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Molecular insights into the identification and phylogenetics of the cosmopolitan marine fish blood parasite, *Haemogregarina bigemina* (Adeleorina: Haemogregarinidae)

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ABSTRACT

Haemogregarina bigemina is one of the most prevalent haemogregarines of marine fishes and has long been considered an enigmatic and cosmopolitan species. However, to determine whether *H. bigemina* truly represents a single global species, or whether it should be partitioned into several species or subspecies, and to confirm its taxonomic status among the *Haemogregarina*, molecular analysis is required. Here, we provide the first molecular characterisation of *H. bigemina* from one of its type hosts, *Lipophrys pholis*, in the UK using 18S rDNA sequences. Phylogenetic and *p*-distance comparisons of the newly generated *H. bigemina* sequences with those published from other haemogregarine taxa and related apicomplexans suggest that *H. bigemina* falls outside of the "*Haemogregarina*" clade as well as the Adeleorina altogether, forming a separate apicomplexan marine clade, and appears to be in fact more closely related to other non-haemogregarine Apicomplexa genera. Further work including sequences of *H. bigemina* and *H. bigemina*-like parasites observed from fishes in other localities is now needed to investigate this further.

1. Introduction

Haemogregarines are adeleorine apicomplexan protists inhabiting the blood cells and tissues of a variety of vertebrates and are especially common in marine fishes (Davies et al., 2004). *Haemogregarina* (sensu lato) *bigemina* Laveran and Mesnil (1901) is probably the most enigmatic of the marine fish haemogregarines, having first been described from intertidal blenniid fishes *Lipophrys pholis* (Linnaeus, 1758) and *Coryphoblennius galerita* (Linnaeus, 1758) in northern France in 1901 (Laveran and Mesnil, 1901) and subsequently reported from the blood of at least 96 species of teleost fishes, across 70 genera and 34 families from across the globe, including South Africa (Smit and Davies, 1999), Australia (Smit et al., 2006) and the Caribbean (Cook et al., 2015a,b). This cosmopolitan distribution appears to be unique among aquatic hosts and the apparent broad dispersal of *H. bigemina*, and its current taxonomic placing within *Haemogregarina* (sensu lato) Danilewsky, 1885, due to its development most closely but not perfectly resembling *Haemogregarina* (sensu stricto), have been questioned previously by the current authors and others (Davies et al., 2004; Davies and Smit, 2001).

Within its fish hosts, *H. bigemina* undergoes intraerythrocytic merogony, as do, similarly, other members of *Haemogregarina* (sensu lato) and members of the genus *Cyrilia* Lainson, 1981, while haemogregarines belonging to *Desseria* Siddall, 1995 lack this process (Smit and Davies, 2006). Unusually for a fish haemogregarine, there is unequivocal evidence from studies in Europe and South Africa that *H. bigemina* undergoes development in and is most likely transmitted by blood-feeding juvenile gnathiid isopods (Davies, 1982; Davies et al., 1994; Davies and Smit, 2001). More recently, life stages of a second haemogregarine species, *Haemogregarina balistapi* Smit et al. (2006), has also been described in detail from another likely gnathiid vector, *Gnathia aureamaculosa* Ferreira et al., 2009 in Ferreira et al. (2009), from the Great Barrier Reef, Australia, which may act as a vector of *H. bigemina* in this region (Curtis et al., 2013). Other species of fish haemogregarine belonging to *Haemogregarina* (sensu lato), *Cyrilia* and *Desseria* are mostly likely transmitted by leeches (Hayes et al., 2006, 2011). *Haemogregarina* (sensu stricto) is comprised of haemogregarines from chelonians and most probably also transmitted by leeches (Davies and Johnston, 2000). The development of *H. bigemina* in juvenile gnathiids has been shown to most closely resemble that of *Haemogregarina* (sensu stricto) in leeches., although, some comparisons of similarities to

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other arthropod-vectored haemogregarines of semi-aquatic and terrestrial vertebrates, including *Hepatozoon* Miller, 1908 and *Hemolivia* Petit, Landau, Baccam and Lainson, 1990 species, in tick and mite hosts, respectively, have also been made (Davies and Johnston, 2000; Davies and Smit, 2001).

Relatively little is known of the biology of fish haemogregarines compared with those of reptiles and other groups of vertebrates, and the current taxonomic classification of fish haemogregarines remains heavily reliant on morphology of developmental stages mainly in the vertebrate and invertebrate hosts. There is also a lack of molecular data on fish haemogregarines in general and all the recent descriptions of new species, such as *H. balistapi*, *Haemogregarina curvata* Hayes et al., (2006), *Haemogregarina koppiensis* Smit and Davies, 2001, and *Desseria zei* Smit and Davies (2006) (see Hayes et al., 2006; Smit and Davies, 2001; 2006), were only based on arbitrary and often tenuous morphological comparisons and morphometrics in comparison with haemoregarines from amphibian and reptilian hosts. For example, those from frogs (Netherlands et al., 2018) and from turtles (Úngari et al., 2018), tortoises and snakes (Cook et al., 2014, 2018; Cook et al., 2015a,b, 2016) which were characterised both morphologically and molecularly using the ribosomal 18S rDNA gene sequences. Recently, Xavier et al. (2018) highlighted the paucity of available molecular data for apicomplexans from aquatic hosts in general, and to date, the only marine fish blood apicomplexans known to be sequenced are the unnamed species from the Caribbean reported by Renoux et al. (2017) and Sikkel et al. (2018).

To confirm its identity and taxonomic status, and then to determine whether *H. bigemina* likely represents a single global species or a complex of more than one species, molecular analysis is required. Here, in the initial step to unravelling the identity of this apicomplexan parasite we report the first molecular characterisation of *H. bigemina* from one of its type hosts, *L. pholis*, in the UK. This study also presents the first molecular characterisation of any of the named marine fish haemogregarines.

2. Methods

2.1. Screening of fish

Giemsa-stained blood films and whole blood samples (fixed in absolute ethanol (≥99.8%) for molecular analysis) from seven *Lipophrys pholis* (Fig. 1) captured in rock pools at College Rocks in Aberystwyth, Wales, which were archived in the collection of the late Professor Angela Davies, were used in the current study. Screening of blood films for parasite stages was undertaken using 100x oil immersion on a Nikon Eclipse 80i photomicroscope with Nikon DN100/DS-5 M digital camera and Nikon NIS 2.10 image analysis system for image capture.

Fig. 1. Photograph of the fish host *Lipophrys pholis*, one of the type hosts of *Haemogregarina bigemina,* screened in this study.

2.2. Molecular and phylogenetic analysis

Total genomic DNA was isolated from blood samples from 3/7 of the *L. pholis* using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer's protocol (QIAGEN Inc.). A whole genome amplification step was performed for each sample using an REPLI-g Mini Kit (QIAGEN Inc.) prior to amplification of partial fragments of 18S rDNA using primers HEP300 and HEP900 by PCR as described by Inokuma et al. (2002). PCR amplicons were visualised in 1% agarose gels with gel red (Cambridge Biosciences™) prior to sequencing using the same PCR primers with Fluorescent Dye Terminator Sequencing Kits (Applied Biosystems™) run on an Applied Biosystems™ 3730XL automated sequencer. Resultant sequences were assembled, and edited manually to resolve ambiguous base calls, in BioEdit (Hall, 1999). BLASTn searches were performed at NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to provide initial identification and to ensure no contamination and sequences were submitted to GenBank (accession numbers: MK393799-MK393801).

The generated sequences were aligned along with published 18S rDNA sequences representing other adeleorine and related apicomplexan species retrieved from GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), using MUSCLE (http://www.ebi.ac.uk) and Gblocks (http://phylogeny.lirmm.fr/phylo_cgi/one_task.cgi?task_type=gblocks).

Maximum parsimony (MP) and maximum likelihood (ML) phylogenies were constructed in MEGA v7 (Kumar et al., 2016) with two *Rhytidocystis* Henneguy, 1907 species as an outgroup. The MP phylogeny was constructed using the Subtree-Pruning-Regrafting (SPR) algorithm. For the ML analysis was performed under the conditions of the T92 model with gamma distribution, which based on the lowest Bayesian information criterion was determined as the best fit model for the data using the model test function in MEGA v7. In both the MP and ML analyses, 1000 bootstrap replicates were used to estimate nodal support values. Bayesian inference (BI) analysis was also performed using the MrBayes 3.2.6 (Huelsenbeck and Ronquist, 2001) plugin in Geneious 9.0.5 (https://www.geneious.com), with the GTR + G model and two independent runs, for 10^6 generations, each with four heated chains (heated chain temp. of 0.2), with a sampling frequency of every 100 generations and a burn-in of 25%.

As a measure of divergence, uncorrected pairwise genetic distance (*p*-distance) between sequences was calculated using Mega v7.

3. Results

3.1. Screening of fish

Intraerythrocytic stages of *H. bigemina*, including trophozoites, meronts, dividing meronts, and characteristic paired gamonts (Fig. 2), were observed in the blood films from all seven *L. pholis* (9.0 ± 2.5 cm TL, range: 4.4–12.0 cm TL) examined. These stages conformed to the standard description of *H. bigemina* from this fish host in this location, where 100% prevalence in fish ≥4.5 cm TL has previously been observed (Davies and Johnston, 1976; Davies, 1982; Davies et al., 2004; Hayes and Smit, personal observations).

3.2. Molecular and phylogenetic analysis

As no other blood parasites were seen in blood films the 565 bp fragment rDNA 18S sequences generated from three of the seven *L. pholis* are considered to correspond to *H. bigemina*, with BLASTn searches identifying a 93–97% similarity to other apicomplexans sequenced from marine fish hosts.

The MP and ML, and BI, phylogenies (final alignment of 914 bp) provided the same overall topologies with high bootstrap support, or posterior probabilities, respectively, and only minor variations between sequence position within specific clades (Fig. 3 A and B; Supplementary

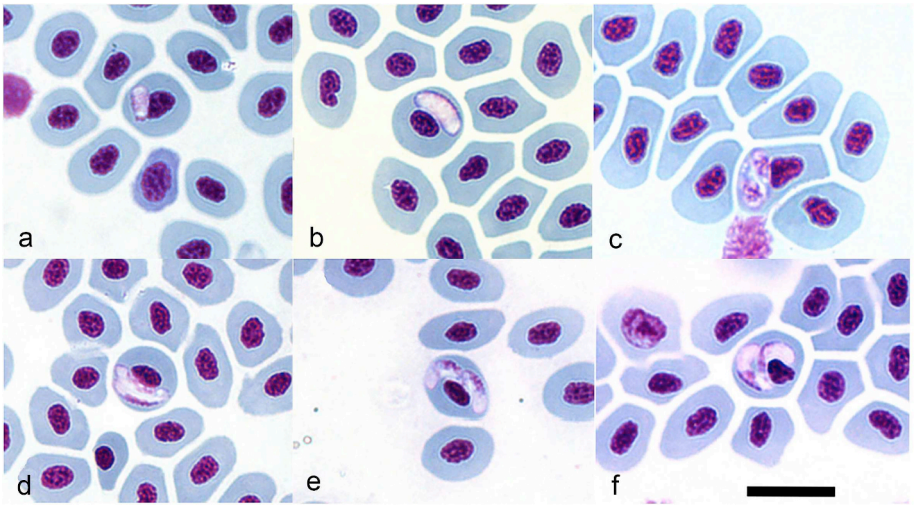

Fig. 2. Stages of *Haemogregarina bigemina* from Giemsa-stained blood films of *Lipophrys pholis* from the UK. (a) trophozoite, (b) meront, (c–e) dividing meronts, and (f) paired gamonts. Scale bar = 10 μm.

data S1). The *H. bigemina* sequences from this study did not cluster with representatives of *Haemogregarina* (sensu stricto) and, also failed to cluster with any of the other haemogregarine or other adeleorine genera, instead forming a unique clade with unspecified apicomplexan parasites of marine fishes (KT806396, KT806397, KT806398) from the Caribbean and an unidentified symbiont of coral (AF238264) outside of the adeleorine clade altogether (Fig. 3 A and B, clade i). Within the large adeleorine grouping, six distinct clades were formed, representing *Adelina* Hesse, 1911, *Dactylosoma* Labbé, 1894, *Haemogregarina* (sensu stricto), *Karyolysus* Labbé, 1894, *Hemolivia* and *Hepatozoon* clades (Fig. 3 A and B, clades ii-vii). A third distinct clade comprised of a single *Choleoeimeria* sp. and several *Goussia* spp., which are apicomplexan parasites of fish tissues, was bracketed by the two former major clades.

The separation of *H. bigemina* from other *Haemogregarina* species was further supported by high *p*-distance values (Table 1) demonstrating high divergence (16–20%), well in excess of related species, between the *H. bigemina* UK sequences and the representative *Haemogregarina* (sensu stricto) species sequences.

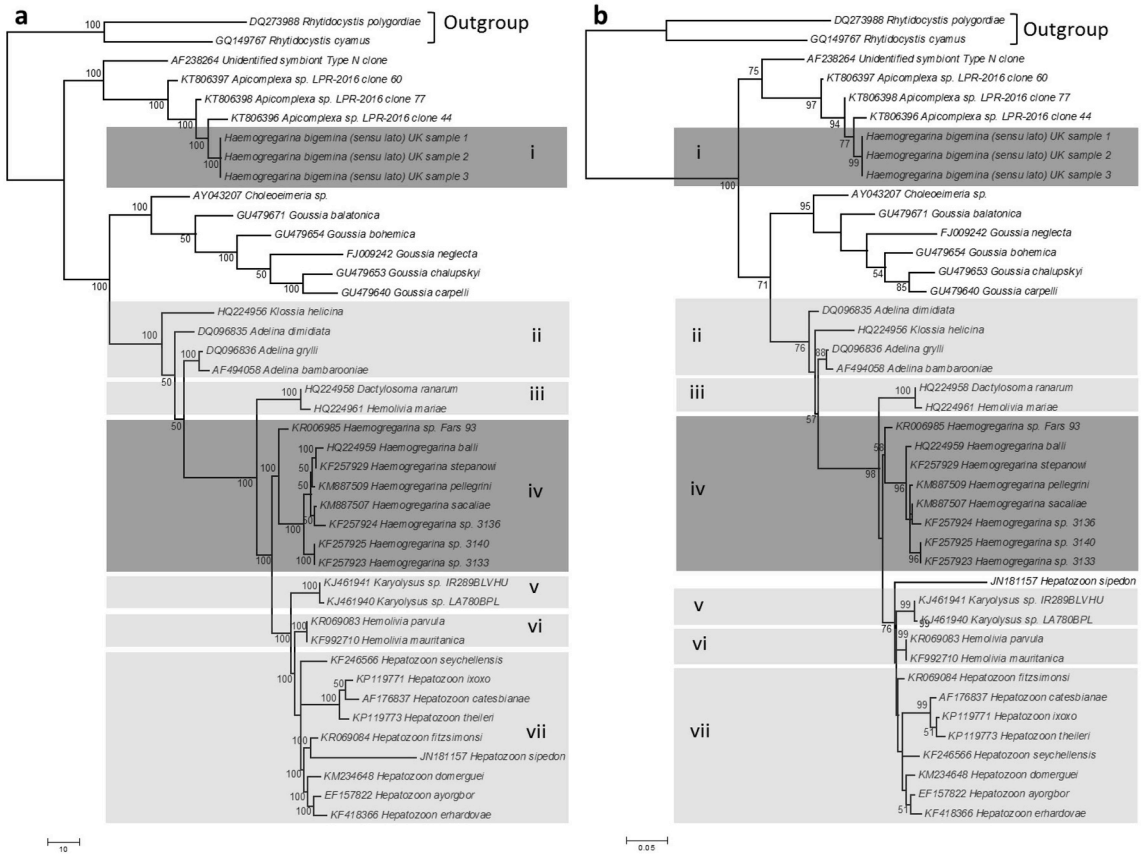

Fig. 3. Phylogenetic identification of *Haemogregarina bigemina* from the UK based on 18S rDNA sequences. (a) Maximum parsimony and (b) Maximum likelihood reconstructions revealing the unique position of UK *H. bigemina* samples outside of the adeleorine groups. For both phylogenies nodal support was calculated using 1000 bootstrap replicates with only values > 50% presented.

Table 1
Uncorrected pair-wise distance (*p*-distance) between *Haemogregarina bigemina* and other *Haemogregarina* spp. based on number of base differences per site between sequences of 18S rDNA.

Species	1	2	3	4	5	6	7	8	9	10	11
1 *Haemogregarina bigemina* UK 1											
2 *Haemogregarina bigemina* UK 2	0.000										
3 *Haemogregarina bigemina* UK 3	0.000	0.000									
4 *Haemogregarina* sp. KR006985	0.208	0.208	0.208								
5 *Haemogregarina sacaliae* KM887507	0.161	0.161	0.161	0.034							
6 *Haemogregarina pellegrini* KM887509	0.166	0.166	0.166	0.030	0.005						
7 *Haemogregarina balli* HQ224959	0.168	0.168	0.168	0.033	0.009	0.007					
8 *Haemogregarina stepanowi* KF257929	0.168	0.168	0.168	0.031	0.007	0.004	0.004				
9 *Haemogregarina* sp. KF257924	0.165	0.165	0.165	0.034	0.011	0.011	0.011	0.011			
10 *Haemogregarina* sp. KF257925	0.161	0.161	0.161	0.034	0.013	0.012	0.013	0.013	0.009		
11 *Haemogregarina* sp. KF257923	0.161	0.161	0.161	0.034	0.013	0.012	0.013	0.013	0.009	0.000	

4. Discussion

The taxonomic status of *H. bigemina* has been under scrutiny for some time, with Davies et al. (2004) questioning its taxonomic placing within the genus *Haemogregarina* (sensu lato) and suggesting that rather than remaining in this genus, *H. bigemina* might be deserving of a genus in its own right. The main argument for their proposed taxonomic re-evaluation of the generic status of *H. bigemina* was based on the finding that gnathiid isopods can act as vectors of *H. bigemina*. This argument was justifiable at the time as the development in a leech vector forms part of the generic characteristics of the *Haemogregarina* (sensu lato). However, the discovery by Hayes et al. (2006) that another marine fish haemogregarine, *H. curvata* is transmitted by a leech, made the establishment of a separate genus for marine fish haemogregarines solely based on the type of vector and development within that vector, basically impossible. Curtis et al. (2013), subsequently demonstrated that gnathiid isopods also act as the vector of at least one other marine haemogregarine.

Considering the questions surrounding its taxonomic placing and given that its development in arthropods does not fully match that of members of *Haemogregarina* (sensu stricto), the *H. bigemina* sequences from this study not clustering with representatives of *Haemogregarina* (sensu stricto) is not entirely surprising and lends support to the suggestion of a new genus for *H. bigemina* by Davies et al. (2004). What is even more interesting is that *H. bigemina* not only grouped outside of the *Haemogregarina* (sensu stricto), which was also supported by high p-distance values, but formed a unique “marine clade” with unspecified apicomplexan parasites of reef fishes, *Stegastes* spp. and *Ophioblennius macclurei* (Silvester, 1915), from the Caribbean (Renoux et al., 2017; Sikkel et al., 2018) and an unidentified apicomplexan symbiont of coral, *Montastrea annularis* (Ellis & Solander, 1786), also from the Caribbean (Toller et al., 2001), completely outside of the Adeleorina altogether. This not only builds on the idea of Davies et al. (2004) that *H. bigemina* could potentially be assigned to a new genus, but even further suggests that *H. bigemina* and *H. bigemina*-like species, as well as other closely related marine apicomplexans, might in fact be worthy of a new family in the order Eucoccidiorida.

It is only now that more molecular data is becoming available for members of the different haemogregarine genera, although still limited, that the relationships amongst and between adeleid apicomplexans is starting to be more fully elucidated and revisions are being made or considered, although issues still remain (Karadijan et al., 2015; Maia et al., 2016; Xavier et al., 2018). Furthermore, as demonstrated here in the current study, future taxonomic revisions may extend beyond the generic and possibly family levels.

Despite its relatively uniform appearance in fishes across continents, something that has been questioned is the cosmopolitan distribution of *H. bigemina*, which is unique among aquatic hosts but is reminiscent of the global occurrence of another extraordinary and well-known apicomplexan, *Toxoplasma gondii* (Nicolle & Manceaux, 1908), found predominantly in land-dwelling mammals, including humans.

Whether distinct populations of so-called *H. bigemina* exist, or whether *H. bigemina* may be a complex of more than one species still needs to be elucidated. Morphological and morphometric comparisons of *H. bigemina* across several continents fail largely to distinguish between different *H. bigemina* samples. *Haemogregarina bigemina* also occurs in non-migratory fishes, mostly confined to rocky shores, or reefs and, therefore, it seems unlikely that natural mixing of fish populations that host this apicomplexan would occur. The results of the present study now make it possible for a molecular comparison of so-called *H. bigemina* and *H. bigemina*-like parasites from other localities worldwide with that of *H. bigemina* from its type host here sequenced in order to determine whether those reports all truly represent a single species or a species complex.

This current study of *H. bigemina* in the UK provides molecular and phylogenetic evidence that this parasite is in fact neither a *Haemogregarina* species nor an adeleorine. This is the first step to unravelling this parasite's true identity and illustrates the need to revisit the classification of this protist and other *H. bigemina*-like parasites within *Haemogregarina* (sensu lato), not only suggesting the need for a new genus but, in fact, a potential new family outside of the Adeleorina. However, this can only be confirmed once a significant number of other marine fish blood parasites from different hosts and localities have been sequenced.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2019.01.006.

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