Detection and characterisation of haplosporidian parasites of the blue mussel *Mytilus edulis*, including description of the novel parasite *Minchinia mytili* n. sp.

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ABSTRACT: The edible mussel *Mytilus edulis* is a major aquaculture commodity in Europe, with 168 000 t produced in 2015. A number of abundant, well characterised parasites of the species are known, though none are considered to cause significant mortality. Haplosporida (Rhizaria, Endomyxa) is an order of protistan parasites of aquatic invertebrates, the best studied of which are the oyster pathogens *Haplosporidium nelsoni* and *Bonamia ostreae*. While these species are well characterised within their hosts, the diversity, life-cycle and modes of transmission of haplosporidians are very poorly understood. Haplosporidian parasites have previously been reported from *Mytilus* spp., however the majority of these remain uncharacterised, and no molecular data exist for any species. In this study, we identified 2 novel haplosporidian parasites of *M. edulis* present in the UK. The first of these, observed by light microscopy and *in situ* hybridisation infecting the gills, mantle, gonadal tubules and digestive connective tissues of mussels in the Tamar estuary, England, we describe as *Minchinia mytili* on the basis of 18S sequence data. The second, observed infecting a single archive specimen collected in Loch Spelve, Mull, Scotland, infects the foot muscle, gills and connective tissue of the digestive gland. Sequence data places this parasite in an uncharacterised clade of sequences amplified from tropical bivalve guts and water samples, sister to *H. nelsoni*. Screening of water and sediment samples collected at the sample site in the Tamar estuary revealed the presence of both sequence types in the water column, suggesting host-free or planktonic life stages.

KEY WORDS: Ascetosporea · Haplosporida · *Haplosporidium* · *Minchinia* · Mussel · *Mytilus* · Parasite

1. INTRODUCTION

The blue mussel *Mytilus edulis* Linneaus, 1798 is of immense significance to aquaculture worldwide, with over 190 000 t produced globally in 2015 (FAO 2017). The majority of these mussels were farmed in Europe (168 000 t), with France (61 000 t), the Netherlands (54 000 t) and the UK (20 000 t) being the largest producers. Outside of Europe, the Atlantic coast of Canada is also a major producer (22 000 t in 2015). In addition to the value of this species to global aquaculture, as filter feeders, mussels play an impor-

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tant role ecologically, filtering waterborne nutrients and particles, and their sessile lifestyle makes them ideal proxies for the detection of local environmental contaminants such as heavy metals and organic chemicals, as demonstrated by the United States' NOAA Mussel Watch program (Kimbrough et al. 2008).

Continued interest in the economic exploitation of mussels for aquaculture has led to a number of attempts to establish baselines for population health, aiming in particular to shed light on the parasites and pathogens of the species, their significance and distribution (e.g. Figueras et al. 1991, Bignell et al. 2008). Such studies predominantly utilize histopathological methods and have identified several highly prevalent, widely distributed parasites, including the copepod *Mytilicola intestinalis* and the gill ciliate *Ancistrum mytili*. Both of these are ubiquitous in *Mytilus* spp. populations across Europe, and though they can be linked to a loss of condition, they are not thought to be associated with increased mortality in the host (Villalba et al. 1997). More recently, studies of baseline population health have incorporated molecular screening of mussel tissues for specific parasite groups, allowing for the more sensitive detection of rarer, low-prevalence parasitic infections (e.g. Lynch et al. 2014).

Over recent decades, the protistan class Ascetosporea (Rhizaria, Endomyxa) has emerged as an important group of parasites of economically significant aquatic species. Ascetosporea is comprised of 5 orders, the most well known of which is the Haplosporida, which includes species known to infect molluscs, crustaceans and annelid worms in both marine and freshwater environments. The orders Mikrocytida, Paramyxida and Paradinida also include important invertebrate pathogens, though their diversity and host range are only very recently becoming apparent (Hartikainen et al. 2014b, Ward et al. 2016, 2018). The fifth order, Claustrosporida, remains very poorly characterised, comprising just 2 described species, *Claustrosporidium gammari* and *C. asellii*, with no molecular data available for either species (Larsson 1987, Cavalier-Smith & Chao 2003).

Haplosporida includes over 40 described species across 4 genera (*Haplosporidium*, *Minchinia*, *Bonamia* and *Urosporidium*) and a number of undescribed species (Arzul & Carnegie 2015). In addition, recent haplosporidian-targeted molecular studies using group-specific primer sets to probe environmental water and sediment (Hartikainen et al. 2014a) and bivalve gut samples (Pagenkopp-Lohan et al. 2016)

have revealed significant uncharacterised molecular diversity within the order.

The most well-studied haplosporidian species are the oyster parasites *Haplosporidium nelsoni* and *Bonamia ostreae*, responsible for mass mortalities in *Crassostrea* spp. and *Ostrea edulis* respectively (Ford & Haskin 1982, Robert et al. 1991). Economic losses associated with *B. ostreae* infection are so significant that the parasite is currently listed as notifiable to the World Organisation for Animal Health (OIE) (http://oie.int/en/international-standard-setting/aquatic-code 2017).

Urosporidium spp. parasitise free-living annelid worms and Platyhelminthes, themselves parasites of marine molluscs and crustaceans. *Minchinia* spp. have been reported only from marine molluscs (Ford et al. 2009), and *Bonamia* spp. from oysters (Engelsma et al. 2014). The most speciose genus, *Haplosporidium*, is comprised of species parasitising molluscs, crustaceans and annelid worms in both marine and freshwater environments (Arzul & Carnegie 2015).

The morphology of haplosporidian spores has frequently been used to assign species to genera, with species belonging to the 'microcell' genus *Bonamia* previously presumed not to form spores (Perkins 2000). Haplosporida form ovoid, walled spores without polar filaments or tubules, with an orifice at one pole. *Urosporidium* spp. produce unornamented spores with an internal flap composed of spore wall material covering the orifice. In *Minchinia* and *Haplosporidium* spp., the orifice is covered by an external hinged lid. Both genera form ornamented spores, though differ in the origin of this ornamentation: in *Minchinia* spp., ornamental extensions are formed of episporum cytoplasm, while the extensions of *Haplosporidium* spp. spores are composed of spore wall material (Burreson & Ford 2004). All *Urosporidium* and *Minchinia* species for which the spore structure is known are concordant with these criteria, but the only species of *Bonamia* known to form spores, *B. perspora*, produces spores matching the criteria for *Haplosporidium* (Carnegie et al. 2006). Further, lineages with the 'haplo' spore structure do not form a monophyletic clade (Burreson & Reece 2006; supported by more recent analyses by Hartikainen et al. 2014a, Pagenkopp-Lohan et al. 2016) and are therefore unsuitable for defining *Bonamia* and *Haplosporidium*; even more so when the necessary taxonomic revisions have been made to *Haplosporidium*. It is likely that more genera must be created in order to resolve the paraphyletic *Haplosporidium*. However, with spore structure and molecular data cur-

rently lacking for the type species, *H. scolopli*, it remains unclear which clade should be considered the true *Haplosporidium* (Arzul & Carnegie 2015).

The life-cycles of haplosporidian parasites are also largely unknown, though evidence exists to suggest direct transmission between hosts in at least some non spore-forming *Bonamia* species, including *B. ostreae* (Engelsma et al. 2014). Numerous unsuccessful attempts have been made at direct transmission of *H. nelsoni*, and so the involvement of intermediate hosts in the parasite life-cycle is suspected, though none have been identified (Powell et al. 1999). The presence of a large diversity of haplosporidian sequence types in the water column and sediment may suggest either free-living transmissible stages or the involvement of planktonic metazoans in haplosporidian life-cycles (Hartikainen et al. 2014a).

Haplosporidians have been reported in *Mytilus* spp. mussels on numerous occasions, however only 2 have ever been formally described: *H. tumefacientis*, causing distinctive tumour-like lesions in the kidney and digestive tissues of *M californiensis* in California, USA (Taylor 1966), and *H. mytilovum*, infecting the ovocytes of *M. edulis*, which was later reassigned to the microsporidian genus *Steinhausia* following microscopic examination of fresh material (Sprague 1972). More recently, haplosporidians have been observed infecting *Mytilus* spp. in Maine, USA (Figueras et al. 1991), southern France (Comps & Tigé 1997) and the Atlantic coast of Canada (Stephenson & McGladdery 2002). However, insufficient morphological data were available in each case to formally describe any of these parasites, and no molecular data exists for any haplosporidian parasite of *Mytilus*.

This study combined traditional histopathological light microscopy with targeted, group-specific environmental and host-focused molecular probing and *in situ* hybridisation (ISH) to characterise a novel haplosporidian parasite infecting *M. edulis* in Devon, England, UK. A further novel haplosporidian is informally described from a single archive specimen of *M. edulis* from the west coast of Scotland.

2. MATERIALS AND METHODS

2.1. Sample collection

A total of 156 *Mytilus edulis* were collected from the River Tamar estuary mouth near Cremyll Ferry, Devon, UK, in June 2013 and incubated overnight (Hartikainen et al. 2014b) in sterile artificial seawater (Culture Collection of Algae and Protozoa recipe) in batches of 10, clustered according to sampling proximity. Transverse sections to include the digestive gland, gonad, gills and mantle were then fixed in Davidson's solution for histological processing and examination, 2.5% glutaraldehyde for transmission electron microscopy (TEM) and 100% molecular-grade ethanol for molecular analyses, as in Ward et al. (2016). Subsequent processing for microscopy and DNA extraction were as described in Ward et al. (2016). Water samples were collected from 3 sites in the Tamar estuary (Cremyll Ferry, Wilcove and Neal Point), processed and DNA extracted as in Ward et al. (2016). A further 153 *M. edulis* were collected from the same site in July 2013 and tissues fixed for microscopy as above. Digestive gland and mantle tissues were preserved in RNALater (Qiagen) for molecular analyses, as in Ward et al. (2016).

Forty *M. edulis* were collected from a farmed population in Loch Spelve, Mull, Scotland, UK, as part of an investigation into reported mortalities in January 2011. DNA was extracted from formalin-fixed material from a single specimen in which haplosporidian plasmodia had been observed during histology screening using the EZNA FFPE DNA extraction kit with the kit's standard xylene deparaffinisation protocol (Omega Biotek).

2.2. PCR and sequencing

All PCR reactions were performed in 20 µl volumes consisting of 1× Promega colourless buffer, 2.5 mM MgCl$_2$, 0.4 mM dNTPs, 0.5 µM of each primer, 0.2 mg bovine serum albumin (BSA), 0.5 U GoTaq G2 (Promega) and 1 µl template DNA. The sequences of all primers used in this study are shown in Table 1. DNA extracted from *M. edulis* tissues from the Tamar estuary was screened using the nested, haplosporidian-specific primer set of Hartikainen et al. (2014a), targeting the V7-V9 variable regions of the 18S rRNA gene. Resultant amplicons were visualised on 2% agarose gels stained with GelRed (Biotium) and Sanger sequenced unidirectionally using primer V5fHapl.

The 2 distinct haplosporidian sequence types (Type 1 and Type 2) amplified from these tissues were aligned with all known haplosporidian sequence types using the MAFFT e-ins-I algorithm (Katoh & Standley 2013). This alignment was used to design specific primer sets for each sequence type to be used for lineage-specific PCR screening of incubation and eDNA samples and for the gen-

Table 1. Sequences of primers used in this study for group- and species-specific haplosporidian probing and 18S sequence extension. Reference indicates original publication of primer sequences; where none is provided, primers were designed for this study

Primer name	Primer sequence (5′–3′)	Purpose	Reference
C5fHap Sb1n	GTA GTC CCA RCY ATA AAC BAT GTC GAT CCH TCY GCA GGT TCA CCT ACG	General haplosporidian 18S nested PCR first round	Hartikainen et al. (2014a)
V5fHapl Sb2nHap	GGA CTC RGG GGG AAG TAT GCT CCT TGT TAC GAC TTB TYC TTC CTC	General haplosporidian 18S nested PCR second round	Hartikainen et al. (2014a)

Hap-M258f
Hap-M412r
	AAC TTT TAG CGT CCA GCC CA
	CGA GGT TGC CAA GTT CTT TCG
		Minchinia mytili-specific tissue and
		  environmental PCR screens and
		  in situ hybridisation probe generation	

Hap-E312f
Hap-E620r
	CAT AGC AGA TGG AAG TTT GAG G
	GGA GCC AAA TCC GAG GAC TT
		Haplosporidium sp.-specific tissue and
		  environmental PCR screens and *in situ*
		  hybridisation probe generation	

Hap-E449r	TTG GAT GCA CTT TCA AGA TTA CC	*Haplosporidium* sp.-specific 18S reverse primer used for SSU sequence extension	
HapGenFor33 HapGenFor84	TTG YCT YAA AGA TTA AGC CAT GCA CTG TGA AAC TGC AKA TGG CTC	General haplosporidian 18S forward primer for SSU sequence extension	

eration of ISH probes. The specificity of each primer set was tested by screening a panel of tissues infected with other haplosporidian species (*Bonamia ostreae*, *Haplosporidium nelsoni*, *H. edule*, *H. littoralis*, *H. costale* and *Minchinia mercenariae*) and uninfected, PCR-negative host DNA samples. In each case, an amplicon was produced only from templates known to be the intended lineage, as confirmed by Sanger sequencing. The first round general haplosporidian primer set of Hartikainen et al. (2014a), C5fHap and Sb1n, was used for the first round for both lineage-specific PCRs. PCR probing for Type 1 used primers Hap-M258f and Hap-M412r and the following cycling conditions: 5 min denaturation at 95°C, followed by 30 cycles of 95°C for 1 min, annealing at 63°C for 1 min and extension at 72°C for 1 min. Amplicons were extended by final incubation at 72°C for 10 min and stored at 4°C. Screens for Type 2 used primers Hap-E312f and Hap-E620r and the same conditions as for Type 1, but with an annealing temperature of 64°C. The single haplosporidian-infected *M. edulis* collected from Loch Spelve, Scotland, was screened using the Type 1 and Type 2 primer sets individually as a single-round PCR only (i.e. no nesting). Mussels collected in the Tamar estuary were screened once more by PCR separately using only Type 1 and Type 2 lineage-specific primers (i.e. not nested) to give an approximation of prevalence of molecular signal.

Prior to phylogenetic analysis, both haplosporidian SSU sequence types were extended by PCR to include the V2-V7 variable regions. To extend

Type 1, first round PCR used primers HapGenFor33 and Hap-M412r, followed by a second round using primers HapGenFor84 and Hap-M412r. For Type 2, first round PCR used primers HapGenFor33 and Hap-E620r, followed by a second round using Hap-GenFor84 and Hap-E449r. All 4 PCRs used the following cycling conditions: 5 min denaturation at 95°C, followed by 40 cycles of 95°C for 30 s, annealing at 55°C for 1 min and extension at 72°C for 90 s. Amplicons were extended by final incubation at 72°C for 12 min and stored at 4°C. Amplicons were then visualised on a 1.5% agarose-TAE gel stained with GelRed, and bidirectionally sequenced using second-round primers. Low-quality base calls were removed before consensus sequences were formed from these reads and initial V7-V9 amplicons.

2.3. Phylogenetic analysis

All available full-length haplosporidian 18S sequence types and those covering the V5-V9 variable regions were downloaded from NCBI GenBank in August 2017. Sequences not overlapping this region or without significant (>200 bp) sequence overlap were excluded from analyses. BLASTn searches of haplosporidian sequences against the GenBank database were used to identify uncharacterised (including environmental) sequences related to known taxa. The extended haplosporidian sequence types generated in this study were aligned with these using MAFFT ver-

sion 7, e-ins-I algorithm (Katoh & Standley 2013). The resulting alignment, using the closest known haplosporidian relatives ENDO-3 (Bass et al. 2009) as an outgroup, was refined by eye and analysed in RAxML BlackBox v.8 (Stamatakis et al. 2008, Stamatakis 2014) (GTR model with CAT approximation; all parameters estimated from the data). A Bayesian consensus tree was constructed using MrBayes v.3.2.5 (Ronquist et al. 2012). Two separate MC^3 runs with randomly generated starting trees were carried out for 2 million generations each with 1 cold and 3 heated chains. The evolutionary model applied a GTR substitution matrix, a 4 category autocorrelated gamma correction and the covarion model. All parameters were estimated from the data. The trees were sampled every 1000 generations and the first 500 000 generations discarded as burn-in (trees sampled before the likelihood plots reached stationarity). A consensus tree was constructed from the remaining sample.

2.4. Histology and *in situ* hybridisation (ISH)

M. edulis tissues were preserved, prepared for histology and screened for pathogens using light microscopy as in Ward et al. (2016). Haplosporidian Type 1-specific probes for ISH were generated by PCR using a sample previously PCR-positive for this sequence type in which haplosporidian plasmodia were visible in histology as template DNA. Type 2-specific probes used a PCR-positive *M. edulis* sample as a DNA template, though no plasmodia were readily visible in the tissue under light microscopy. Probes were labeled using digoxigenin(DIG)-11-dUTP in reactions of 100 µl volume with a final concentration of 1× Promega colourless buffer, 2.5 mM $MgCl_2$, 20 µM PCR DIG labeling mix (Roche), 5 U of Promega GoTaq G2 polymerase, 6 µl of template DNA and 0.5 µM each of primers Hap-M258f and Hap-M412r (Type 1) or Hap-E312f and Hap-E620r (Type 2). Cycling conditions were as detailed in Section 2.2 for lineage-specific PCR. Tissue sections 4 µm thick were mounted onto Poly-L lysine slides, de-paraffinised by immersion in Clearene (Leica Biosystems), rehydrated and treated with Proteinase K solution (45 µg ml^{-1}) for 20 min at 37°C in a humid chamber. Proteolysis was terminated by incubation in distilled water and slides rinsed in ice-cold 12% acetic acid for 45 s, 70% industrial methylated spirits (IMS) for 3 min, 100% for 3 min and 2× saline sodium citrate (SSC) buffer for 1 min with agitation. Sections were overlaid with hybridisation solution (4× SSC buffer, 50% formamide, 1× Denhardt's solution, 10% dextran sulphate, 250 µg ml^{-1} salmon sperm DNA) containing 10 ng µl^{-1} probe (negative controls lacked the DIG-labelled probe in the hybridisation buffer). Slides were heated to 94°C for 7 min and hybridised overnight at 47°C. After hybridisation, sections were washed in room temperature washing buffer (6 M urea, 0.5× SSC and 20 µg ml^{-1} BSA), followed by 2 further washes at 47°C and immersion in 1× SSC at 47°C. Slides were blocked with 6% non-fat milk in Tris buffer (pH 7.5) before incubation with anti-DIG antibodies conjugated with alkaline phosphatase (Roche) in Tris pH 7.5 (1:300) for 1 h in darkness. Reactions were developed with 20 µl ml^{-1} nitroblue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate (NBT/BCIP) (Roche) in Tris pH 9.5 until staining developed. Sections were then rinsed in Tris pH 9.5 and counterstained with 0.5% Bismarck Brown Y in 30% ethanol, rinsed with tap water and dehydrated in IMS before they were coverslipped with Eukitt (Sigma). Sections were examined under light microscopy for the presence of haplosporidian stages.

3. RESULTS

3.1. Histology and *in situ* hybridisation

Haplosporidian plasmodia were observed by light microscopy in 6 out of 309 individual *Mytilus edulis* collected in the Tamar estuary. The same haplosporidian sequence type, Type 1, was amplified from all 6 individuals using a nested, haplosporidian-specific PCR. Haplosporidian plasmodia were observed associated with the gonadal tubules in the mantle, gill epithelium, vascular spaces and in the connective interstitial tissues of the digestive gland. In the mantle, unicellular stages were occasionally seen (Fig. 1A) occurring within the cytoplasm of the tubule epithelial cells. More frequently, larger multinucleate plasmodia containing several cells (up to approximately 12) were observed within the epithelium. In heavily infected tubules, the epithelium was disrupted or destroyed. Limited haemocyte infiltration with release of plasmodia into the lumen was seen in a few cases (Fig. 1B). Evidence of parasite division by cleavage and plasmotomy could be discerned (Fig. 1A,B,D) and migration of plasmodia through the epithelium of the gonadal tubule into the lumen was also detected (Fig. 1C). Gill infections were associated with the presence of numerous plasmodial stages within the respiratory epithelium and
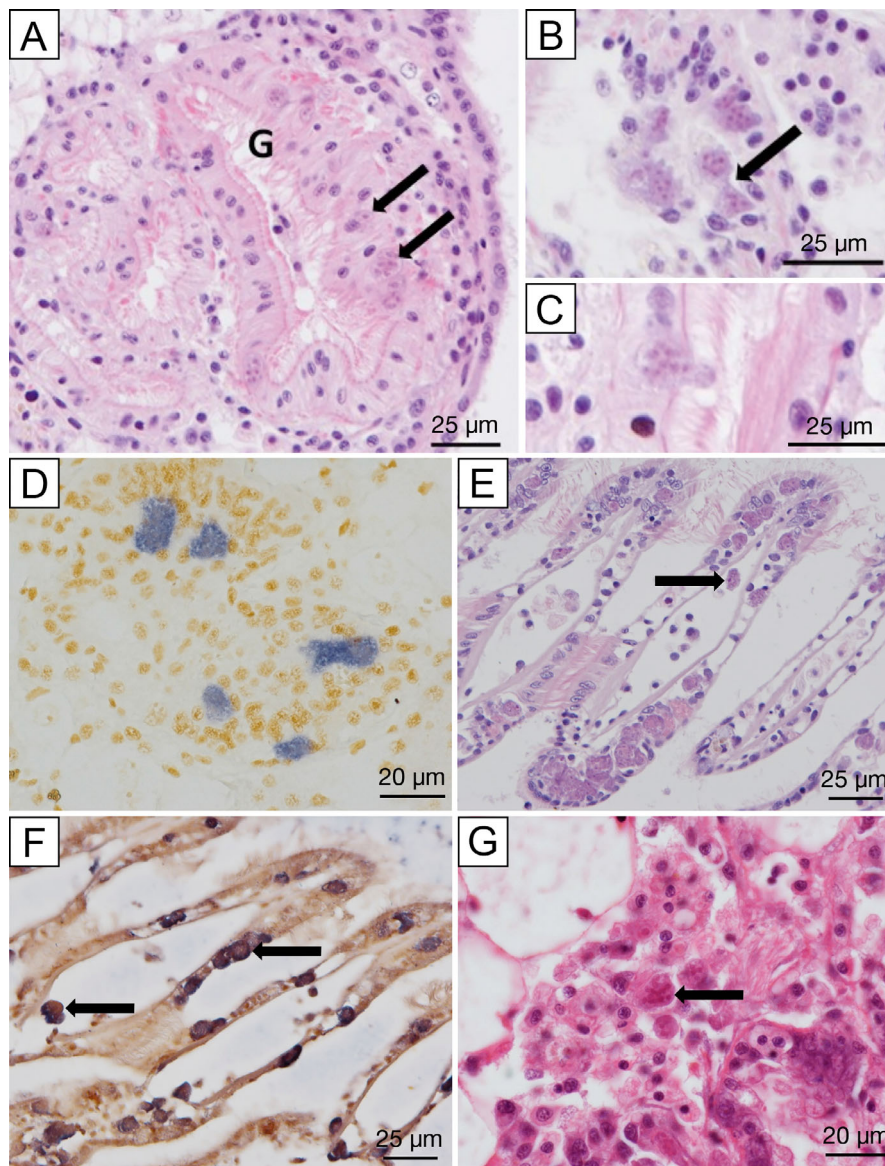
Fig. 1. Light micrographs showing *Minchinia mytili* cells in the gill, mantle and digestive gland tissues of host *Mytilus edulis*. (A) Section through several gonadal tubules (G) in the mantle of *M. edulis*, 2 of which contain unicellular stages and multinucleate intracellular plasmodia (arrows). (B) Plasmodial division by plasmotomy, each section containing several cells. (C) Migration of a single plasmodium through the epithelium of the gonadal tubule to the lumen. (D) Section from the same specimen as (B) showing labelling of plasmodia in a gonadal tubule. (E) Plasmodia within the gill epithelium with a few also present in the vascular space (arrow). (F) Same specimen as (E), showing specific labelling of haplosporidian plasmodia (arrows). (G) Interstitial tissue of the digestive gland with several plasmodia present (arrow). H&E stain in (A–C, E, G); *in situ* hybridization labelling of plasmodial cytoplasm in (E,F)

occasionally in the vascular spaces (Fig. 1E). No direct evidence of parasite migration through the epithelium was seen. Unlike infections in the gill and mantle, digestive gland infections were associated with mild haemocyte infiltration (Fig. 1G). TEM examination of digestive gland tissues from infected animals was unable to locate haplosporidian life stages for ultrastructural characterisation. ISH using

the Type 1-specific probe bound to haplosporidian plasmodia in the mantle (Fig. 1D), gill (Fig. 1F) and digestive gland (not shown).

Of 40 *M. edulis* collected during the investigation of a mortality episode in farmed mussels on the west coast of Scotland, a severe haplosporidian infection was noted in a single specimen (2.5% of sampled population). Numerous plasmodia were observed

within the foot muscle, in the gills and throughout the connective tissue surrounding the digestive gland (Fig. 2). No sporulation stages were observed. Hae-mocytic infiltration and multifocal granulocytomas were observed in this individual and others sampled. Necrotic cells were present within the granulocytomas. DNA was extracted from formalin-fixed tissue from this individual, and subsequent PCR screens using haplosporidian-specific primer sets amplified a different 18S sequence type than the samples from the Tamar estuary (Type 2).

A subset of PCR-positive mussels from the Tamar estuary from which the Type 2 sequence was amplified by a single-round (30 cycle) PCR were screened by ISH using a Type 2-specific probe. In each case no staining of plasmodia in any tissues was observed. Haplosporidian cells were also not observed in H&E-stained tissues, and so no microscopic evidence of this haplosporidian infecting *M. edulis* in the Tamar estuary was observed.

3.2. PCR screening of tissues and environmental samples

A summary of all nested and non-nested PCR screens of tissue and environmental samples is shown in Table 2. PCR screens of DNA extracted from the digestive gland tissues of *M. edulis* collected in the River Tamar estuary in June 2013 using general nested haplosporidian primers (Hartikainen et al. 2014a) produced an amplicon in 20 of 153 samples. Screening of DNA from digestive gland and mantle tissues from *M. edulis* collected at the same site in July 2013 produced amplicons in 63 of 156 and 12 of 156 samples respectively. In total, 11 of 56 mussels sampled produced amplicons from both tissues. Sanger sequencing of amplicons
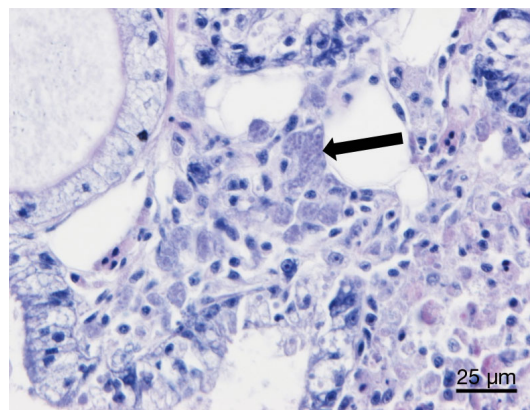

Fig. 2. Light micrograph showing several pleomorphic multinucleate haplosporidian plasmodia (arrow) in the interstitial tissue of the digestive gland of *Mytilus edulis*

from all mussels produced 2 distinct haplosporidian sequence types. Subsequent screens of the same sample sets using separate Type 1 and Type 2-specific primers in a single-round (30 cycle) PCR produced amplicons in 13 of 309 digestive gland and 7 of 156 mantle samples for Type 1, and 14 of 309 digestive gland and 0 of 156 mantle samples for Type 2. Screening of *M. edulis* incubation filters (16 total; June data set only) with 'Type'-specific primer pairs resulted in 8 positives from the Type 1 primers when nested with the first round primers of Hartikainen et al. (2014a) and 6 positives when the same primers were used as a single round, 30-cycle PCR. The Type 2 primers produced no positives as either a nested or a single-round PCR. Screens of filtered water samples collected at 3 sites within the Tamar estuary in June 2013 using the same primer sets and nested and non-nested strategies resulted in amplification from all sites by both primer sets.

Table 2. Prevalence of haplosporidian sequence types in *Mytilus edulis* digestive gland and mantle tissues by haplosporidian-targeted nested, and specific single-round PCR. Numbers in brackets following sample type indicate total number of samples screened. For nested PCR, a total of 60 cycles of amplification were used (30 each in first and second round); for non-nested PCRs, 30 amplification cycles were used

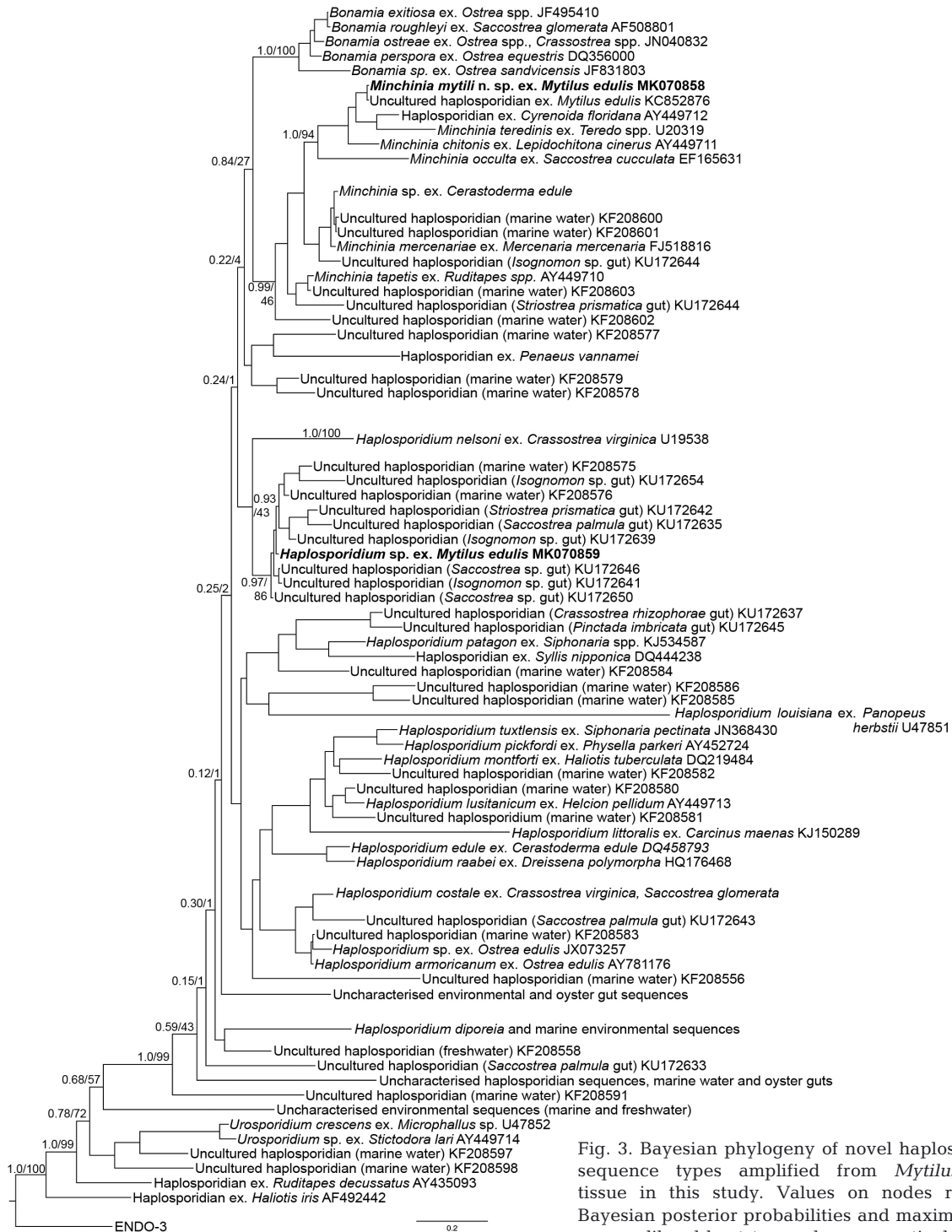| Dataset	*Minchinia mytili* (Type 1)		*Haplosporidium* sp. (Type 2)	
	Nested	Specific	Nested	Specific
June 2013 digestive gland only (153)	9	5	11	2
July 2013 digestive gland (156)	22	8	41	12
July 2013 mantle (156)	13	7	2	0
M. edulis incubation filters (June dataset only) (16)	8	6	0	0
Cremyll water column (6)	1	1	6	4
Wilcove water column (14)	5	3	11	6
Neal Point water column (18)	2	0	17	9

Fig. 3. Bayesian phylogeny of novel haplosporidian sequence types amplified from *Mytilus edulis* tissue in this study. Values on nodes represent Bayesian posterior probabilities and maximum likelihood bootstrap values respectively

3.3. Phylogenetic analyses

Phylogenetic analyses including both nearly full-length 18S haplosporidian sequences with all available haplosporidian 18S rRNA sequence types places Type 1 in a strongly supported monophyletic clade comprising all known *Minchinia* spp. plus related environmental sequences (Fig. 3). As expected from initial BLAST searches of the partial sequence, the closest relative of Type 1 is a previously uncharacterised haplosporidian sequence type derived from mussel tissue collected in the Menai Strait, Wales, UK.

Type 2 falls within a group of uncharacterised sequences from marine water column and tropical oyster gut samples, sister to the oyster parasite *Haplosporidium nelsoni*, with strong support. As in previous studies, branches described as *Haplosporidium* and their relatives do not form a monophyletic clade.

3.4. Taxonomic summary: *Minchinia mytili* n. sp.

Specific diagnosis: Plasmodia associated with the gonadal tubes in the mantle, gill epithelium, vascular spaces and in the connective interstitial tissues of the digestive gland. Unicellular stages occasionally present within the cytoplasm of the tubule epithelial cells of the mantle. This species is distinguishable from all other haplosporidians by its unique 18S rRNA gene sequence, which can be specifically amplified by the diagnostic PCR using primers Hap-M258f/Hap-M412r or the *in situ* hybridisation probe generated by them.

Type host: Blue mussel *Mytilus edulis*

Type locality: Cremyll Ferry, Tamar estuary, Devon, United Kingdom (50° 21′ 34.2″ N, 4° 10′ 24.9″ W)

Site of infection: Systemic, with plasmodia observed associated with the gonadal tubules in the mantle, gill epithelium, vascular spaces and in the connective interstitial tissues of the digestive gland.

Prevalence: Observed in 6 of 309 individuals examined histologically.

Etymology: The species epithet refers to its infection in the bivalve mollusc host *Mytilus edulis*

Type material: Reference tissue blocks (histology) and digoxigenin-stained *in situ* hybridisation slides and ethanol-fixed tissue are deposited in the Registry of Aquatic Pathology (RAP), held at the Centre for Environment, Fisheries and Aquaculture Science (Cefas), Weymouth, UK. Reference RA13082-39

Gene sequence: The 18S rRNA gene sequence has the GenBank Accession Number MK070858

Zoobank registration: urn:lsid:zoobank.org:act:BC4E1F46-FB74-48BB-B716-98054432BB2A

4. DISCUSSION

This study demonstrated the presence of 2 novel haplosporidian parasites infecting *Mytilus edulis* in the UK. The first of these, described here as *Minchinia mytili*, was observed infecting the gill, gonadal tubules and digestive connective tissues of mussels in the Tamar estuary, Devon, England. The

second, which we informally refer to as *Haplosporidium* sp., was found infecting a single *Mytilus edulis* collected in Loch Spelve, Scotland.

Phylogenetic analyses place the *Minchinia mytili* 18S sequence type within the haplosporidian genus *Minchinia* with strong support, along with other parasites of molluscs including the bivalves *Cyrenoida floridana* (*Minchinia* sp.), *Mercenaria mercenaria* (*Minchinia mercenariae*) and *Saccostrea cucculata* (*M. occulta*). Though spore ornamentation is not available for this species, its infection of a molluscan host and strong phylogenetic placement within the genus is sufficient to describe this novel parasite as *M. mytili*.

The absence of sporogonic stages in all infected individuals examined is unusual for *Minchinia* spp., but it is not without precedent. The hard clam parasite *M. mercenariae* has recently been reported infecting cockles *Cerastoderma edule* in Galicia, Spain, with no observed sporogonic stages (Ramilo et al. 2018). As is true for *M. mytili*, *M. mercenariae* was present only at very low levels in the sampled cockle population, and so it is possible that in both cases too few infected individuals were available for examination with sufficiently advanced infection.

M. mytili infections were observed in the gill, mantle and digestive gland. Though no direct evidence of parasite migration through the gill epithelium was observed, the presence of haplosporidian plasmodia in the gills may indicate this tissue to be the route of entry into the host, as is the case for other ascetosporean parasites including *Haplosporidium nelsoni* (Lauckner 1983) and *Marteilia* spp. (Kleeman et al. 2002, Carrasco et al. 2008), with the eventual spread of proliferative (plasmodial) stages systemically through the host.

Another *Minchinia* species, *M. occulta*, infects the gill, mantle, reproductive follicles and digestive diverticulae of the rock oyster *Saccostrea cuccullata*. Sporulation has been observed only in the connective tissues of the digestive gland (Bearham et al. 2008). Infection in this case is presumed to develop in the gills before disseminating to the mantle and then the digestive gland, which may also be the case for *M. mytili*. Only a single individual was examined where *M. mytili* infection had progressed to the digestive connective tissues. It may be that examination of more infected mussels will reveal sporulation of *M. mytili* within the bivalve host. However, it cannot be ruled out that *M. mytili* does not sporulate in *Mytilus edulis*, but does so outside of this host.

Lynch et al. (2014) amplified a haplosporidian sequence type identical to *Minchinia mytili* from the

gill tissue of *Mytilus edulis* collected in the Menai Strait, Wales. Though 'unidentified organisms' were observed in the tissue of the mussel from which the sequence was amplified, they could not be identified as haplosporidian. However, given the presence of the same sequence type in mussels in the Menai Strait, it is likely the same parasite is present in both populations.

Comps & Tigé (1997) reported a haplosporidian producing *Minchinia*-like spores infecting *Mytilus galloprovincialis* in the Thau lagoon, southern France. Infection was limited to the connective tissues surrounding the digestive tubules, with little information to describe plasmodial stages. More recently, Matozzo et al. (2018) observed sporocysts containing haplosporidian-like spores in the digestive gland, gonad and mantle of a single specimen of *M. galloprovincialis* collected in Porto Venere, Gulf of La Spezia, Italy. In this case infection was systemic, with necrosis and atrophy of the digestive tubule epithelium associated with granular haemocytes. *In situ* hybridization using a general haplosporidian probe confirmed the affiliation of this parasite with the order. While it is possible both reports are of the same parasite, no sequence data exist in either case to confirm this.

An undescribed haplosporidian observed by Figueras et al. (1991) infecting *M. edulis* in Maine, USA showed similarities to *Minchinia mytili*. This parasite was observed infecting the tips of the gills, mantle epithelium and digestive connective tissues. Only plasmodial stages were observed, with no spore stages present. As with other reports of haplosporidian parasites of *Mytilus* spp., no molecular data are available for this parasite, and so it is not possible to state with any certainty their relation to *Minchinia mytili*.

We observed a further novel haplosporidian, infecting the digestive connective tissues, foot muscle and gills of a single mussel collected in Loch Spelve, Scotland, during a mortality episode not attributed to the parasite. As with *M. mytili*, spores were not observed in any tissue. Haemocytic infiltration and multifocal granulocytomas were observed in the infected individual and others from the same batch, with necrotic cells present within the granulocytomas. However, scuticociliate, bacterial and other infections were observed within the population (including the haplosporidian-infected individual), which are more likely to be the cause of these pathologies than haplosporidian infection.

The novel sequence type associated with this parasite was amplified from mussel digestive gland tissues from the Tamar estuary, though no haplosporid-

ian life stages were observed by light microscopy or ISH. Phylogenetic analyses place this parasite in a clade comprising uncharacterised sequence types amplified from water column samples and tropical bivalve guts, sister to oyster parasite *H. nelsoni*. This clade is sister to *Bonamia* + *Minchinia*, and so species assigned to the genus *Haplosporidium* are paraphyletic, as in previous studies (Burreson & Reece 2006, Hartikainen et al. 2014a, Pagenkopp-Lohan et al. 2016).

The haplosporidian infecting this specimen differs greatly from the only *Haplosporidium* species described from mussels, *H. tumefacientis* (Taylor, 1965). Though no sequence data is available for this species, tumefactions observed in the kidney and digestive gland of *Mytilus californianus* infected with *H. tumefacientis* are absent in the specimen from Loch Spelve, and so they are unlikely to be the same species.

This parasite is distinct from other haplosporidians observed in mussels, and the sequence type associated with the parasite is also novel; however, we lack sufficient data to fully describe this species and so refer to it informally as *Haplosporidium* sp.

The haplosporidian sequence type associated with *Haplosporidium* sp. was amplified from 41 of 156 of mussels collected in the Tamar estuary in July 2013. Screening the same samples using type-specific primers in a single-round, 30-cycle PCR reduced this number to 12. No haplosporidian life stages were observed in any individual using light microscopy or ISH. Burreson (2008) drew attention to the misuse of PCR assays in molluscan disease diagnosis and rightly stated that PCR-based detection methods must be validated against established microscopic techniques such as histology and *in situ* hybridisation. As such, we have no irrefutable evidence of *Haplosporidium* sp. infecting *Mytilus edulis* in the Tamar estuary. PCR positives indicate only the presence of *Haplosporidium* sp. DNA and in this case may indicate passage of parasite cells or DNA through the gut tubules by filter feeding rather than established infection. Barber & Ford (1992) noted the presence of ingested *H. nelsoni* spores by light microscopy in the digestive lumen of *Crassostrea virginica* collected in Delaware Bay, USA (mean frequency 0.5 spores section^{-1}). Their presence appeared to be negatively correlated with *H. nelsoni* infection, suggesting they were unable to directly infect the oyster. In the case of *Haplosporidium* sp. a single histology slide was read for each individual, so any ingested haplosporidian spores may not be readily apparent. PCR assays are more sensitive than light microscopy, and will also detect haplosporidian DNA from digested cells. This highlights the impor-

tance of validating PCR data with complementary light microscopy and ISH when inferring infection in bivalves.

Both novel haplosporidian sequence types were amplified from water column samples collected at the sample site. This finding is not unexpected; Hartikainen et al. (2014a) detected a wide diversity of haplosporidian sequence types in water and sediment samples, including both characterised and novel lineages. Though haplosporidians have previously been detected in planktonic metazoans by PCR (e.g. *Bonamia ostreae*, Lynch et al. 2007; *H. nelsoni*, Messerman & Bowden 2016), no complementary microscopy or ISH was carried out, and so it is not possible to say whether this is representative of actual infection or trophic interactions. Similarly, it remains to be established how long these molecular signals can persist in the water column. *B. ostreae* has been shown to survive at 15°C in laboratory tests for at least 1 wk (Arzul et al. 2009); however, the effects of predation and other external environmental factors are as yet untested. Further investigation is needed to understand the significance of eDNA findings, for example determining ribosomal activity of haplosporidian sequence types in environmental samples (and therefore which lineages represent living, active cells), and elucidating the role of planktonic metazoans and other invertebrates in haplosporidian life-cycles.

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