The Journal of Eukaryotic Microbiology

ISOP Published by the International Society of Protistologists

Journal of Eukaryotic Microbiology ISSN 1066-5234

SHORT COMMUNICATION

Fish Parasite Dinoflagellates *Haidadinium ichthyophilum* and *Piscinoodinium* Share a Recent Common Ancestor

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Keywords
Dinophyceae; endemic stickleback; refugium; SSU rRNA; taxonomy.

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Received: 23 January 2017; revised 19 April 2017; accepted May 12, 2017.

doi:10.1111/jeu.12430

ABSTRACT

The dinoflagellate *Haidadinium ichthyophilum* Buckland-Nicks, Reimchen and Garbary 1997 is an ectoparasite of the spine-deficient, three-spine stickleback *Gasterosteus aculeatus* L. Reimchen 1984, a fish endemic to Rouge Lake, Haida Gwaii. *Haidadinium ichthyophilum* proved difficult to assign taxonomically because its morphology and complex life cycle exhibited defining characteristics of both autotrophic and heterotrophic dinoflagellates, and was tentatively assigned to the Phytodiniales. Here, we characterized a 492 bp fragment of the small subunit ribosomal RNA (SSU rRNA) from preserved *H. ichthyophilum* cysts. In SSU phylogeny, *H. ichthyophilum* branches with the fish parasites, *Piscinoodinium* sp., strongly supporting the inclusion of *H. ichthyophilum* within the Suessiales.

HAIDADINIUM ichthyophilum Buckland-Nicks, Reimchen and Garbary 1997 is an ectoparasite of an endemic population of the three-spine stickleback, *Gasterosteus aculeatus* L., currently known only in the acidic Rouge Lake on the island of Haida Gwaii (Reimchen and Buckland-Nicks 1990). Infection of the fish by dinospores induces epithelial hyperplasia, during which epithelial cells migrate to the surface of the fish and enclose the infective dinospores causing them to encyst. This results in a thick, jelly-like layer of cells on the fish, in which the vegetative cysts are embedded, but no obvious behavioural signs of stress or loss of reproductive activity even in cases of extensive infection have been reported from the stickleback (Buckland-Nicks et al. 1990). *Haidadinium ichthyophilum* cysts are very unusual compared with other dinoflagellate fish parasites. The cysts retain their plastids and contain a rigid fenestrated matrix penetrated by cytoplasmic processes that extend outwards from the dinokaryotic nucleus. These traits, in addition to amoeboid stages and a variety of resting cysts, at the time suggested associations with the Phytodiniales (Fensome et al.1993), while a temporary dinokaryon, palintomic sporogenesis and a trophont stage suggested affinities with the Blastodiniales (Buckland-Nicks and Reimchen 1995; Buckland-Nicks et al. 1997). The Blastodiniales has since been shown to be polyphyletic and reevaluation has been proposed (Saldarriaga et al. 2001; Skovgaard et al. 2007). In light of its autotrophic nature and other specific characteristics, the species was provisionally assigned to the Phytodiniales (Buckland-Nicks et al. 1997), awaiting confirmation by molecular analysis. Here, we describe a partial SSU rDNA sequence from individually isolated cysts, which resolves the taxonomic affinities of *H. ichthyophilum*.

MATERIALS AND METHODS

DNA extraction, PCR amplification and sequencing

Three-spine sticklebacks were collected from minnow traps placed overnight in Rouge Lake, Haida Gwaii (54 02.008, -131 52.558) and fixed in 95% ethanol. *Haidadinium ichthyophilum* vegetative cysts were manually isolated from the skin of those fixed samples, as the host is

relatively rare and endemic to one remote lake. Since that time the lake has completely dried, and the resulting drop in fish populations means *H. ichthyophilum* can no longer be sampled. Cysts were identified microscopically and removed with surrounding skin by microdissection. Residual skin tissue was removed by additional microdissection, and the resulting cysts were washed in distilled water twice (Fig. 1A). Total DNA was prepared from cleaned cysts either individually or in a pool of five cysts with the Epicentre Masterpure Complete DNA and RNA purification Kit (Madison, WI). Small subunit ribosomal RNA (SSU rRNA) gene sequences were amplified from the pool of five cysts as previously described (Gile et al. 2011; Saldarriaga et al. 2011). Nested PCR reactions, using the initial PCR product as a template from the five-cyst DNA sample, were completed as previously described with the following PCR primers: 18SComR1/Dino18sF1, 18sComF1/18SComR1, GGF/18sComR1, 18SComF1/GGR and Dino787F/18sComR1 (Lin et al. 2006). PCR products were cloned, using the StrataClone PCR Cloning Kit (Stratagene, Mississauga, ON, Canada), and sequenced on both strands, using BigDye Terminator v3.1.

Phylogenetic analysis

Sequencing products were assembled into a consensus sequence of 492 bp with Geneious version 10.0.2 (http://www.geneious.com; Kearse et al. 2012), and added to the taxonomic sampling in the SSU rRNA tree of the recently described genus *Yihiella* (Jang et al. 2016), together with sequences for *Oodinium pouchetii*, another fish parasite with unknown taxonomic affinity (Gómez and Skovgaard 2015). The *Haidadinium* product was also compared to existing databases, using BLASTN (Altschul et al. 1990) to identify closely related sequences (> 90% similarity), which were also added to the dataset. The GenBank accession numbers for the sequences used are indicated in Fig. 1B.

For phylogenetic analysis, the sequences were aligned with the auto option of MAFFT (Katoh and Standley 2013; Katoh et al. 2005) and poorly aligned regions were eliminated, using trimAl (Capella-Gutierrez et al. 2009) with a gap threshold set at 30% and a similarity threshold of 0.001, resulting in a 477 bp sequence for *Haidadinium* employed for phylogenetic reconstruction. The best of 1,000 maximum likelihood trees was calculated, using RAxML assuming the GTR-CAT substitution model, and a nonparametric bootstrap analysis was performed with 1,000 replicates (Stamatakis 2006, 2014). The initial tree was pruned to remove 100% identical sequences, using the clustering function of usearch (Edgar 2010), retaining only the longest representative each, and tree reconstruction was repeated, using the strategy described above. Sequences from *Oodinium pouchetii* (accession numbers KM879217–KM879219) were found to be extremely divergent and were removed from the analysis. Trees including *Oodinium pouchetii* did not differ significantly with respect to the position of *H. ichthyophilum* (Fig. S1).

RESULTS AND DISCUSSION

Characterization of *Haidadinium ichthyophilum* SSU rRNA gene

The pool of five vegetative cysts yielded several products, which were sequenced and assembled. Single-cyst DNA yielded no product, even when treated by whole-genome amplification, all suggesting the DNA was not of high quality in the ethanol-preserved samples. The assembled product from five cysts was found to be most similar to sequences from the fish parasite *Piscinoodinium* (93,67% pairwise identity) and to contain a deletion of approximately 1,200 bp. Using five additional primer combinations (including universal and dinoflagellate-specific primers) again only this deletion product could be recovered, however, the regions framing this deletion contain the hypervariable regions V1 and V2 as well as V9, with region V2 conferring the highest phylogenetic resolution within the SSU rRNA of dinoflagellates in general (Ki 2012).

The partial *Haidadinium* SSU rRNA plus the most similar SSU sequences retrieved by BLASTN were added to the taxonomic sampling of a recently published SSU rRNA phylogeny (Jang et al. 2016) and its phylogenetic position was inferred. The *Haidadinium* sequence was specifically related to the fish parasite *Piscinoodinium* sp. with strong support (Fig. 1B).

Taxonomy of *Haidadinium*

When *H. ichthyophilum* was initially described, its possible relationships to other dinoflagellate lineages that were known at the time to contain fish parasites were unclear. *Haidadinium* has a complex life cycle including swarmer, trophont, vegetative cyst and four amoeboid stages. Also it is autotrophic with chloroplasts present in both swarmers and vegetative cysts and with proplastids in some resting cysts and amoeboid stages (Buckland-Nicks and Reimchen 1995; Buckland-Nicks et al. 1997). Buckland-Nicks et al. (1990) noted a resemblance to *Piscinoodinium,* which was at that time assigned to the Blastodiniales, but based on overall characteristics, *H. ichthyophilum* was tentatively assigned to the Phytodiniales and it became the only fish parasite of that order (Buckland-Nicks et al. 1997).

The SSU rRNA phylogeny presented here shows *H. ichthyophilum* to branch with the Suessiaceae, and within that group it is specifically related to *Piscinoodinium* to the exclusion of *Phytodinium* sp., which represents the Phytodiniales in our tree. Similar to a recent phylogeny of the Suessiaceae (Jang et al. 2016), the strongly supported clade formed by *H. ichthyophilum* and *Piscinoodinium* sp. is in turn sister to another highly supported clade containing the freshwater dinoflagellate *Asulcocephalium miricentonis* and several unidentified dinoflagellates. Support for this topology that the *Haidadinium*/*Piscinoodinium* clade is sister to the *Asulcocephalium*-containing clade is, however, low in our tree, confirming the decision of Takahashi

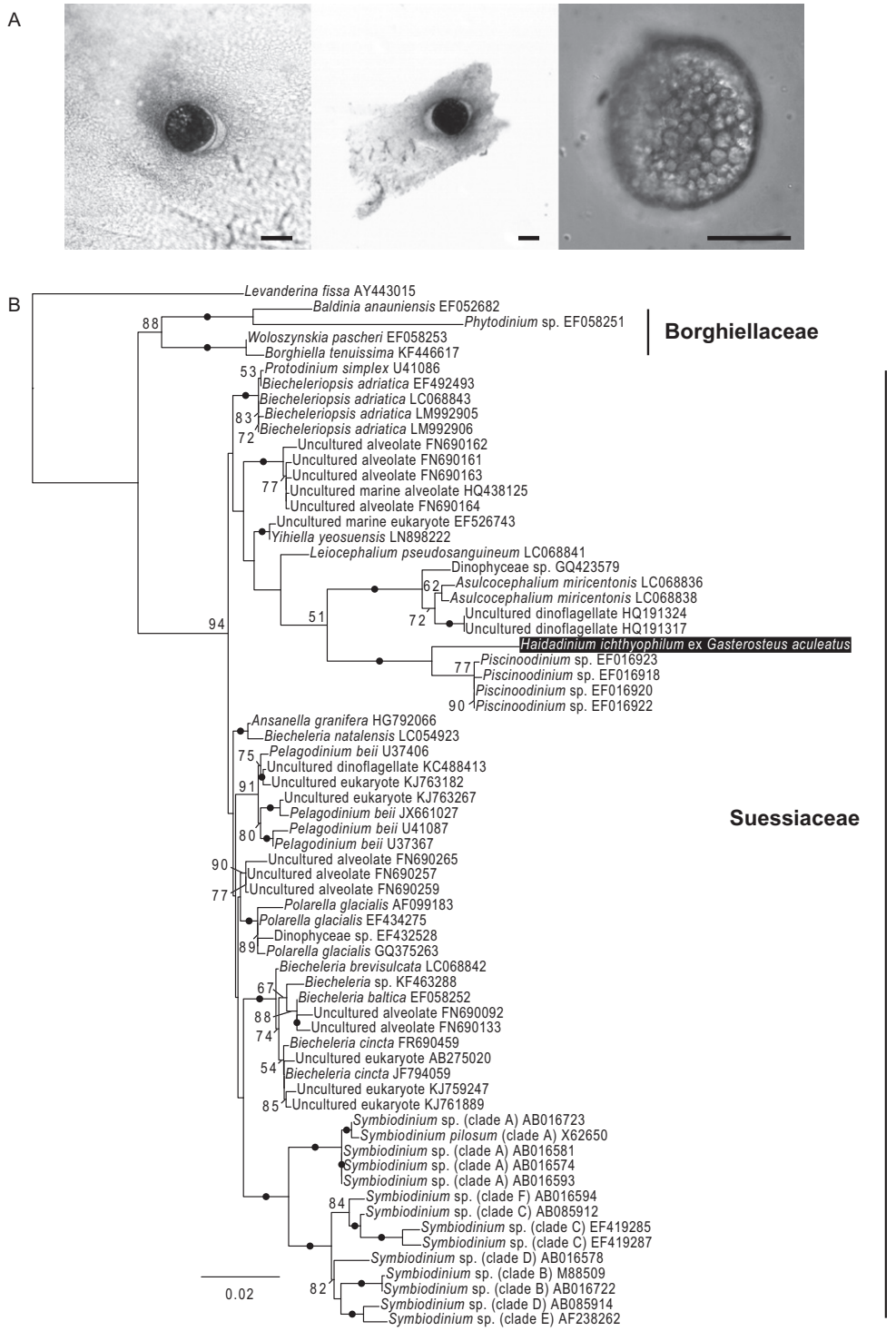

Figure 1 Vegetative cyst isolation and phylogenetic position of *Haidadinium ichthyophilum* SSU rRNA isolated from these cysts. (**A**) Light micrographs of *H. ichthyophilum* cysts from ethanol-preserved samples: whole preserved tissue sample with dense cyst embedded in the tissue (left), partially microdissected cyst with attached tissue (centre), and washed cyst (right). Scale bar = 20 μm. (**B**) Phylogeny of 1,779 (477 for *H. ichthyophilum*) aligned positions of SSU rRNA as inferred by ML (GTR-CAT-I) and 1,000 bootstrap replicates, depicting the position of *H. ichthyophilum* within dinoflagellates (shaded in black). *Levanderina fissa* comprised the outgroup. Black dots correspond to > 95% ML bootstrap support. Numbers at nodes represent bootstrap supports of > 50%. The scale bar represents the estimated number of nucleic acid substitutions per site.

et al. (2015) to assign *A. miricentonis* to its own genus, separate from *Piscinoodinium*.

Analysis of mitochondrial DNA of three-spine stickleback populations from the northern hemisphere shows two highly divergent lineages, the most common Euro-North American lineage and a second Trans-Pacific lineage (Orti et al. 1994). Haida Gwaii stickleback are primarily the Euro-North American lineage apart from four watersheds where both the Euro-North American and Trans-Pacific lineages occur. Rouge Lake stickleback is the only Haida Gwaii population that is monomorphic for the Trans-Pacific lineage (Deagle et al. 1996). Recent genome-wide assessment of the Haida Gwaii stickleback populations (Deagle et al. 2013) shows also that the Rouge Lake stickleback occur in an uncommon clade restricted to the northeastern corner of Haida Gwaii. It remains uncertain whether the presence of *H. ichthyophilum,* the distinctive genomic lineage of the Rouge Lake stickleback, as well as their highly derived loss of defensive armour (Reimchen et al. 2013) are merely a coincidence that occurred after postglacial colonization, or whether these parameters comprise an extended historical legacy. The latter option can potentially be explained by an ice-free refugium that is suspected to have remained between Haida Gwaii and the continental mainland during the Pleistocene (Reimchen and Byun 2005; Warner et al. 1982**)**. It would accordingly be interesting to look for *Haidadinium*-like infections in other geographically separated members of the Trans-Pacific lineages to perhaps even date the origin of the infection and further illuminate the history of fish parasitism in the *Haidadinium*/*Piscinoodinium* clade.

ACKNOWLEDGMENTS

We are grateful to Dr. Rob Fensome and Dr. Juan Saldarriaga for connecting our laboratories. This work was supported by NSERC Discovery grants to PJK (#227301), JB-N (#46205) and TER (#2354). EH and JdC were supported by a grant to the Centre for Microbial Diversity and Evolution from the Tula Foundation. JdC was supported by Marie Curie International Outgoing Fellowship grant (FP7-PEOPLE-2012-IOF – 331450 CAARL). PJK is a Senior Fellow of the Canadian Institute for Advanced Research.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Phylogenetic position of *Haidadinium ichthyophilum* SSU rRNA.