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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.

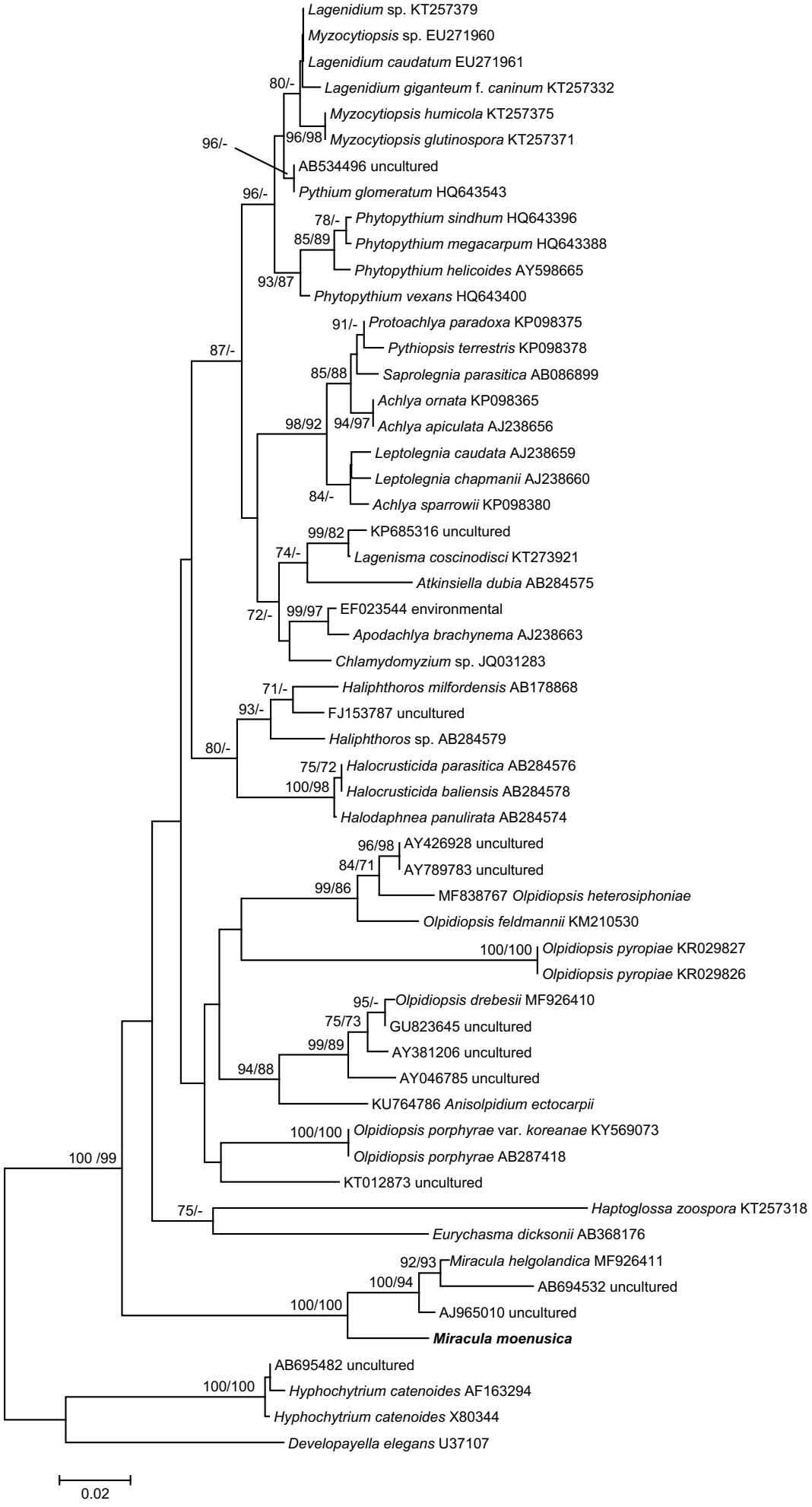
Diagnosis: Differs from *Miracula helgolandica* by its lemon-shaped maturing thallus, its more elongated discharge tube, its host in *Coscinodiscophyceae*, and its occurrence in a freshwater habitat.

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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