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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.

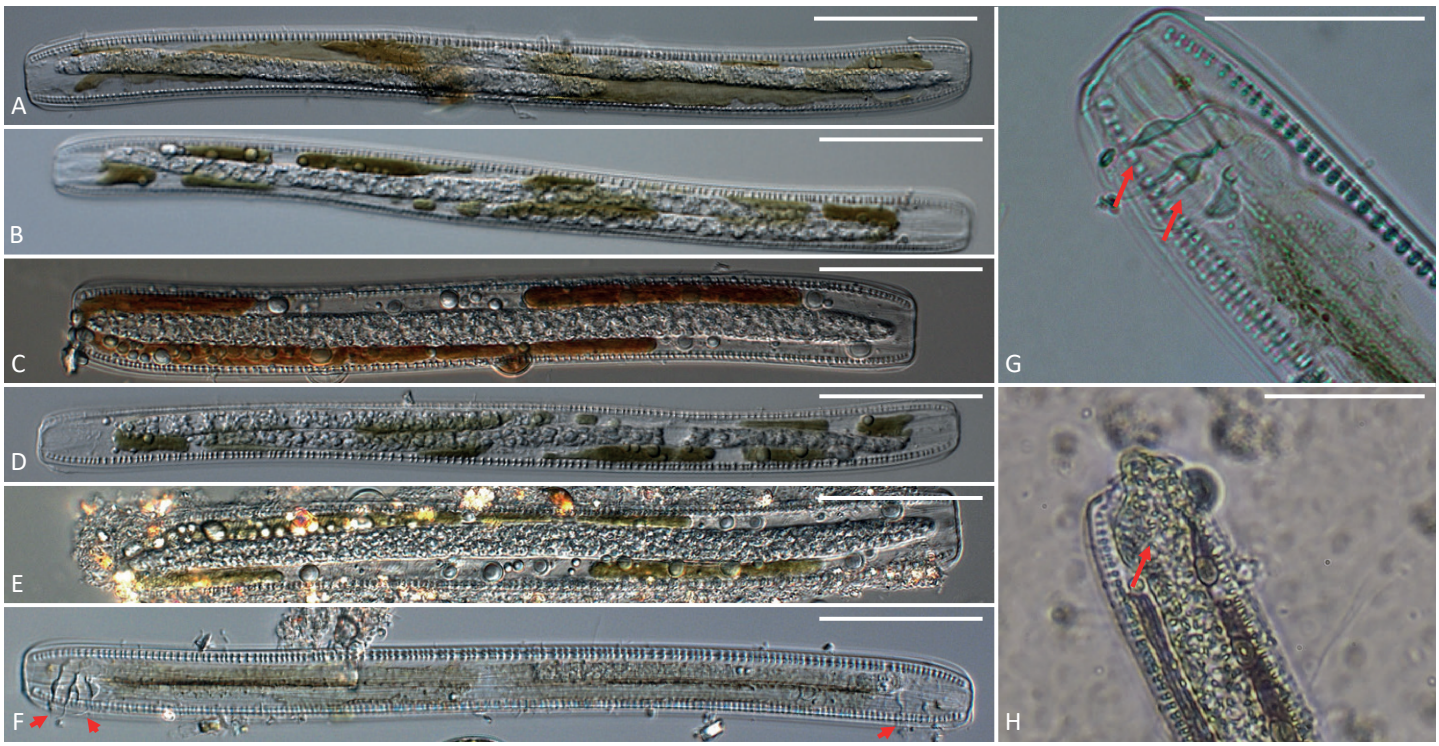

Fig. 1. Light micrographs of *Ectrogella bacillariacearum*, at different developmental stages on *Nitzschia sigmoidea*. **A.** Early infection of *N. sigmoidea* cell, with two developing endobiotic thalli. **B.** Two mature sporangia with emerging multiple vacuoles. **C.** Zoospore differentiation following 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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