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Bolbea parasitica gen. et sp. nov., a cultivable holocarpic parasitoid of the early-diverging *Saprolegniomycetes*

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Abstract: Holocarpic oomycetes convert their entire cytoplasm into zoospores and thus do not form dedicated sporangia or hyphal compartments for asexual reproduction. The majority of holocarpic oomycetes are obligate parasites and parasitoids of a diverse suite of organisms, among them green and red algae, brown seaweeds, diatoms, fungi, oomycetes and invertebrates. Most of them are found among the early diverging oomycetes or the *Peronosporomycetes*, and some in the early-diverging *Saprolegniomycetes* (*Leptomitales*). The obligate parasitism renders it difficult to study some of these organisms. Only a few members of the genus *Haliphthoros* s. l. have been cultured without their hosts, and of the parasitoid *Leptomitales*, some transient cultures have been established, which are difficult to maintain. Here, the cultivation of a new holocarpic oomycete genus of the *Leptomitales*, *Bolbea*, is presented. *Bolbea* is parasitic to ostracods, is readily cultivable on malt extract agar, and upon contact with water converts its cytoplasm into zoospores. Its morphology and phylogenetic relationships are reported. Due to the ease of cultivation and the ready triggering of zoospore development, similar to some lagenidiaceous oomycetes, the species could be a promising model to study sporulation processes in detail.

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INTRODUCTION

Holocarpic oomycetes convert their entire cytoplasm into zoospores during sporulation (Sparrow 1960, Dick 2001). This mode of asexual reproduction is found among the early-diverging lineages (Beakes & Sekimoto 2009), as well as in the lagenidiaceous *Peronosporomycetes* and the early-diverging *Saprolegniomycetes* (Beakes & Thines 2017). The early-diverging *Saprolegniomycetes* (*Leptomitales*) are probably primarily holocarpic, as the extended hyphal network present in the *Haliphthorales* is holocarpic (Chukanhom *et al.* 2003) as well. However, the holocarpy of the lagenidiaceous *Peronosporomycetes* is probably secondary in nature, as the eucarpic *Albuginales* (Spies *et al.* 2016) and the eucarpic *Rhipidiales* (Bennett & Thines 2018) branch below the holocarpic *Peronosporales*. Alternatively, it would have to be assumed that the eucarpic nature has evolved in these groups independently from *Peronosporales*. While the lagenidiaceous *Peronosporomycetes* have received considerable attention in the past decades (Golkar *et al.* 1993, Glockling & Dick 1997, Blackwell 2011, Mendoza *et al.* 2016, Spies *et al.* 2016), and several species were successfully cultivated, the primarily holocarpic oomycetes have received much less research effort.

The primarily holocarpic oomycetes have been extensively studied in the last quarter of the 19th century and the first two thirds of the 20th century (Schenk 1859, Cornu 1872, Zopf 1884, Maurizio 1895, de Wildeman 1896, Petersen 1905, Magnus 1905, Barrett 1912, Coker 1923, Scherffel 1925, Tokunaga 1933, Couch 1935, Sparrow 1934, 1936, 1950, 1960, Shanor 1939, McLarty 1941, Whiffen 1942, Karling 1942, 1944, Canter 1949, Sparrow & Ellison 1949, Friedmann 1952, Aleem 1952, Kobayashi & Ookubo 1953, Vishniac 1955, Miller 1962, Johnson 1966, Drebes 1968). Since then, the interest in these organisms has declined and only a few articles on their ecology, evolution, and taxonomy were published until the second decade of the 21st century (*e.g.* Müller *et al.* 1999, Dick 2001, Chukanhom *et al.* 2003, Sekimoto *et al.* 2008a, b, Beakes & Sekimoto 2009, Gachon *et al.* 2009, Sekimoto *et al.* 2009, Hanic *et al.* 2009). Interest in these organisms resurged when it was realised that while only few species have a severe adverse effect on economy, their ecological importance in natural ecosystems has been largely underestimated (Gachon *et al.* 2010, Beakes *et al.* 2012, Fletcher *et al.* 2015, Scholz *et al.* 2016, Hassett *et al.* 2019, Garvetto *et al.* 2019). Despite some earlier notions (*e.g.* Canter & Heaney 1984, Müller *et al.* 1999), the key publications raising new interest in the neglected group

were those that reported the finding that algal parasites were the earliest-diverging oomycete lineages (Küpper *et al.* 2006, Sekimoto *et al.* 2008a) and that a pathogen of the toxic diatom *Pseudo-nitzschia pungens* was abundant and thus a potential regulator of harmful algal blooms (Hanic *et al.* 2009). Since then, efforts have been taken to rediscover the holocarpic parasites of brown algae (Tsirigoti *et al.* 2013, Strittmatter *et al.* 2016, Gachon *et al.* 2017), red algae (Sekimoto *et al.* 2008b, 2009, Fletcher *et al.* 2015, Klochkova *et al.* 2016, 2017, Kwak *et al.* 2017, Badis *et al.* 2018, Buaya *et al.* 2019c), diatoms (Thines *et al.* 2015, Scholz *et al.* 2016, Buaya *et al.* 2017, 2019a, d, Garvetto *et al.* 2018, Buaya & Thines 2019b, 2020, Garvetto *et al.* 2019), invertebrates (Muraosa *et al.* 2009, Beakes *et al.* 2012, Molloy *et al.* 2014, Lee *et al.*, 2017, Mendoza *et al.* 2018) and oomycetes (Buaya *et al.* 2019c). With the exception of the *Haliphthorales* (Vishniac 1958, Dick 2001, Sekimoto *et al.* 2007, Beakes & Thines 2017) most primarily holocarpic pathogens seem to be obligate biotrophic and, thus, unable to live apart from a living host. Even though successful cultivation has been reported for *Chlamydomyrium* (Glockling & Beakes 2006) and *Crypticola* (Frances 1991), it seems to be difficult to cultivate the holocarpic pathogens of pathogenic *Leptomitales* over long periods of time. This renders detailed studies regarding the physiology, genetics and behaviour very difficult, and apart from *Lagenisma coscinodisci* (Drebes 1966, Schnepf & Drebes 1977, Schnepf *et al.* 1978a, b, c, Thines *et al.* 2015, Buaya *et al.* 2019d, Vallet *et al.* 2019) and *Diatomophthora perforans* (Buaya & Thines 2020) no host/pathogen co-culture of this group seems to have been maintained over several years.

During a survey for limnic holocarpic oomycetes, infested ostracods were found in a pond in Frankfurt am Main, Germany. Due to their extensive growth after the death of the host, cultivation of the pathogen was attempted and successfully achieved. Here, its morphology, life-cycle stages, and phylogeny are reported, and a new genus and species, *Bolbea parasitica*, is described to accommodate the organism. Due to the ready induction of sporulation upon contact with water, *Bolbea parasitica* might be a well-suited organism to study oomycete sporulation.

MATERIALS AND METHODS

Material acquisition

Limnic invertebrates were caught in the Rebstock lake in Frankfurt am Main, Germany, using a plankton net with 20 µm mesh size (Hydrobios, Kiel), by throwing the net from the lake shore into the water, about 5 m away, and towing the net slowly to the shore. Samples were put into 500 mL plastic bottles filled half with lake water. Samples were poured into 12 cm Petri dishes and screened using a compound inverted light microscope (Type AE31, Motic, Xiamen). Infested ostracods were removed from the samples using 10 µL micropipettes (Brandt, Wertheim) and transferred to a new Petri dish containing sterile water. Pictures of oomycete-infested animals were taken as described for diatoms (Buaya *et al.* 2019a). For DNA extraction, around 20 infected ostracods were collected and preserved in 0.5 mL RNA*later* (Invitrogen, Thermo Fisher, Lithuania) for subsequent DNA extraction as described previously (Buaya & Thines 2020). Samples preserved in 70 % ethanol were deposited in herbarium collection of Senckenberg Museum of Natural History, Frankfurt am Main (accession number: FR-0046116).

DNA extraction, PCR and sequencing

DNA, PCR and sequencing were carried out as described in Buaya *et al.* (2017) and Buaya & Thines (2020). In short, DNA was extracted from infested animals and mycelium cultivated on modified malt extract agar (MEA) (Carl Roth GmbH, Karlsruhe) supplemented with yeast extract (Carl Roth GmbH, Karlsruhe) (malt extract 12 g/L, yeast extracts 5 g/L, bacteriological agar 15 g/L), using a commercial kit (innuPREP Plant DNA Kit) according to the instructions of the manufacturer (Analytik Jena AG, Germany). Sequencing was done by the laboratory centre of the Senckenberg Biodiversity and Climate Research Centre using the primers employed in PCR.

Alignment and phylogenetic analyses

Sequences obtained for the ostracod parasite were identical in sequence, thus, only one was added to the dataset of (Buaya & Thines 2020). The sequence obtained from the ex-type culture was deposited in GenBank under the accession number (MN688695). Sequences were aligned using MAFFT v. 7 (Katoh & Standley 2013), employing the G-INS-i algorithm. Phylogenetic analyses were done using MEGA v. 5.0 (Tamura *et al.* 2011) for Minimum Evolution (ME) and using RAxML, v. 8 (Stamatakis 2014), as implemented on the TrEase webserver (thines-lab.senckenberg.de/trease) for Maximum Likelihood (ML) analysis. For ME default settings were chosen except for using the Tamura-Nei substitution model, which is the most complex standard model available in MEGA, running 1 000 bootstrap replicates and considering pairwise deletions. For ML analyses, the GTRGAMMA model was used and 1 000 bootstrap replicates were performed.

RESULTS

Morphology and culture

The parasitoid started its development in the body cavity and upon maturation consumed the infested ostracods completely (Fig. 1A, B). Irregularly bent hyphae then extended beyond the infected individuals (Fig. 1B, C) and in older ones, a fragmentation of the cytoplasm could be observed (Fig. 1C). In contact with water, the hyphal cytoplasm converted entirely into zoospores (Fig. 1C–E), which were released through short, wide discharge openings mostly forming at the apices of hyphae (Fig. 1F).

The oomycete grew readily at 12–16 °C when transferred to 1 % MEA supplemented with yeast extract (Fig. 2A, B). In culture it formed mycelium both on the surface and within the agar, with hyphae showing an undulating growth pattern (Fig. 2C). Hyphae increased in diameter to a certain degree during maturation, and vacuolisation increased. After about 2 wk of culture, radial growth slowed down until coming to an almost complete stop. After cutting agar plugs with hyphal tips and transferring them to new media plates, hyphae resumed their growth until the colonies reached a similar diameter. To fulfil Koch's postulates (Loeffler 1884, Evans 1976), some individuals from a clonal ostracod culture were exposed to the pathogen. They were apparently attracted strongly to the media blocks from which the zoospores were released (Fig. 2D). All ostracods were infected by the pathogen and died within 2–4 d after inoculation due to the parasitoid growth (Fig. 2E). In addition, the pathogen

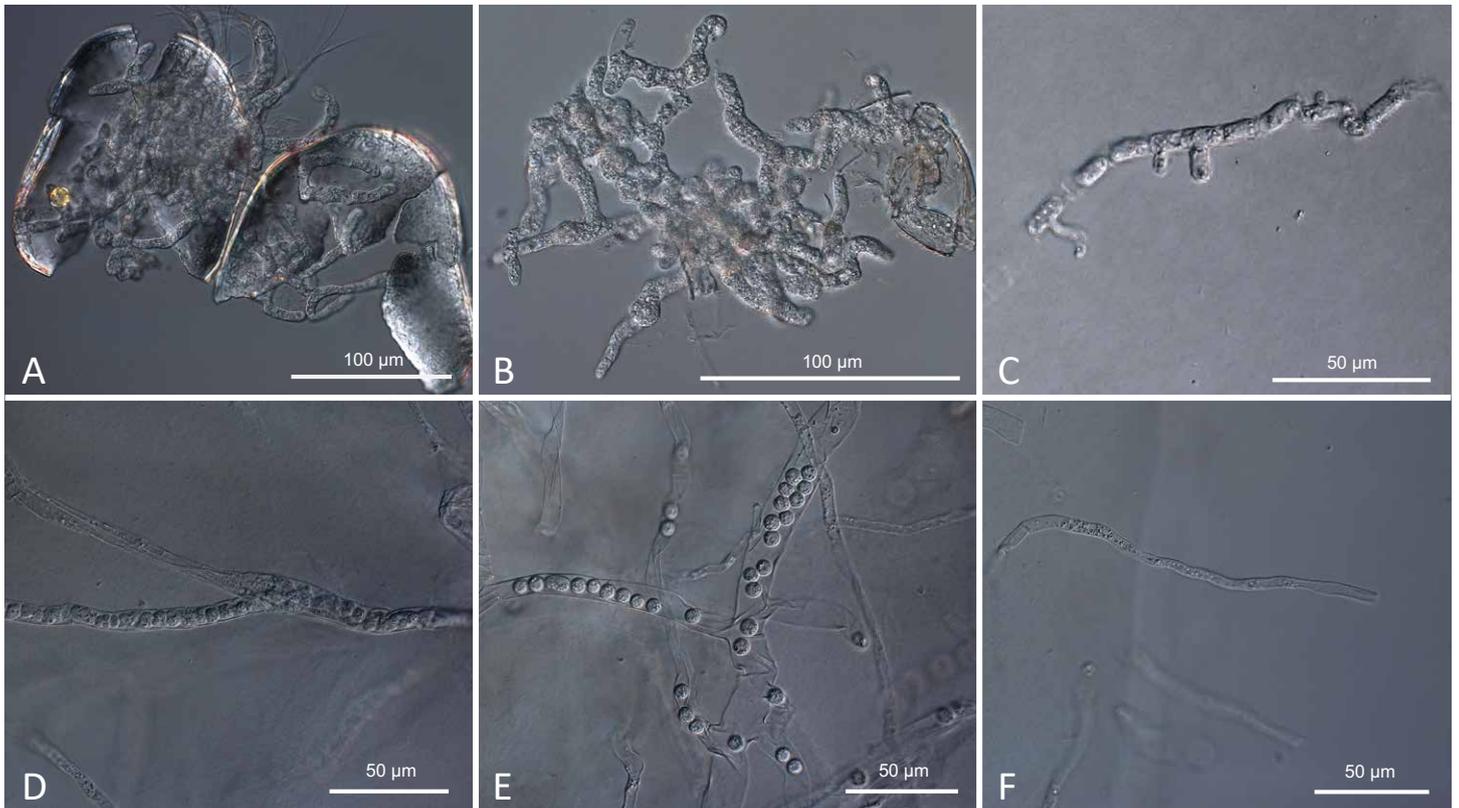


Fig. 1. Morphology and asexual cycle of the parasitoid. **A** Open carapace of the host containing an almost mature parasitoid thallus. **B** Mature parasitoid thallus. **C** Detail of an older, mature hyphal branch with some fragmentation of the cytoplasm. **D** Zoospores differentiation. **E** Just-differentiated zoospores inside a hyphal branch. **F** Empty hyphae with discharge tubes.

was also inoculated in the same manner into clonal cultures of three species of *Daphnia* (*D. pulicaria*, *D. pulex*, *D. galeata*) provided by Klaus Schwenk. However, no infection was observed in any of the strains. The ostrocod culture that was isolated from Rebstock lake and the *Daphnia* clonal cultures were cultivated using ADaM (Aachener Daphnien Medium) (Klüttgen *et al.* 1994).

Phylogenetic analysis

Phylogenetic analyses based on partial 18S nrRNA gene (nrSSU) sequences did not reveal support for conflicting topologies in Minimum Evolution (ME) and Maximum Likelihood (ML) analyses. Thus, only the tree from the ME analyses is shown in Fig. 3, with the support values from the ML analysis added on the branches. The parasitoid infecting ostracods clustered without support with *Atkinsiella dubia*, a parasitoid of marine crustaceans, with both being separated by long genetic distance. Both pathogens were embedded in the clade of early-diverging *Saprolegniomycetes* identified in previous studies (e.g. Buaya & Thines 2020), which also includes holocarpic parasitoids of diatoms. The cluster with the early-diverging *Saprolegniomycetes* was present in both the phylogenies based on Minimum Evolution (ME) and on Maximum Likelihood (ML) analyses, but did not receive statistical support in either. Also, the clustering with the crown *Saprolegniomycetes* present in the ME analysis did not receive statistical support. The clustering of both the crown *Saprolegniomycetes* and the *Peronosporomycetes* received strong support in ME analyses, while in ML analysis the *Peronosporomycetes* were not resolved as a group. Apart from *Saprolegniales*, *Olpidiopsidales*, and *Miraculales*, no other

order-level clade was resolved as monophyletic with strong support, and also the order of divergence of these clades did not receive strong support.

TAXONOMY

Due to its distinct phylogenetic position, morphology, and life-cycle characteristics, the parasitoid of freshwater ostracods isolated from the Rebstocksee in Frankfurt am Main is described as new below.

Bolbea A.T. Buaya & Thines, **gen. nov.** MycoBank MB833722.

Etymology: Named after the Greek lake nymph Bolbe, whose parents were of oceanic origin, reflecting the marine origin of the host group and likely also the pathogen group of the organism.

Diagnosis: Differs from *Atkinsiella* by the lack of sacculate hyphae in culture and from *Blastulidium* by its much more infrequent hyphal constrictions and more regular hyphae. Differs from other members of the *Leptomitales* by its crustacean host.

Description: Thallus holocarpic in crustaceans, killing the host while growing, finally consuming it entirely; hyphae regular, usually 5–15 µm broad, with their cytoplasmic contents maturing into zoospores; zoospores roundish within hyphae, dipanetic; zoospore discharge from mature hyphae by short discharge tubes; asexual resting spores or oospores not observed.

Type species: *Bolbea parasitica* A.T. Buaya & Thines

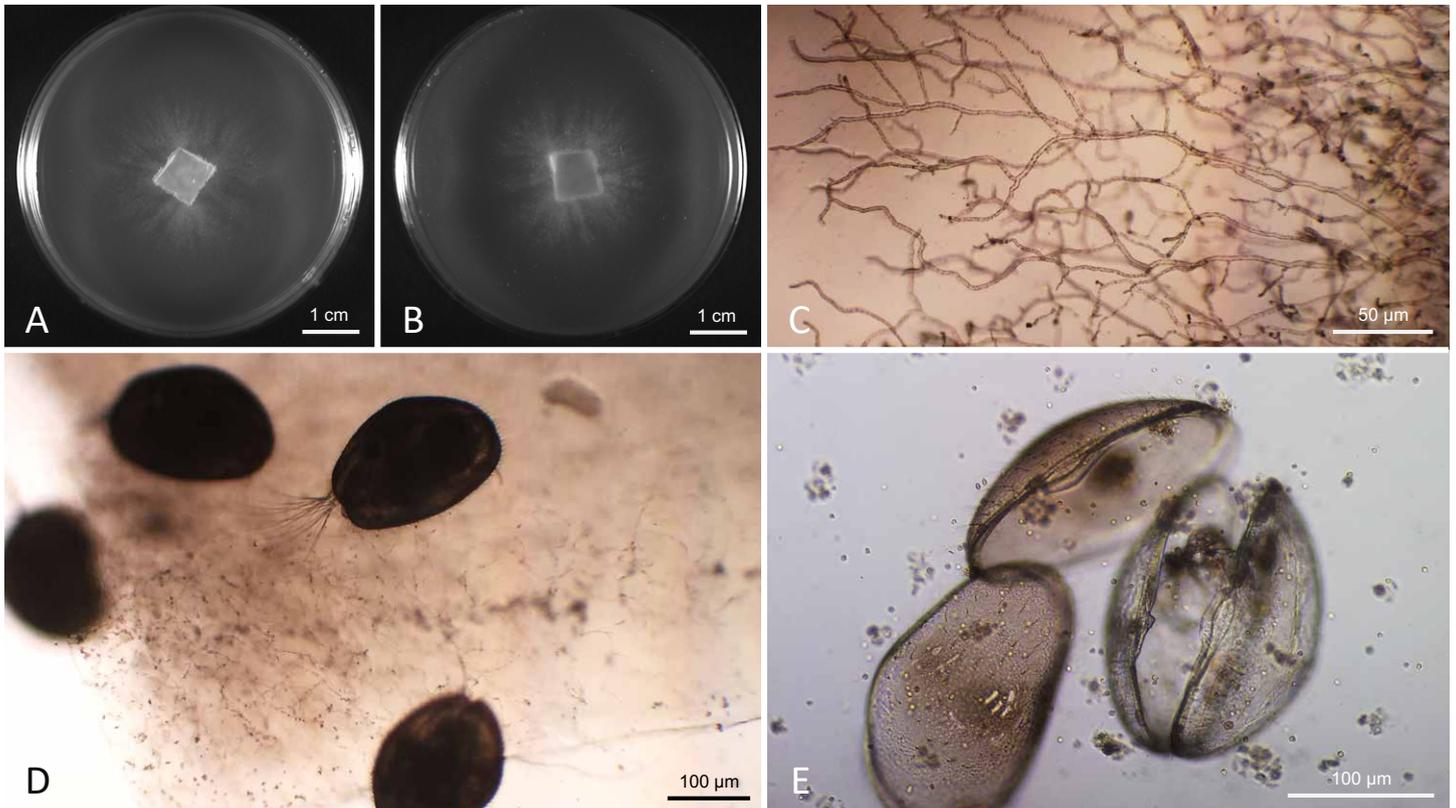


Fig. 2. Cultural characteristics and fulfillment of Koch's postulates. **A.** Top view of a culture plate. **B.** Bottom view of the same plate. **C.** Close-up of the colony margin with contorted hyphae. **D.** Re-inoculation to living individuals of a clonal ostracod culture for fulfilling Koch's postulates. **E.** Sacrificed individuals with abundant zoospores attached to the host carapace.

Bolbea parasitica A.T. Buaya & Thines, *sp. nov.* MycoBank MB833723. Figs 1–2.

Etymology: Named for its parasitic behaviour in ostracods.

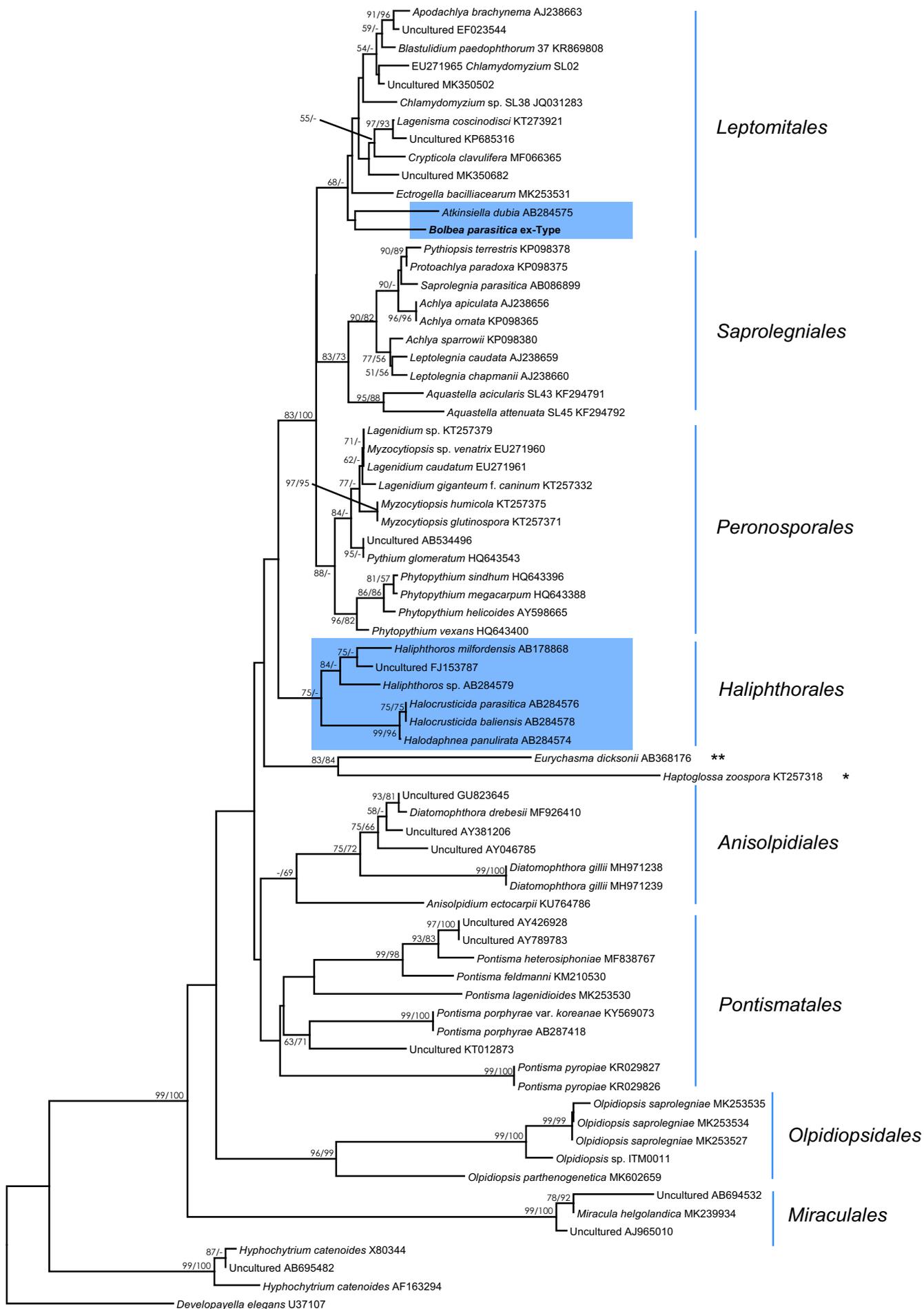
Description: Thallus holocarpic in ostracods, killing the host while growing, finally consuming it entirely; hyphae regular, but cytoplasm fragmentation observable in older hyphae or upon contact with water, with infrequent constrictions, usually 5–15 µm broad on modified MEA, with their cytoplasmic contents maturing into zoospores; zoospores roundish within hyphae, about 6 µm diam, diplanetic with secondary zoospores being more elongate in shape and forming after a rest period, without pronounced cyst development; zoospore discharge from mature hyphae by the dissolution of hyphal apices and the formation of short discharge tubes; growth on modified MEA of intermediate density with contorted hyphae, slowing down growth after several days; upon contact with water producing zoospores; resting spores and oospores not observed.

Typus: **Germany**, Hessen, Frankfurt am Main, Rebstocksee, July 2018, A.T. Buaya (**holotype** FR-0046116, ex-type partial nrSSU sequence MN688695).

DISCUSSION

Primarily holocarpic oomycetes are diverse and abundant in both freshwater and marine ecosystems (Karling 1942, 1981, Sparrow 1960, Dick 2001) and are important organisms in natural ecosystems, *e.g.* for global carbon cycling (Beakes & Sekimoto 2009, Strittmatter *et al.* 2009, Skovgaard 2014, Scholz *et al.* 2016, Beakes & Thines 2017, Hassett *et al.* 2019). Despite their ecological importance, only a few of them cause direct economic losses. The most direct impact of holocarpic oomycetes is probably seen in aquaculture, especially of crustaceans. There, the *Haliphthorales* can cause yield losses, affecting a variety of shrimps (Tharp & Bland 1977, Hatai *et al.* 1980, 1992, Hideki 2001, Chukanhom *et al.* 2003). But *Atkinsiella dubia* has also been reported as a serious pathogen in some cases (Vishniac 1958, Kitancharoen *et al.* 1995, Nakamura & Hatai 1995, Wallace Martin 1977, Bian & Egusa 1980). Often *Atkinsiella* is seen as holocarpic (Vishniac 1958, Nakamura & Hatai 1995), but Sparrow & Gotelli (1969) and Sparrow (1973) have contested this view, emphasising the fact that not all thallus parts simultaneously engage in zoospore formation. But as there does not seem to be a specialisation between hyphae that do and do not produce zoospores at a specific point in time, and all hyphae apparently have the potential to convert their cytoplasm into zoospores, we prefer to refer to *Atkinsiella dubia* as a holocarpic pathogen. Cultures of *A. dubia* have been reported (Sparrow 1973) and also

Fig. 3. Phylogenetic reconstruction derived from Minimum Evolution analysis based on partial nrSSU sequences. Numbers on branches denote support in Minimum Evolution and Maximum Likelihood analyses, in the respective order. Blue underlay indicates holocarpic pathogens affecting crustaceans.



0.02 substitutions / site

* *Haptoglossales*

** *Eurychasmales*

deposited in ATCC, but attempts to revive material sent by ATCC failed several times (unpubl. data). In our phylogenetic analysis, *Bolbea parasitica* grouped loosely with *A. dubia*, with both species being separated by a large genetic distance. However, the typical sacculate hyphal swellings and the “elephant hyphae” (Sparrow 1973) were never observed in *B. parasitica*, which is the reason, together with the rather weak clustering with *A. dubia*, why we chose to describe a new genus for the parasitoid.

Several oomycete species attacking invertebrate animals are not primarily holocarpic (Schikora 1903, Coker 1923, Atkins 1954, Seymour 1984, Cock *et al.* 1987, Willoughby *et al.* 1995, Molloy *et al.* 2014, Spies *et al.* 2016, Mendoza *et al.* 2018) or belong to the early-diverging oomycetes (Nakamura & Hatai 1995, Leaño 2002, Chukanhom *et al.*, 2003, Hakariya *et al.* 2007, Sekimoto *et al.* 2007, Glockling & Beakes 2002, Muraosa *et al.* 2009, Glockling & Serpell 2010, Beakes *et al.* 2014). But there are a few species in *Atkinsiella*, *Blastulidium*, *Chlamydomycium*, and *Crypticola* that also belong to the primarily holocarpic *Leptomitales* and form a hyphal network, an important feature for successful cultivation. However, all of these species form very irregular hyphae or hyphae with very frequent narrow constrictions between thallus segments (Frances *et al.* 1989, Frances 1991, Glockling & Beakes 2006, Duffy *et al.* 2015) and cultivation seems to be more difficult than for the secondarily holocarpic lagenidiaceous *Peronosporomycetes*. Even though constrictions are present in *Bolbea parasitica*, they are less frequent and often less narrow (the dark, septa-like lines that are visible in Fig. 1E are the results of a collapsing of the hyphae after zoospore release has started) leading to a rather regular appearance of the hyphae, but in older hyphae, or upon contact with water, a fragmentation of the cytoplasm may occur. In line with this, *Bolbea parasitica* does not form a supported sister lineage to any of these genera in the phylogenetic analyses. In terms of colony morphology, *B. parasitica* is similar to *Synchaetophagus balticus* (Apstein 1911). However, *S. balticus* is parasitic to a largely divergent invertebrate group, rotifers, and thrives in brackish to marine waters. In addition, according to Apstein (1911), it has more regular hyphae and apparently produces zoospores usually in a non-synchronised manner, while in *B. parasitica* usually the entire thallus produces zoospores simultaneously. This behaviour of synchronised zoospore formation could also be interesting for studying sporulation and especially zoospore formation processes in oomycetes, as similar to some lagenidiaceous oomycetes (Domnas *et al.* 1982), it is possible to trigger zoospore formation in a well-defined manner, which should render it easier to obtain homogenous time-points for certain stages of the sporulation process.

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

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