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SHORT COMMUNICATION

A Revised Taxonomy of Diplonemids Including the Eupelagonemidae n. fam. and a Type Species, *Eupelagonema oceanica* n. gen. & sp.

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ABSTRACT

Recent surveys of marine microbial diversity have identified a previously unrecognized lineage of diplonemid protists as being among the most diverse heterotrophic eukaryotes in global oceans. Despite their monophyly (and assumed importance), they lack a formal taxonomic description, and are informally known as deep-sea pelagic diplonemids (DSPDs) or marine diplonemids. Recently, we documented morphology and molecular sequences from several DSPDs, one of which is particularly widespread and abundant in environmental sequence data. To simplify the communication of future work on this important group, here we formally propose to erect the family Eupelagonemidae to encompass this clade, as well as a formal genus and species description for the apparently most abundant phylotype, *Eupelagonema oceanica*, for which morphological information and single-cell amplified genome data are currently available.

HETEROTROPHIC flagellates remain one of the most poorly studied fractions of microbial diversity, consistently and substantially less well-studied than their parasitic or photosynthetic cousins. They tend to be hard to culture in the laboratory and often possess relatively few morphological characters that would allow easy identification. As culturing and morphological identification were the twin pillars of traditional protistology up to the late twentieth century, the challenges in both areas have left the diversity of heterotrophic flagellates "a neglected majority" (Caron et al. 2017). One result of this is that molecular surveys of protist diversity have revealed a great deal of previously unsuspected diversity in clades most likely to be heterotrophic flagellates (de Vargas et al. 2015).

Diplonemids are one example of such heterotrophic protists with tremendous diversity that had slipped under our radar. Diplonemids are group of heterotrophic flagellates that are sister to the kinetoplastids within the Euglenozoa. They have been known for a little over a century, but until recently, only three genera of diplonemids had been formally described, namely, *Diplonema* Griessmann (1913), *Rhynchopus* Skuja (1948), and *Hemistasia* Griessmann (1913) (Cavalier-Smith 2016; Yabuki and Tame 2015). A fourth proposed genus, *Isonema*, is generally considered a junior synonym of *Diplonema* (Triemer and Ott 1990). In 2018, three new diplonemid genera were reported (*Lacrimia*, *Sulcionema*, and *Flectonema*) (Tashyreva et al. 2018a), and novel morphological and behavioral features,

as well as endosymbionts, were described for the genus *Diplonema* (Tashyreva et al. 2018b), demonstrating the under-explored diversity of this group.

These genera were mostly collected from marine environments: *Diplonema* and *Rhynchopus* are primarily benthic, while *Hemistasia* is found among marine plankton in coastal waters (Cavalier-Smith 2016; Elbrächter et al. 1996; Roy et al. 2007; Yabuki and Tame 2015). The newly described genera are also marine, though their habitats are yet to be clarified (Tashyreva et al. 2018a). Some of these diplonemids have been studied mostly due to their relationship to the more famous kinetoplastids, and because of their baroque mitochondrial genome architecture and posttranscriptional editing characteristics (Kiethega et al. 2013; Marande et al. 2005; Valach et al. 2016; Yabuki et al. 2016), as well as some curious metabolic and molecular traits (Morales et al. 2016; Qian and Keeling 2001). But overall, the group has not been extensively described, with multiple undetermined phylotypes present in environmental molecular surveys (Tashyreva et al. 2018a,b).

Diplonemids were not found to be particularly common in early molecular surveys based on the 18S rRNA gene, although one enigmatic sister group of other diplonemids, dubbed the 'deep-sea pelagic diplonemids' (Lara et al. 2009; also referred to as DSPD I and DSPD II), was observed (López-García et al. 2001, 2007; Scheckenbach et al. 2010). More recently, however, analyses based on the Tara Oceans data (de Vargas et al. 2015) demonstrated that DSPDs are not restricted to the deep sea and are more common than previously thought (Flegontova et al. 2016; Lukeš et al. 2015). They are present at various depths ranging from surface water to deep oceans, with the majority found in mesopelagic waters (200–1,000 m). They are also present in different geographic locations, ranging from tropical to temperate to high latitude regions, as well as coastal to open ocean environments. In addition to this wide distribution, they are one of the most abundant and diverse protist groups yet characterized based only on 18S rRNA gene sequences (Flegontova et al. 2016; Lukeš et al. 2015).

Despite the diversity, ubiquity, and abundance of DSPDs in amplicon data, direct information about the biology of these organisms has been unavailable until a recent characterization of ten marine diplonemid cells that included basic microscopy and single-cell amplified genomic (SAG) data (Gawryluk et al. 2016). Of the phylotypes characterized, "Cell 37" was found to be particularly highly represented in Tara Oceans data; with more than 6,000,000 mapped amplicon reads, this phylotype is more abundant in amplicon data than all ciliate phylotypes combined.

With the emerging evidence for the diversity and ecological importance of DSPDs, one would expect a series of new discoveries on their biology, genomics, and ecological interactions. But, as we have seen from the history of study of other protist groups, the absence of a formal name for this group also sets the stage for a great deal of unnecessary confusion stemming from the use of multiple "unofficial" names or acronyms for the same lineage. To circumvent this confusion as early as possible, we here formally erect the family Eupelagonemidae n. fam. to encompass the abundant and diverse "DSPD I" lineage based on their phylogenetic coherence and distribution in nature. We also formally describe the Cell 37 phylotype as the type species *Eupelagonema oceanica* n. gen. & sp. for the group, based on its unique molecular phylogenetic position, in addition to 160.6 Mbp of genomic data comprising 531 identifiable protein coding genes, as well as the morphological information currently available from this uncultured taxon.

MATERIALS AND METHODS

A single cell of *Eupelagonema oceanica* was collected from 100 m depth at 33°18.08 N, 29°24.03 on October 7, 2013 as previously described (Gawryluk et al. 2016). The cell was photographed live using a Leica DM IL LED inverted microscope equipped with a Canon D5100 camera, then isolated into 10 μl of DNase-free water. The isolated samples were immediately frozen at −80°C, and kept frozen at or below −20°C. The same cell was subsequently used for a single cell genomic survey, from which the 18S rRNA genes and 160.6 Mbp of genomic sequence was obtained, as previously described (Gawryluk et al. 2016), and deposited in GenBank (18S RNA genes: #KY947154; genomic sequence: #SRX2014516). The 18S gene sequence from *E. oceanica* was assembled from a single amplified genome (Gawryluk et al. 2016). Mapping 40 reads of 250 bp each over the contig, we detected variable sites at 1.5% of alignment positions. Diplonemid 18S rRNA genes were retrieved from NCBI and aligned with 18S rRNA genes from 10 diplonemid cells reported in Gawryluk et al. (2016) using MAFFT v.7.212 with the L-INS-i iterative refinement method (Katoh and Standley 2013). Alignments were trimmed automatically with trimAl (Capella-Gutiérrez et al. 2009), with -gt and -st equal to 0.3, and 0.001, respectively. Excessively short sequences or sequences with long branches in a preliminary tree were also removed. ML trees were reconstructed with RAxML v.8.1.6 (Stamatakis 2014), under the GTR model of substitution rates, the gamma model of rate heterogeneity, and an estimated proportion of invariable sites [GTRGAMMAI]. Bootstrap support values derived from 1000 replicates were mapped onto the highest likelihood ML tree generated from 100 independent heuristic searches. Since diplonemids are not ever known to be photosynthetic and there is no evidence that any of the members of the DSPD lineage is photosynthetic, descriptions are according to the International Code of Zoological Nomenclature.

RESULTS AND DISCUSSION

Taxonomy of diplonemids

In phylogenetic analyses based on 18S rRNA, the Diplonemea families Diplonemidae and Hemistasiidae are typically

monophyletic, but most of the molecular diversity falls in another well-supported and more diverse clade, formerly known as DSPD I (Fig. 1). Based on this phylogenetic distinctiveness, we erect the new family, Eupelagonemidae to encompass this DSPD I lineage.

The substantial molecular diversity apparent in the Eupelagonemidae suggests the group will likely display substantial morphological diversity as well; indeed, the morphological variation already evident in the 10 cells that have been observed is noteworthy (Gawryluk et al. 2016). Consistent with this, eupelagonemid sequences in clone libraries have been obtained from pico-/nano- and microplankton fractions (Lukeš et al. 2015). Interestingly, Tara Oceans 18S rRNA gene amplicon sequences were retrieved both from the small planktonic as well as the mesoplanktonic fractions. In fact, one-fifth of the Tara Oceans hits for *E. oceanica* were found in the mesoplanktonic fractions (Flegontova et al. 2016). This implies that some members of Eupelagonemidae are physically associated with larger particles or mesoplankton (Amacher et al. 2009), potentially through parasitism (Lima-Mendez et al. 2015), though the frequency may be low (Lukeš et al. 2015). The idea that other diplonemids have some parasitic tendencies has also been discussed (Flegontova et al. 2016; Kent et al. 1987; Tashyreva et al. 2018a), although there is no direct data in support of this for the eupelagonemids specifically. Since diplonemids, and especially eupelagonemids, are so poorly known and what we do know suggests they are probably very diverse, it is premature to propose a simple morphological synapomorphy for the whole group. Nevertheless, we do note that to date metaboly has not been observed in live eupelagonemid cells, suggesting presence or absence of metaboly may be a distinguishing feature between Eupelagonemidae and both Diplonemidae or Hemistasiidae, which do show metaboly.

The *E. oceanica* cell that we observed was motile, despite the lack of a visible flagellum. It showed a pivoting movement with the round end settled on the surface. It is unclear what is the anterior–posterior direction of the cell, as we did not witness a linear propulsion of the cell. In some members of Diplonemidae, such as *Rhynchopus*

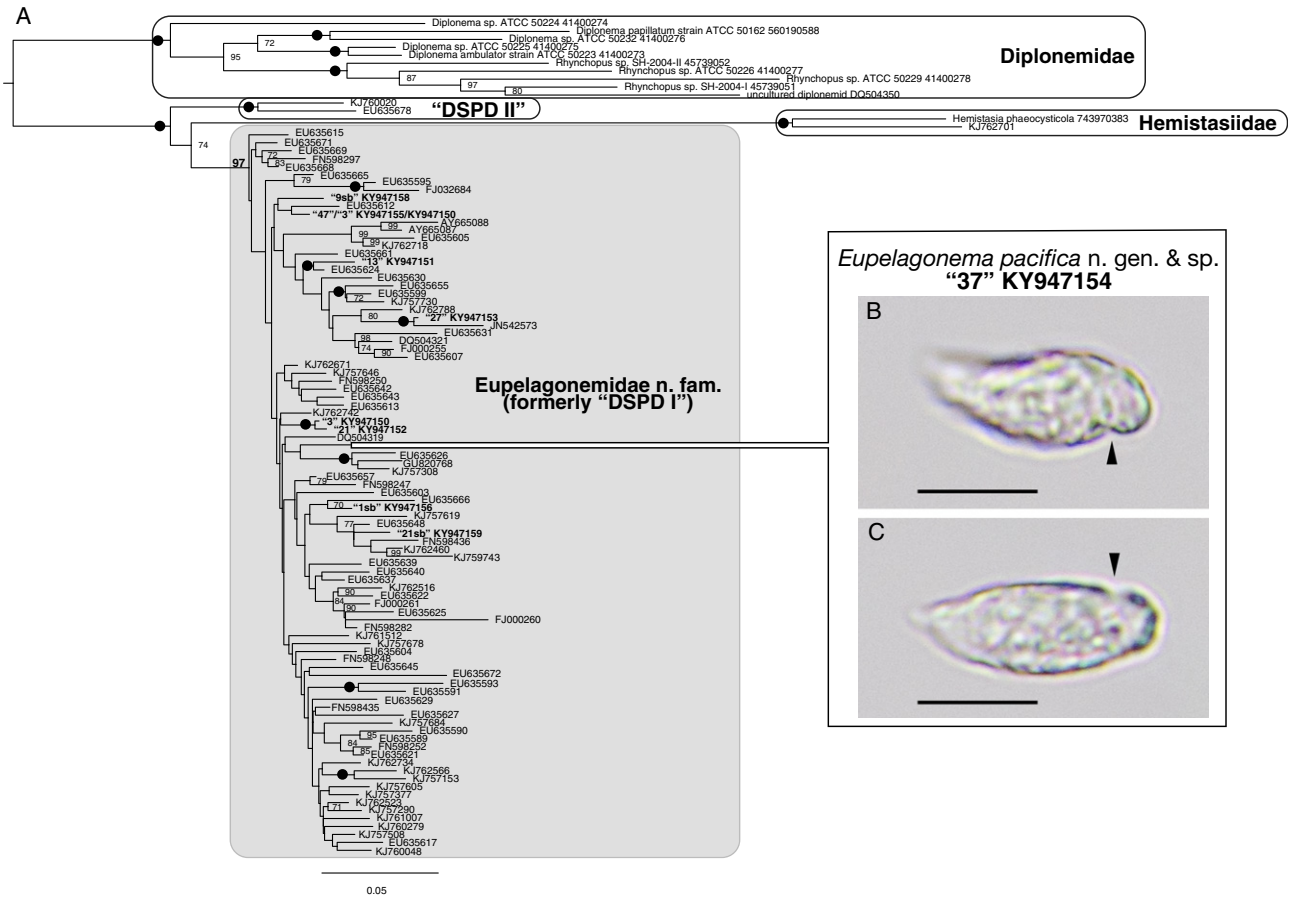

Figure 1 *Eupelagonema pacifica* n. gen. & sp. **A**. Maximum Likelihood phylogenetic tree of the SSU rRNA gene from diplonemids. Bootstrap values less than 70% are not shown. **B–C**. Light micrographs of a live cell of *E. pacifica*. The cell has a round end and an acute end, with a subterminal indentation (arrowhead). The images were taken ca. 1 min apart, when the cell showed a pivoting movement about the rounded end. Scale = 10 μm. These are the only two photographs of the original isolation (and **B** is a reprocessed version of the same raw photo used in a plate in Gawryluk et al. 2016), which took place under difficult conditions at sea.

spp., the flagella are only visible on the swarmer cells but not on cells that exhibit gliding movement and active metaboly (Lee 2015; Roy et al. 2007; Schnepf 1994; Simpson 1997). Although *E. oceanica* maintains its cell shape and did not show metaboly, it may use a similar gliding mechanism for the pivoting movement we observed. Some other Eupelagonemidae (e.g. Cell 9sb in Gawryluk et al. 2016) are reminiscent of *Pronoctiluca*, a mysterious genus that has been controversially assigned to the dinoflagellates (Fabre-Domergue 1889; Gómez 2013). Their morphology also shows some differences, such as the reported motility of the *Pronoctiluca* rostrum, which has yet to be observed in eupelagonemids. It is entirely possible that a cell closely matching the description of *Pronoctiluca* will ultimately be shown to fall within the Eupelagonemidae clade by molecular analyses in the future, which will solve the placement of this enigmatic genus. One remaining small subgroup of diplonemids is still only known from environmental data, DSPD II, and will remain undescribed until more data are available.

Considerations for the taxonomy of uncultivated and isolated single cells

Taxonomy and systematics of uncultivated lineages is a challenging problem, but one that must be considered carefully as culture-free methods give us ever-greater access to information about these lineages. Uncultivated protist species have been formally described on many occasions based on a wide spectrum of data; historically a single line drawing was not uncommon (e.g. the original description of genus *Pronoctiluca* by Fabre-Domergue 1889), and it is similarly not unheard of for a current description to be based on a micrograph and corresponding DNA sequence (e.g. Boscaro et al. 2017; Del Campo et al. 2017).

Protist taxonomy is in the awkward position of often relying in practice on molecular data to identify and define species and other lineages, but then attempting to fit this philosophically into the framework of a morphological species concept. It is accordingly possible for a description to be based strongly on molecular phylogenetic information, but with post hoc morphological rationale. This problem is exacerbated in small heterotrophic flagellates that are hard to culture; light microscopical morphology of these species may hold very little information and the ultrastractural characters maybe difficult to obtain without culture (Massana 2011). These are potentially some of the biggest gaps in our understanding of protist diversity—and accordingly include some of the most important groups where a better understanding of their diversity and a better classification system is needed. This also makes them a rich source of interesting new species. However, they are also the protists least likely to easily yield a lot of morphological characteristics suitable for taxonomy, leading us to wonder if these in particular are well suited to the use of molecular data for taxonomy. For such species, including *E. oceanica* for example, we feel that a taxonomic argument openly and strongly based on the molecular data is preferable to a weak or vague argument based on morphology, and should be encouraged. Such data are more likely to prove to be unambiguous characters in any re-identification of the same species in future samples, and similarly in distinguishing closely related or morphologically similar species that are in fact evolutionarily distinct. This is not to say we feel morphological data should be discouraged: for descriptions of new species, the more information the better, but there is no clear argument that a large amount of genomic data holds an inferior standard of information than a large amount of morphological data. We argue describing such species based on molecular data are entirely justified, given the data are high quality and informative so that it improve reproducibility.

While these issues apply to species descriptions, they can even more often apply to higher order groups. For example, the recognition of the monophyly of the Rhizaria through their formal naming was very useful, but it was entirely based on molecular phylogeny for a long time (and arguably still is) (Burki and Keeling 2014; Pawlowski 2008). If higher order taxa are often described based purely on molecular data, it raises the question of why species-level descriptions might not also be based entirely on molecular sequence, as is currently under discussion in bacterial taxonomy (e.g. Garrity 2016). Our opinion is that there is no compelling reason against this, but since protists have much more morphological variation than do bacteria (Keeling and del Campo 2017), it would seem reasonable to argue instead for 'as much of all kinds of data as possible' other than proscribing one kind of data in favor of another. That being said, we believe it would be timely to begin an open discussion on how we shall evaluate the molecular evidence as a type material of a species.

Here in the case of *E. oceanica*, we describe this new species primarily based on the genome analyses and molecular phylogeny. We do have a micrograph for the species that, albeit less informative on its own, serve to show that the genome data do come from the single cell, so we include both in the description.

Given the global distribution and potential abundance of the Eupelagonemidae, we are confident that future work will result in cultured model representatives and detailed information on their molecular biology, morphology, and ecology, all of which will help to increase our understanding of the diversity and roles of free-living heterotrophs in natural ecosystems. We are also confident that the literature generated from such studies will benefit from the early proposal of a name for the group.

TAXONOMIC SUMMARY

Euglenozoa Cavalier-Smith, 1993
Class Diplonemea Cavalier-Smith, 1993
Order Diplonemida Cavalier-Smith, 1993
Family Diplonemidae Cavalier-Smith, 1993
Genus *Diplonema* Griessmann, 1913
Genus *Rhynchopus* Skuja, 1948
Genus *Lacrimia* Tashyreva, Prokopchuk, Horák & Lukeš, 2018

Genus *Flectonema* Tashyreva, Prokopchuk, Horák & Lukeš, 2018
Genus *Sulcionema* Tashyreva, Prokopchuk, Horák & Lukeš, 2018
Family Hemistasiidae Cavalier-Smith, 2016
Genus *Hemistasia* Griessmann, 1913
Family Eupelagonemidae Okamoto & Keeling
Genus *Eupelagonema* Okamoto & Keeling
Species *Eupelagonema oceanica* Okamoto & Keeling

Descriptions: Eupelagonemidae n. fam. Okamoto & Keeling.
Zoobank ID: urn:lsid:zoobank.org:act:E9BD5F75-D3EE-4324-AFD8-C9F034C3E33A.
Diagnosis: Form a distinct and well-supported clade in phylogeny based on 18S rRNA gene sequences. The members of Eupelagonemidae include a free-living motile stage without photosynthetic pigment. Cells are oblong to spindle-shaped, motile with or without flagella. Some members possess a rostrum on one end of the cell. Globally distributed in the eupelagic water.
Type genus: *Eupelagonema* n. gen. Okamoto & Keeling.

Eupelagonema n. gen. Okamoto & Keeling

Zoobank ID: urn:lsid:zoobank.org:act:71C83C59-E329-4DBC-B4E3-B72FC712D491.
Diagnosis: Elongated colorless cells are motile with or without flagella; metaboly not observed. Globally distributed in the eupelagic water of various depth, latitude, and ocean provinces.
Type species: *Eupelagonema oceanica* n. sp. Okamoto & Keeling.
Etymology: eu- [true (g)] + pelages [sea (g)] + -nema [thread (g)] (n).

Eupelagonema oceanica n. sp. Okamoto & Keeling

Zoobank ID: urn:lsid:zoobank.org:act:B150096C-7BFB-4620-B22E-549BD4307A91.
Diagnosis: Cell displays a unique SSU rRNA sequence phylotype represented by the type sequence. Elongated, elliptical shaped cell, round on one end and with an acute tip on the other. Near the round end of the cell, an indentation in the cell contour was observed (arrowhead). Cell length is ca. 20 μm; metaboly not observed.
Type material: The specimen shown in Fig. 1B–C is the holotype. The actual specimen was by necessity as a single cell destroyed in the process of single-cell genome sequencing (see International Code of Zoological Nomenclature, Art. 72.5.6, Declaration 45).
Type sequence: The SSUrRNA gene sequence is Genbank # KY947154.
Type locality: The cell was obtained from 100 m depth at 33°18.08 N, 129°24.03 W during CANON cruise CN13ID (October 7-17, 2013; R/V *Western Flyer*).
Etymology: oceanica [from the ocean].

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