB831325.

Obligate parasitic in diatoms, thallus endobiotic, holocarpic, thin-walled at maturity; discharge tube usually single, without basal thickening or with a slightly thickened base; zoospores numerous, without clear-cut diplanetism; resting spores not known.

*Type genus*: *Diatomophthora* A.T. Buaya & Thines

***Diatomophthora*** A.T. Buaya & Thines, ***gen. nov.*** MycoBank MB831326.

*Etymology*: *Diatomophthora* refers to the known host range of the genus and its destructive effect on host populations.

Obligate parasitic in diatoms; thallus endobiotic, holocarpic, broadly tubular, fusiform, ellipsoidal or spherical, colourless, thin-walled at maturity, often pushing apart the host valves, sometimes with equatorial swelling; discharge tubes often single, mostly elongating, tubular to slightly tapering, without a strongly thickened base; zoospores escaping after the dissolution of the tip of the discharge tube, numerous, moving or swarming within the thallus prior to release, without clear-cut diplanetism; resting spores not observed.

*Type species*: *Diatomophthora drebesii* (A.T. Buaya & Thines) A.T. Buaya & Thines

***Diatomophthora drebesii*** (A.T. Buaya & Thines) A.T. Buaya & Thines, ***comb. nov.*** MycoBank MB831327.
*Basionym*: *Olpidiopsis drebesii* A.T. Buaya & Thines, *Mycol. Prog.* **16**: 1048. 2017.

*Typus*: **Germany**, Helgoland Roads, 29 Jun. 2017, *A.T. Buaya* (**holotype** FR-0247058). Ex-type partial nrSSU sequence MF926410.

***Diatomophthora gillii*** (de Wild.) A.T. Buaya & Thines, ***comb. nov.*** MycoBank MB831328.
*Basionym*: *Olpidium gillii* De Wild., *Ann. Soc. Belge Microscop.* **20**: 41. 1896.

*Typus*: *J. Royal. Microsc. Soc. (London)* **1893, part 1**, plate I, fig. 3, *H. Gill* (**lectotype designated here** from the figures cited in the description of the species by De Wildeman, MBT388917).

*Epitype*: **Germany**, Hessen, Frankfurt am Main, river Main, *A.T. Buaya,* 2017, deposited in 70 % ethanol in the Herbarium Senckenbergianum (FR-0046005, **epitype designated here**, MBT387362). GenBank MH971238 (ex-epitype, partial nrSSU).

## DISCUSSION

Despite their widespread occurrence and recent efforts by researchers, holocarpic parasitoids of diatoms are poorly studied compared to other biotrophic oomycetes (Thines *et al.* 2015, Scholz *et al.* 2016, Buaya *et al.* 2017, 2019c, Buaya & Thines 2019). In fact, the present understanding of these inconspicuous parasitoids is still fundamentally based on descriptions made almost a century ago, and several species have only been observed once or a few times since their discovery. Also, the taxonomic affinity of several species and genera is still unresolved because most have not yet been included in molecular phylogenies (Beakes & Thines 2017, Buaya *et al.* 2019a). To date, the majority of the diatom-parasitic oomycetes included in molecular phylogenetic investigations or studied for cellular ultrastructure are from marine environments (Schnepf *et al.* 1978a, b, Chakravarty 1978, Raghu Kumar 1980, Hanic *et al.* 2009, Thines *et al.* 2015, Buaya *et al.* 2017). So far, only two diatom infecting species from freshwater, *Diatomophthora gillii* and *Miracula moenusica*, have been investigated for their molecular phylogenetic affinities (Buaya & Thines 2019, Buaya *et al.* 2019a). Scherffel (1925), Karling (1942), Sparrow (1960), and Dick (2001) all agree with the placement of *Ectrogella* in the *Saprolegniales* (*Ectrogellaceae*), in line with the current study. However, because of heterogeneity in zoospore size, shape and formation, assessment of the delimitation of *Ectrogella* was variable, leading to several taxonomic revisions over time and several attempts have been made to restructure the holocarpic oomycetes, sometimes by describing new genera (Karling 1942, Cejp 1959, Sparrow 1960, Dick 2001). However, zoospore formation and thallus development might differ, depending on physiochemical properties, similar to the situation found in some terrestrial pathogens (Runge *et al.* 2012).

According to Dick (2001), the genus *Ectrogella* contains 13 species (*E. bacillariacearum*, *E. besseyi*, *E. brachystoma*, *E. cyclotellae*, *E. dicksonii*, *E. eunotiae*, *E. eurychasmoides*, *E. gomphonematis*, *E. lauderiae*, *E. licmophorae*, *E. marina*, *E. monostoma*, *E. perforans*), all forming single-celled, unbranched, endobiotic thalli, mostly producing zoospores with diplanetism, which we assume as the key diagnostic feature of the genus (Zopf 1884, Petersen 1905, Scherffel 1925, Sparrow & Ellison 1949, Friedmann 1952, Feldmann & Feldmann 1955, Dick 2001). The majority of the species in the genus *sensu* Dick (2001) are parasitoids of diatoms (*E. bacillariacearum*, *E. monostoma*, *E. gomphonematis*, *E. eunotiae*, *E. brachystoma*, *E. cyclotellae*, *E. licmophorae*, *E. perforans*, *E. eurychasmoides*), others parasitise algae (*E. marina*, *E. lauderia*, *E. dicksonii*) and one is an endobiotic oomycete hyperparasite (*E. besseyi*). Within the group, zoospore morphology, development and movement, as well as discharge pattern, differ. For example, in *E. perforans* zoospores swarm within the sporangia prior to discharge (Petersen 1905), while in *e.g*. *E. monostoma*, non-motile spores are discharged. A similar situation was described for *E. besseyi*, which, unlike *E. bacillariacearum*, also produces non-flagellated primary aplanospores, encysting at the orifice of the discharge tube and forming a cluster of spores similar to *Achlya*. After encystment, they germinate, producing secondary zoospores (Scherffel 1925). Also, the normal number of exit tubes varies for several species within the genus. For example, *E. bacillariacearum*, *E. licmophorae* and *E. perforans* have multiple exit tubes, while *E. monostoma*, *E. gomphonematis*, *E. eunotiae*, *E. marina*, *E. besseyi* and *E. eurychasmoides* produce only one or two (Zopf 1884, Petersen 1905, Scherffel 1925, Friedmann 1952, Feldmann & Feldmann 1955). Additional species were added to the genus, some with incomplete life-cycle descriptions and unclear zoospore diplanetism, *e.g. E. brachystoma*, *E. cyclotellae*, *E. dicksonii, E. eunotiae*, *E. eurychasmoides*, *E. lauderiae,* and *E. marina*, *E. perforans* (Sparrow 1960, Dick 2001). It has been speculated that *Ectrogella* belongs to the basal oomycetes (Garvetto *et al.* 2018), but the phylogenetic reconstructions of this study places *Ectrogella* among the early-diverging *Saprolegniomycetes* to which also another diatom parasitoid, *Lagenisma coscinodisci*, belongs. Therefore, *Ectrogella* is unrelated to the diatom parasitoids previously in *Olpidiopsis*, which are placed in a new genus, *Diatomophthora*, in this study. The inclusion of the *Ectrogellaceae* into the deep-branching *Saprolegniales* is in line with the formation of zoospores with diplanetism, and confirms earlier treatments of *Ectrogella* in the *Saprolegniales* (Scherffel 1925, Coker & Mathews 1937, Karling 1942, Sparrow 1960, Dick 2001). As this phylogenetic and morphological study further confirms the importance of zoospore development for evaluating the taxonomy of oomycetes, only those species with a clear-cut diplanetism should be attributed to the *Saprolegiomycetes*, while the species that produce monomorphic and monoplanetic zoospores are unlikely to belong to *Ectrogella* or even to the *Saprolegiomycetes*, and should be carefully scrutinised to infer their phylogenetic position. Whether the sole endobiotic hyperparasite in the genus *Ectrogella*, *E. besseyi*, is a *bona fide* member of the genus remains to be shown, but it also has an achlya-like pattern of zoospores discharge similar to other diatom infecting species (Sparrow & Ellison 1949). Until more data become available for these elusive pathogens, it remains unclear, if the different modes of zoospore discharge by *Ectrogella* species (*i.e.* saprolegnia-like *vs*. achlya-like) have phylogenetic significance.

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**Fig. 2.** Molecular phylogeny using minimum evolution analyses inferred from partial 18S sequences. Numbers on branches denote bootstrap values from maximum likelihood and minimum evolution analyses, in respective order. A dash "-" indicates less than 50 % bootstrap support for the presented or a conflicting topology.

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## STATEMENT OF ORIGINAL AUTHORSHIP

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This thesis has also not been previously submitted for a degree or diploma in any other higher academic institution. To the best of my knowledge, this thesis contains no material previously published or written by any other person.

Student signature & date: 09.02.20 Place: Frankfurt am Main