Single and multi-gene phylogeny of *Hepatospora* (Microsporidia) – a generalist pathogen of farmed and wild crustacean hosts

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SUMMARY

Almost half of all known microsporidian taxa infect aquatic animals. Of these, many cause disease in arthropods. *Hepatospora*, a recently erected genus, infects epithelial cells of the hepatopancreas of wild and farmed decapod crustaceans. We isolated *Hepatospora* spp. from three different crustacean hosts, inhabiting different habitats and niches; marine edible crab (*Cancer pagurus*), estuarine and freshwater Chinese mitten crab (*Eriocheir sinensis*) and the marine mussel symbiont pea crab (*Pinnotheres pisum*). Isolates were initially compared using histology and electron microscopy revealing variation in size, polar filament arrangement and nuclear development. However, sequence analysis of the partial SSU rDNA gene could not distinguish between the isolates (~99% similarity). In an attempt to resolve the relationship between *Hepatospora* isolated from *E. sinensis* and *C. pagurus*, six additional gene sequences were mined from on-going unpublished genome projects (RNA polymerase, arginyl tRNA synthetase, prolyl tRNA synthetase, chitin synthase, beta tubulin and heat shock protein 70). Primers were designed based on the above gene sequences to analyse *Hepatospora* isolated from pea crab. Despite application of gene sequences to concatenated phylogenies, we were unable to discriminate *Hepatospora* isolates obtained from these hosts and concluded that they likely represent a single species or, at least subspecies thereof. In this instance, concatenated phylogenetic analysis supported the SSU-based phylogeny, and further, demonstrated that microsporidian taxonomies based upon morphology alone are unreliable, even at the level of the species. Our data, together with description of *H. eriocheir* in Asian crab farms, reveal a preponderance for micro-variants of this parasite to infect the gut of a wide array of decapods crustacean hosts and the potential for *Hepatospora* to exist as a cline across wide geographies and habitats.

Key words: edible crab, pea crab, Chinese mitten crab, microsporidian, *Hepatospora*, multi-gene phylogeny, taxonomy, Enterocytozoonidae.

INTRODUCTION

Microsporidia are single-celled eukaryotic intracellular parasites known to infect a range of vertebrate and invertebrate hosts (Mathis *et al*. 2005). Since the inception of the phylum 'Microsporidia', both phylogenetic placement of the group and classification within the group have proven problematic [reviewed in (Corradi and Keeling, 2009)]. Early phylogenies used to place microsporidia within the tree of life failed to account for rate heterogeneity among gene sites, base-compositional biases and the overall accelerated evolutionary rate characteristic of microsporidian genomes (Hirt *et al*. 1999) and thus microsporidia were for a long-time considered basal eukaryotes. They are now, along with the Cryptomycota, considered the most basal fungi group, a new classification based on the phylogenetic analysis of 200 genes (James *et al*. 2013).

Within the phylum, in the past, classification of taxa has been based on structural characteristics, ultrastructural morphology and karyotypic evidence (Canning, 1953; Vavra and Undeen, 1970; Shadduck *et al*. 1990; Cali *et al*. 1993). Now there is a compounding body of evidence supporting the idea that morphological and developmental features in this phylum are plastic between both closely and distantly related microsporidia (Vossbrinck and Debrunner-Vossbrinck, 2005; Stentiford *et al*. 2013). Stentiford *et al*. (2013) observed that microsporidians isolated from marine decapod crustaceans that would have been classed as distantly related taxa (*Nadelspora* and *Ameson*) under a morphology-based classification system are in fact close relatives on rDNA-based phylogenetic trees and are potential life-cycle variants of the same taxon. An example of a highly plastic morphological character used in classification is nuclear configuration. Two configurations are known to exist in microsporidia: a

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monokaryon (an individual nucleus) and a diplokaryon (two apposed nuclei). Whilst some species retain the same nuclear configuration throughout their life cycle, others switch between the nuclear stages depending upon life-cycle stage, and polymorphic species shift between the nuclear stages when changing hosts or tissues (Vávra and Larsson, 2014).

For many years the alternative to morphological characters has been the SSU rRNA gene, which has now been amplified and sequenced from over 1000 species of microsporidia. However, the rDNA sequences amplified are generally only able to resolve taxonomic relationships down to the genus level (Vossbrinck *et al*. 1998; Vossbrinck and Debrunner-Vossbrinck, 2005). The most commonly used primers, F18 and 1492R (Vossbrinck *et al*. 1993; Kent *et al*. 1996) amplify a 1400 bp fragment of the most conserved region of the SSU rDNA and omit the internal transcribed spacer (ITS) (Vossbrinck and Debrunner-Vossbrinck, 2005), a region reported to be highly variable even within species and hence highly informative for intra-species differentiation analyses (Gresoviac *et al*. 2000; Sak *et al*. 2011). However, even if this region was commonly amplified for microsporidia, there are other issues with using SSU as a marker for closely related species. In at least some microsporidia, the process of concerted evolution that typically keeps rDNA copies uniform within the genome, does not seem to be present. For example, Ironside (2013), O'Mahony *et al*. (2007) and Tay *et al*. (2005) observed a higher ITS sequence variation between repeats in the same genome than between different genomes for some *Nosema* species, which means that caution needs to be used when employing rDNA to discriminate between closely related microsporidia.

One of the issues emerging from the difficulty in resolving close phylogenetic relationships and morphological plasticity in microsporidians is the assignment of appropriate species names. This is an important issue because taxonomic names of pathogenic species are fed into legislative frameworks that are used to inform policy making (Stentiford *et al*. 2014). Therefore there is a growing need to find alternative molecular markers to resolve questions in microsporidia taxonomy. There is currently whole-genome data for 20 microsporidian species on the publicly available MicrosporidiaDB database. With cheaper thorough-put sequencing technologies and the advent of single cell genome sequencing, we can only expect this number to increase. This provides a minable resource for molecular characters for multi-locus phylogenies (Capella-gutiérrez *et al*. 2012).

We isolated putative *Hepatospora* sp. parasites from three different crustacean hosts, inhabiting different habitats and niches; marine edible crab (*Cancer pagurus*), estuarine and freshwater Chinese mitten crab (*Eriocheir sinensis*) and the marine mussel symbiont pea crab (*Pinnotheres pisum*). Isolates were initially compared using histology and electron microscopy revealing not only a similar life cycle, but also the variation in size, polar filament arrangement and nuclear development (Table 1). However, sequence analysis of the partial SSU rDNA gene could not distinguish between the isolates (≈99% similarity). Here we take advantage of the current microsporidian whole-genome database and our in-house genomic data to construct a six-gene concatenated phylogenetic tree for *Hepatospora eriocheir*, a parasite of the invasive Chinese mitten crab (Stentiford *et al*. 2011), a pea crab (*P. pisum*) infecting microsporidium (Longshaw *et al*. 2012) and a novel microsporidium that infects commercially important edible crabs (*C. pagurus*), all from European waters. Our results revealed that *Hepatospora* isolates from the three different crustacean hosts are likely to be microvariants of a single species. Further, they support the concept that microsporidian taxonomies based upon morphology are not only unreliable, but can also be deceptive, even at the level of the species. Our data, together with description of *H. eriocheir* as an agent of emergent disease in Asian crab aquaculture (Ding *et al*. 2016) reveal a preponderance for microvariants of *H. eriocheir* to infect the gut of a wide array of decapods crustacean hosts across wide geographic boundaries and in a range of habitats.

MATERIALS AND METHODS

Eriocheir sinensis, Cancer pagurus *and* Pinnotheres pisum *sampling*

Chinese mitten crabs (*E. sinensis*) were sampled from two locations in the Thames Estuary; a site near to the Millennium Dome (51:27:12N, 00-00-44E) and another at Tilbury Power Station (51:27:12N, 00-23-10E). Crabs were collected using Fyke nets and from the screens of water intake pipes at the power station. Edible crabs (*C. pagurus*) were captured using baited pots in the Weymouth and Portland area of the English Channel, UK (50°32′50″N, 002°11′00″W) as previously described (Bateman *et al*. 2011). Live crabs were transported to the Cefas laboratory in Weymouth and anaesthetized by chilling on ice for 30 min before dissection. The hepatopancreas, gill, gonad, central nerve ganglia, heart and body muscle was removed from the crabs and fixed in Davidson's sea water fixative for histology. Additional hepatopancreas samples were fixed in 2·5% glutaraldehyde in 0·1 M sodium cacodylate buffer for electron microscopy, and 100% ethanol and frozen, for molecular analyses.

For *P. pisum* sampling, blue mussels (*Mytilus edulis*) were collected from a range of sites around the UK. Mussels were opened by cutting the

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Table 1. Comparison of host and structure [histological, electron microscopy and molecular data (SSU 18s rRNA)] from the *Hepatospora* isolates

			
Host			
Species	*Pinnotheres pisum*	*Eriocheir sinensis*	*Cancer pagurus*
Family	Pinnotheridae	Varunidae	Cancridae
Habitat	Symbiont of marine mussels	Estuarine	Marine
Microsporidian			
Species	*Hepatospora* sp.	*Hepatospora eriocheir*	*Hepatospora sp.*
Pathology			
Tissue	Hepatopancreas	Hepatopancreas	Hepatopancreas
Site of development	Cytoplasm of hepatopancreatic epithelial cells	Cytoplasm of hepatopancreatic epithelial cells	Cytoplasm of hepatopancreatic epithelial cells
Ultrastructure			
Spore	Ellipsoid	Ellipsoid	Ellipsoid
Size	1·9 × 0·9 μm	1·8 × 0·9 μm	1·8 × 0·9 μm
Polar filament	Isofilar	Isofilar	Isofilar
Polar filament turns	5–6 polar filament coils in a single rank	7–8 polar filament coils in a single rank	7–8 polar filament coils in a single rank
Nuclear status of spore	Diplokaryotic	Unikaryotic	Diplokaryotic
Development	Syncronous development within a parasitophorous vacuole	Syncronous development within a parasitophorous vacuole	Syncronous development within a parasitophorous vacuole
Molecular analysis			
GenBank Accession	–	HE584635·1	HE584633·1
SSU similarity between isolates	99%	100%	100%

abductor muscle and separating the valves. Symbiotic pea crabs (*P. pisum*) found habituating the mantle of the host mussel were removed and anaesthetized on ice prior to bilateral dissection and removal of the hepatopancreas. Small portions of the organ were fixed in Davidson's sea water fixative, 2·5% glutaraldehyde in 0·1 M sodium cacodylate buffer and 100% ethanol for histology, electron microscopy and molecular techniques, respectively.

Histology and transmission electron microscopy

For histology, fixation was allowed to proceed for 24 h before samples were transferred to 70% industrial methylated spirit. Fixed samples were processed to wax in a vacuum infiltration processor using standard protocols. Sections were cut at a thickness of 3–5 μm on a rotary microtome and mounted onto glass slides before staining with haematoxylin and eosin (H&E) and Feulgen stains. Stained sections were analysed by light microscopy (Nikon Eclipse E800) and digital images and measurements were taken using the LuciaTM Screen Measurement System (Nikon, UK). For electron microscopy, tissues were fixed in 2·5% glutaraldehyde in 0·1 M sodium cacodylate buffer (pH 7·4) for 2 h at room temperature and rinsed in 0·1 M sodium cacodylate buffer (pH 7·4). Tissues were post-fixed for 1 h in 1% osmium tetroxide in 0·1 M sodium cacodylate buffer. Samples were washed in three changes of 0·1 M sodium cacodylate buffer before dehydration through a graded acetone series. Samples were embedded in Agar 100 epoxy (Agar Scientific, Agar 100 pre-mix kit medium) and polymerized overnight at 60 °C in an oven. Semi-thin (1–2 μm) sections were stained with Toluidine Blue for viewing with a light microscope to identify suitable target areas. Ultrathin sections (70–90 nm) of these areas were mounted on uncoated copper grids and stained with 2% aqueous uranyl acetate and Reynolds' lead citrate (Reynolds, 1963). Grids were examined using a JEOL JEM 1400 transmission electron microscope and digital images captured using an AMT XR80 camera and AMTv602 software.

Spore isolation and DNA extraction

Hepatopancreas samples from histologically confirmed microsporidian-infected crabs were crushed with a sterile pestle and mortar in PBS. The homogenous mash was filtered through a 100

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μm mesh followed by cell sieving through 40 μm filter. Filtrate was topped up to 50 mL using PBS/triton-X (0·1%) and pelleted at 3220 g in an Eppendorf centrifuge precooled at 4 °C, for 10 min. The supernatant was removed and the pellet (containing host cell debris and microsporidian spores) was resuspended in 2·5 mL of ice-cold water. Homogenate was added to the top of a Percoll density gradient and centrifuged at 1000 g in a pre-cooled centrifuge for 45 min. Phase contrast and fluorescence microscopy were used to visualize the spores and to examine for purity following Percoll gradient purification. Spores were stained with the chitin marker calcoflour white (Darken, 1962).

For extraction of DNA, spores of the Pea crab and Mitten crab parasites were diluted 1 in 10 (w/v) in G2 buffer and Proteinase K was added at a concentration of 2 mg mL^{-1} (Qiagen, UK). The spores were subsequently disrupted in a Matrix D FastPrep cell disrupter (FastPrep, UK) by shaking on a homogenizer for 2 min at highest setting. Homogenized samples were incubated for 4 h at 56 °C. Total DNA was extracted using an EZ1 DNA tissue kit and EZ1 Advanced XL BioRobot (Qiagen) following manufacturers' instructions. For the edible crab parasite, aliquots of purified spores suspended in 1 × PBS were mixed with liquid nitrogen in a sterile mortar. The mixture was slowly stirred until it solidified, after which it was ground with a sterile pestle for 10 min. Liquid nitrogen was again added to the powder and the mix was ground for an additional 10 min. This step was repeated three times before dissolving the resulting powder in 800 μL of phenol (pH 8·0). The homogenate was transferred to an Eppendorf tube and mixed by inversion and subsequently centrifuged for 10 min at 10 000 × ***g***. The recovered supernatant was mixed by inversion with 400 μL of chloroform and centrifuged for another 10 min at 10 000 × ***g***. Genomic DNA was precipitated from the aqueous solution using a standard ethanol precipitation protocol (Ausubel *et al*. 2002) and sent to the University of Exeter sequencing service, UK for library preparation and Illumina sequencing.

Identification of six marker genes from unpublished genome projects of H. eriocheir *and the edible crab microsporidian*

Orthologues of the six marker genes [amino acyl tRNA synthetases (Brown and Doolittle, 1999): arginyl tRNA synthetase and prolyl tRNA synthetase, beta-tubulin (Edlind *et al*. 1994), chitin synthase (Hinkle *et al*. 1997), heat shock protein 70 (HSP70) (Hirt *et al*. 1997) and RNA polymerase II (Hirt *et al*. 1999)] for publicly available microsporidian genomes were obtained from the MicrosporidiaDB database (Aurrecoechea *et al*. 2011) by initially performing word searches for the individual marker gene names for *Encephalitozoon cuniculi* GB-M1 and then using the 'transform by orthology tool' to find orthologues for all the other microsporidians in the database. To identify the desired marker genes in the newly sequenced genomes of *H. eriocheir* and the edible crab microsporidian, predicted open reading frames (ORFs) were queried using the microsporidian proteins obtained from MicrosporidiaDB (Aurrecoechea *et al*. 2011) using command line blastn (Mount, 2007) with an e-value cutoff of 1e-5. The top ORF hits were selected as the orthologous genes and orthology was verified by assessment of phylogenies of single genes.

Primer design, PCR and sequencing of the six-marker gene from the pea crab parasite

Due to the small amount of pea crab parasite genomic DNA recovered from the extraction procedure, gene-specific PCRs and subsequent sequencing was performed to retrieve the corresponding sequences for the six genes of the pea crab parasite rather than full genome sequencing. We designed gene-specific primers by using the first and last 18 nucleotides of the selected orthologues from *H. eriocheir*. A two-step nested PCR was done to amplify longer genes such as Arginyl tRNA and RNA polymerase (Table 2).

Ribosomal DNA phylogenetic analyses

Universal primers were used to amplify rDNA regions from genomic material extracted from *H. eriocheir*, pea crab and edible crab parasites (Table 3). The sequencing results for the PCR products were subsequently aligned and used to construct a neighbour-joining tree with MEGA software (Tamura *et al*. 2011). All PCR reactions were performed in a 50 μL reaction mix consisting of 1× Green Go Taq buffer, 2·5 mM $MgCl_2$, 0·25 mM dNTPs, 100 pM each of the forward and reverse primer sets, 0·25 units Go Taq Flexi (Promega, UK) and 2·5 μL of extracted genomic DNA. Amplifications were performed on a Peltier PTC-225 thermal cycler with the following settings: Initialization step at 94 °C for 5 min, 40 cycles of 1 min denaturation at 95 °C, a 1 min annealing step (see Tables 1 and 2 for temperatures) and a 1 min extension step at 72 °C. A final elongation step was carried out for 10 min at 72 °C. Amplification products were resolved on 2% agarose gels stained with ethidium bromide and visualized using a UV illuminator.

Correct size products (Tables 2 and 3) were excised from the gels and purified using the Wizard SV gel and PCR purification system (Promega, UK). PCR products were sequenced using Sanger technology, ABI PRISIM Big Dye

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Table 2. Gene-specific primers were designed using the first and last 18 nucleotides of the selected orthologues from *H. eriocheir*

Gene	Primers second round	Sequence	Annealing temp (°C)	Size of product (bp in Mitten crab)
Beta tubulin first round	F1	GTAAGTGATACAGTTGTAGAACC	55	683
	R1(R&C)	CCTTCACCAGTGTACCAGTG		
Beta tubulin second round	F1	GTAAGTGATACAGTTGTAGAACC	55	523
	R2(R&C)	CATTATTAGGAATCCACTCAAC		
	F2	GTTGAGTGGATTCCTAATAATG	55	182
	R1(R&C)	CCTTCACCAGTGTACCAGTG		
Prolyl tRNA first round	F1	ATGAAGATTTATTAGCTGTGCC	55	496
	R1(R&C)	GGAATACCTTTAAGTTCGCAG		
Prolyl tRNA second round	F1	ATGAAGATTTATTAGCTGTGCC	55	200
	R3(R&C)	CTCAGAAGCTACGTCATCAC		
	F2	GTGATGACGTAGCTTCTGAG	55	316
	R1(R&C)	GGAATACCTTTAAGTTCGCAG		
Arginyl tRNA first round	F1	ATGGAAGAAGGTATAAATGAGG	55	668
	R1(R&C)	GGTCCCAGTGATTCACCTTT		
Arginyl tRNA first & second round	F2	TTAGTTACAGGCATGTCAACC	55	242
	R1(R&C)	GGTCCCAGTGATTCACCTTT		
	F1	ATGGAAGAAGGTATAAATGAGG	55	447
	R2(R&C)	GGTTGACATGCCTGTAACTAA		
HSP70 first round	F1	AGAGACAAGCAACAAAAGATGC	57	336
	R2(R&C)	CAGCTGATATTTTCAACTTATTCAA		
HSP70 first & second round	F1	AGAGACAAGCAACAAAAGATGC	57	240
	R1(R&C)	CACTATCAAAATCTTCTCCTCC		
	F2	GGAGGAGAAGATTTTGATAGTG	57	118
	R2(R&C)	CAGCTGATATTTTCAACTTATTCAA		
RNA polymerase first round	F1	AGTGAGATTAGATCTGTACCTG	57	1400
	R1(R&C)	GGATGTATTTCACAATGTGTATAT		
RNA polymerase first & second round	F1	AGTGAGATTAGATCTGTACCTG	57	779
	R2(R&C)	GGTGTATTTACACGTCTTAAATG		
	F2	CATTTAAGACGTGTAAATACACC	57	644
	R1(R&C)	GGATGTATTTCACAATGTGTATAT		
Chitin synthase first round	F1	TGACAGGATGAGTGATGTGG	55	254
	R1(R&C)	GACTAATATAATACTCAAACACTT		

Terminator v3·1 cycle sequencing kit (Thermo Fisher Scientific, UK) with relevant primers (Tables 2 and 3) and the following PCR thermal cycling program: 94 °C × 30 s followed by 30 cycles of 96 °C × 10 s, 50 °C × 10 s and 60 °C × 4 min and held at 4 °C. Analyses of the sequenced PCR product were done using the Sequencher software (Gene codes corporation).

Construction of a six-gene phylogenetic tree

Maximum likelihood (ML) analyses. The orthologues for each gene set were individually aligned with the command line MUSCLE program (v3·8·31) (Edgar, 2004) using the default settings, and then subsequently masked with the automatic command line tool TrimAl (Capella-Gutierrez *et al.* 2009). *Saccharomyces cerevisiae* was used as an out-group in each of the microsporidian datasets.

A GTR substitution model with a gamma model of rate heterogeneity was used to create ML trees for the individual gene sets with the RaxML program (Stamatakis, 2014). These were pilot trees to check for unusually long-branch lengths indicative of unlikely orthologues. The masked genes from each microsporidian species were subsequently manually concatenated using SeaView (v4) (Gouy *et al.* 2010). For the final construction of the ML concatenated gene tree, a partition file that contained the positions of the individual genes within the alignment was manually created and passed to the RaxML program using the '–q' option (Stamatakis, 2014). This was to enable the program to treat each gene set in the concatenated alignment separately and allow it to estimate individual nucleotide substitution rates. These estimations were also performed with the GTR + GAMMA nucleotide substitution model.

Bayesian inference analysis on six-gene concatenated alignment. To check for reliability of the phylogenetic relationships estimated by ML analyses, a Bayesian inference method was also used to reconstruct the six-gene concatenated phylogenetic tree using MrBayes program (v3·2) (Ronquist *et al.* 2012). A partition file containing positions of the individual genes in the alignment was created according to the program manual. The program was run

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Table 3. rDNA primers used in this study

Gene	Primer name	Sequence	Annealing temp (°C)	Size of product approx. bp	Reference
SSU	530 F	GTGCCATCCAGCCGCGG	55	1350–1550	Docker *et al.* (1997)
	580 R	GGTCCGTGTTTCAAGACGG			
SSU	MF1	CCGGAGAGGGAGCCTGAGA	55	848	Tourtip *et al.* (2009)
	MR1	GACGGGCGGTGTGTACAAA			
SSU	Medlin B (used with MF1)	GATCCTTCTGCAGGTTCACCT	55	1500	Medlin *et al.* (1988)

using a GTR + GAMMA model and probability distributions were generated using the Markov Chain Monte Carlo Methods. A total of 1 020 000 generations were run, the first 25% of sampled trees were discarded as 'burn-in' and a consensus tree was constructed.

RESULTS

Pathology, ultrastructure and SSU-based phylogeny

Infected hepatopancreatic tubules from all three crab hosts displayed varying proportions of infected epithelial cells consistent with previous descriptions of *Hepatospora*-associated pathology (Stentiford *et al.* 2011). Infection was confined to the epithelial cells of the hepatopancreas, with no other organ seemingly infected. However, in some severe cases where hepatopancreatic tubules were disrupted, liberated parasite spores could be observed within the lumen of affected tubules and, in the haemolymph. Histologically, the disease caused by this microsporidian was indistinguishable between Chinese mitten crab (where the parasite has been confirmed as *H. eriocheir*), edible crab and pea crab (Fig. 1).

Ultrastructural analysis revealed multiple stages of a microsporidian parasite within the cytoplasm of hepatopancreatic epithelial cells of each host crab species. The earliest stage observed was the meront (Fig 2A, C and E). *Hepatospora eriocheir* (infecting Chinese mitten crab) possessed uni-nucleate meronts, whereas the parasite infecting pea crab and edible crab possessed bi-nucleate meronts. A similar observation was made in the spore stages with spores (Fig 2B, D and F) from the parasite infecting edible crab and pea crab appearing bi-nucleate, while spores from the Chinese mitten crab were of uni-nucleate karyotype. Mature spores in all cases possessed a trilaminar wall consisting of a plasma membrane, an electron lucent endospore and an electron-dense exospore. Spores measured 1·8–1·9 × 0·9 μm but contained varying turns of an isofilar polar filament. Spores from the parasite-infecting Chinese mitten crabs and edible crabs possessed seven to eight turns of the polar filament while those from the parasite-infecting pea crabs possessed five to six turns of the polar filament. A summary of these shared and distinctive features is given in Table 1.

Partial sequencing of the SSU rDNA gene obtained from the parasites infecting the three host crab species revealed an apparent synonymy (≈99% similarity over 890 bp), at least based upon this portion of the SSU, between the three parasites. Despite some ultrastructural and karytopic distinctions between the three isolates (Table 1), SSU-based phylogeny did not support erection of distinctive taxa for the parasites infecting edible crab and pea crab. Based upon SSU phylogeny, these parasites would therefore be classified as *H. eriocheir*, with edible crab and pea crab representing an extended host range for this parasite.

Phylogeny of Hepatospora *isolates based upon six concatenated genes*

Taking in to account the potential weakness of SSU-based phylogenies for discriminating closely related microsporidian (and other) taxa, phylogenies based upon alternative (coding) regions of the *Hepatospora* genome were constructed to investigate their potential as taxonomic discriminators (GenBank accession nos. of *Hepatospora* genes used: KU695715, KU695716, KU695717, KU695718, KU695719, KU695720, KU695721, KU695722, KU695723, KU695724, KU695725, KU695726, KU695727, KU695728, KU695729, KU695730, KU695731, KU695732). The resulting alignment of the six masked concatenated genes consisted of 18 232 sites. Phylogenetic trees based on ML and BI methods displayed identical topologies and strongly supported the grouping of the Chinese mitten crab parasite *H. eriocheir*, the edible crab and pea crab parasites, and the placement of *E. cuniculi* strains and *Nematocida* spp. as distinct clades with high confidence values (Fig. 4). The clade consisting of *H. eriocheir*, the edible crab parasite and the pea crab parasites branched as a sister group to *Enterocytozoon bieneusi* as consistent with previously phylogenies based upon SSU gene sequences (Stentiford *et al.* 2011). Our tree is also consistent with a previous multi-protein microsporidian phylogeny showing the Enterocytozoonidae forming a clade with *Vittaforma corneae* and with the *Nosema*/*Encephalitozoon* clade as a sister group to these (Nakjang *et al.* 2013). Within our tree, all resequenced strains are retrieved as clades with few

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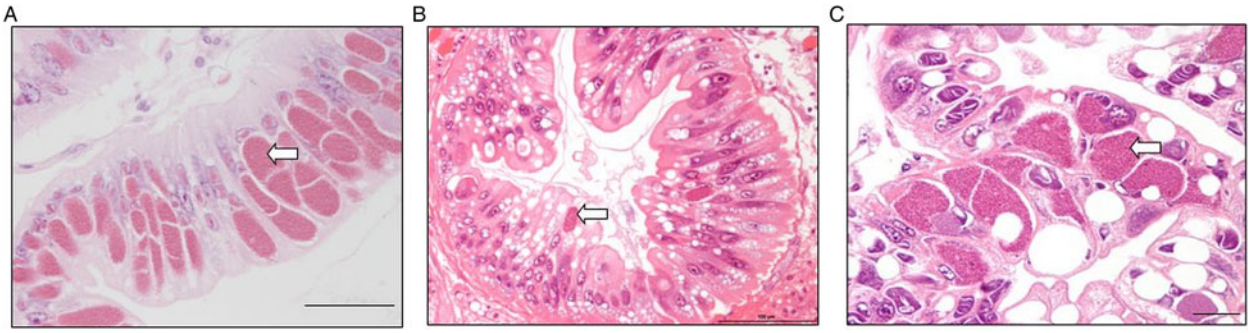

Fig. 1. (A) Histopathology of *Hepatospora* sp. in the hepatopancreas of *E. sinensis*. Scale = 50 μm. (B) Histopathology of *H. eriocheir* in the hepatopancreas of *Cancer pagurus*. Scale = 100 μm. (C) Histopathology of *Hepatospora* sp. in the hepatopancreas of *P. pisum*. Scale = 25 μm. Tubule epithelial cells contained multiple granular inclusions (arrows) All images H&E histology.

nucleotide differences separating them. As observed for our crab pathogens, no nucleotide differences separated strains of *Nematocida* sp. or *Nematocida parisii*, in our analysis. Nucleotide differences do however separate *E. cuniculi* EC1-3 strains from *E. cuniculi* GB and *E. hellem* swiss from *E. hellem* ATCC. The six gene concatenated phylogeny supports the proposition from our SSU-based phylogeny that the parasites infecting Chinese mitten crabs, European edible crabs and, pea crabs can all be classified as *H. eriocheir*. Further, it provides firm evidence that *H. eriocheir* (or very closely related microvariants thereof) can infect a wide variety of decapods crustaceans from different aquatic habitats.

DISCUSSION

Phylogenetic analyses performed with rDNA have been pivotal in erecting new taxa and in the discovery of morphological plasticity within the microsporidian phylum but have however been unsuccessful in resolving the branching relationships between closely related species. The *Hepatospora* genus is a recent taxa erected as a result of modern phylogenetic techniques (Stentiford *et al*. 2011). It has been defined as a genus that encompasses microsporidia with infective life stages that develop within the hepatopancreas of marine and brackish water hosts. With the use of rDNA-based phylogenetic analyses, Stentiford *et al*. (2011) renamed and updated the description of *Endoreticulatus eriocheir* (Wang and Chen, 2007) to form the type species *H. eriocheir*. Unlike Wang and Chen who isolated their spores from native Chinese mitten crabs, the spores in the Stentiford *et al*. (2011) study were isolated from invasive Chinese mitten crabs (*E. sinensis*) which had been caught in the Thames estuary, UK. In both studies, the spores were described as ellipsoid, measuring ~1·8 × 0·9 μm and containing seven to eight polar filaments. Recently, Ding *et al*. (2016) have shown that not only is the pathogen infecting the Asian population of Chinese mitten crabs *H. eriocheir*, but also the infection is associated with an emerging disease condition causing significant proportions in aquaculture production of this species in China. In their original taxonomic paper, Stentiford *et al*. (2011) also highlighted an unassigned microsporidian infecting the hepatopancreas of edible crabs, *C. pagurus*, and suggested at the time that this was also likely a member of the genus *Hepatospora* based upon high similarity with the partial SSU gene sequence from *H. eriocheir*.

In 2012, Longshaw *et al*. produced a disease profile of the pea crab (*P. pisum*) revealing the presence of two uncategorized microsporidian parasites. One of these appeared to be cytoplasmic, residing in hepatopancreatocytes and inducing a necrotizing effect that resulted in the degeneration of hepatopancreatic tubules. However, in this study, the microsporidian was not assigned a taxon and no further data was presented in order to aid its molecular or morphological characterization.

In the present study, we have shown that infection with the parasite of the hepatopancreas of edible crabs displays a similar pathology to that caused by infection with *H. eriocheir* in Chinese mitten crabs from London (Stentiford *et al*. 2011) and from China (Wang and Chen, 2007; Ding *et al*. 2016), and to the infection described in pea crabs by Longshaw *et al*. (2012). These parasites also appear to share a broadly similar morphological development within host gut epithelial cells albeit with some distinctive differences in karyo-status (*H. eriocheir* is unikaryotic whereas the pea crab and edible crab parasites are dikaryotic) and some minor differences in polar tube-coiling patterns (summarized in Table 1). Based upon our analysis of rDNA gene sequence-based phylogenetic relationship between *H. eriocheir* and the two novel parasites, all three are virtually indistinguishable, forming a monophyletic group immediately adjacent to the Enterocytozoonidae clade (Stentiford *et al*. 2013) (see Fig. 3).

Based upon the somewhat surprising finding that the same parasite taxon appeared to infect not only

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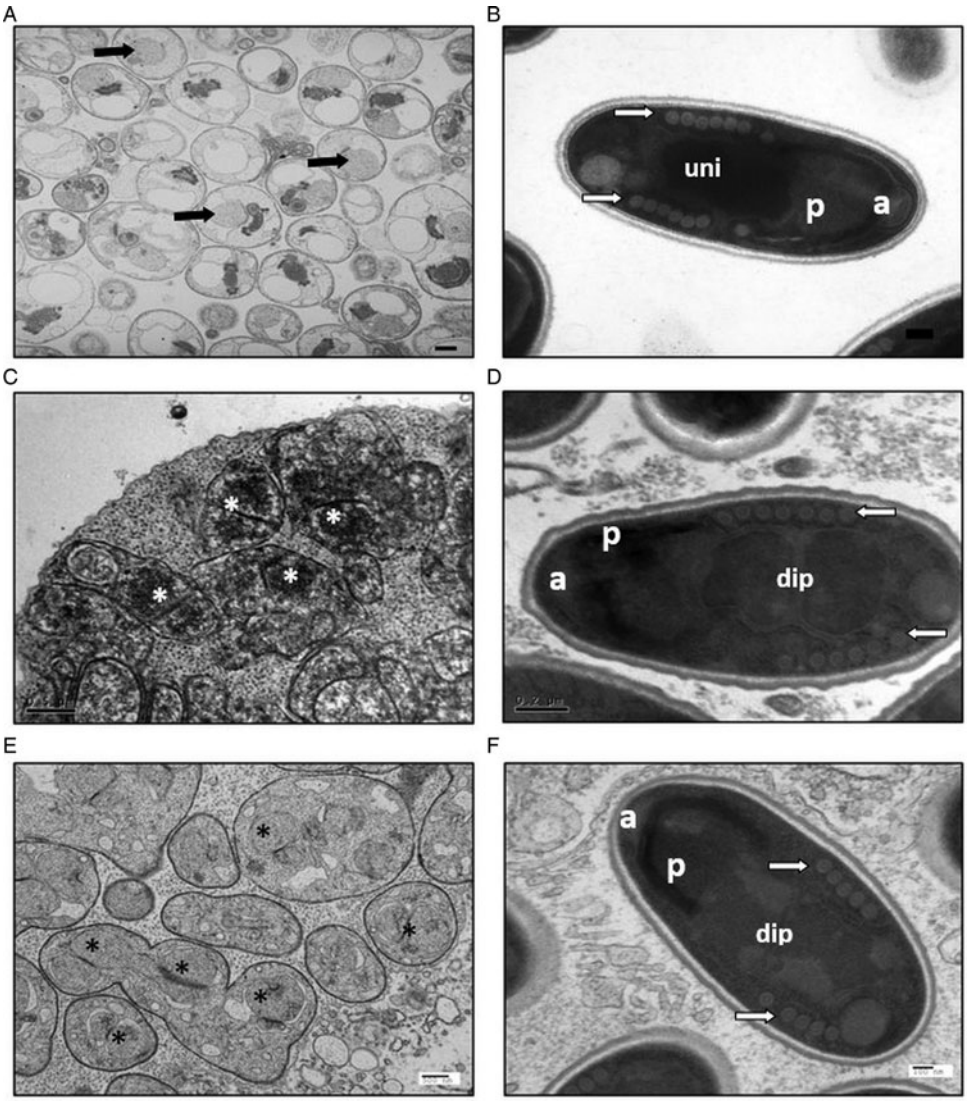

Fig. 2. Electron micrographs of *H. eriocheir* from *E. sinensis* (A and B) and *Hepatospora* sp. from *Cancer pagurus* (C and D) and *P. pisum* (E and F). (A) Developing sporonts within a parasitophorous vesicle. Sporonts contain multiple unikaryotic nuclei (arrows) which then divide and mature to form spores. Scale bar = 500 nm. (B) Mature spore containing seven to eight turns of the polar filament (arrow) in single file, unikaryotic nucleus (uni), anchoring disc (a) and polaroplast (p). Scale bar = 100 nm. (C) Developing sporonts within a parasitophorous vesicle. Sporonts contain multiple diplokaryotic nuclei (*) which then divide and mature to form spores. Scale bar = 0·5 μm. (D) Mature spore containing five to six turns of the polar filament (arrow) in single file, diplokaryotic nucleus (dip), anchoring disc (a) and polaroplast (p). Scale bar = 0·2 μm. (E) Developing sporonts within a parasitophorous vesicle. Sporonts contain multiple diplokaryotic nuclei (*) which then divide and mature to form spores. Scale bar = 500 nm. (F) Mature spore containing five to six turns of the polar filament (arrow) in single file, diplokaryotic nucleus (dip), anchoring disc (a) and polaroplast (p). Scale bar = 100 nm.

Chinese mitten crabs from different parts of their invasive/native range, but also occurred in niche-separated crab hosts from the European marine environment we used these three microsporidians as a model system to assess how a concatenated multi-gene phylogenetic approach could be applied as refined tool for discriminating taxa which cannot be separated by rDNA-based phylogenetic approaches. As outlined in our recent Opinion piece, appropriate application of such techniques may be suitable where significant biological reasoning exists for potential separation of taxa (e.g. one causing disease and another not) and, may be particularly important where pathogens are to be listed to prevent their international trade in animals and their products (Stentiford *et al*. 2014). The model system presented by *Hepatospora* was appropriate given the potential for geographical, host and habitat distinction between known isolates, some reasonably significant differences in morphology (i.e. karyotstatus) and, the availability of draft genome data for the best studied of these, *H. eriocheir*. The rationale behind this concatenated phylogeny approach was to look for alternatives or additional data with which to resolve the branching relationship between very closely related

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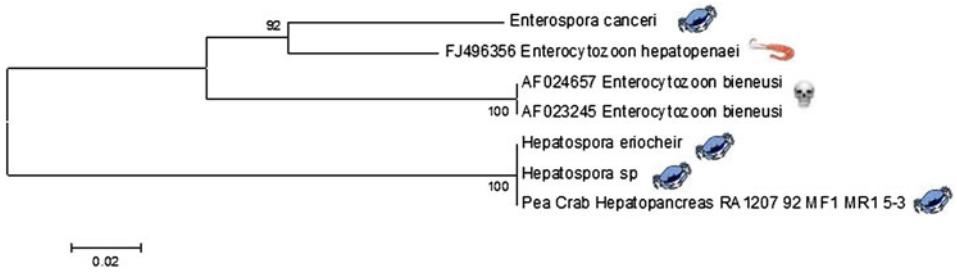

Fig. 3. Neighbour – joining tree based on a 511 bp nucleotide partial SSU 18s sequence from parasites isolated from hepatopancreas of *E. sinensis* (*H. eriocheir*), *Cancer pagurus* (*Hepatospora* sp.) and *P. pisum*. The phylogenetic analysis was performed using Mega version 5·05. Analysis was done using 1000 bootstrapped datasets and values >70% are shown on the tree. The scale bar represents substitutions per nucleotide site.

microsporidia. Despite this reasoning, our concatenated phylogenies resulted in almost identical tree topologies in both the ML and BI probabilistic approaches used with nodes supported by high bootstrap and posterior probability values, respectively. This, in addition to the retrieval of well-known relationships like the grouping of *Encephalitozoon* and *Nematocida* strains (in Fig. 4) and overall tree topology similar to previously published work based on rDNA genes (Troemel *et al*. 2008; Stentiford *et al*. 2011; Cuomo *et al*. 2012) increases our confidence in the phylogenetic relationships inferred by our study.

Whilst our multi-gene phylogeny confirms the close relationship of the three isolates, it does not discriminate well between them. This is in part due to the use of highly conserved genes for our concatenated tree phylogenetic analyses. Even though protein-coding genes have been successfully used in previous studies to infer deep phylogenetic relationships within the microsporidian phylum and placing microsporidians within the tree of life (Fast *et al*. 1999; Hirt *et al*. 1999; Keeling, 2003; Nakjang *et al*. 2013), the selected genes were unable to properly resolve branching relationships in our analyses due to the high level of nucleotide sequence similarity between the three *Hepatospora* strains (Table 1 and Fig. 3). Due to the small size of the pea crab hepatopancreas and very low infection levels in this host, we were unable to extract a large quantity of parasite genomic DNA. This in turn limited the number of marker genes we could amplify from the pea crab parasite for this study. Given more material we would have amplified further genes, for example polar tube and spore wall protein-coding genes. These have been used in previous studies to distinguish between *Encephalitozoon* and *Nosema* strains (Peuvel *et al*. 2000; Polonais *et al*. 2010; Chaimanee *et al*. 2011), and could have been added in our analyses to improve the resolution of branching relationships between the *Hepatospora* strains. It must however be noted that even though Polonais *et al*. (2010) were successful in differentiating between *Encephalitozoon hellem* strains by looking at nucleotide polymorphisms at the spore wall protein gene, this gene was unsuccessful in differentiating between *Nosema ceranae* geographical isolates in similar recent studies (Roudel *et al*. 2013; Van der Zee *et al*. 2014). This highlights the different evolutionary pressures acting on the genes and possibly genomes of different microsporidians and that a gene successfully used to differentiate between strains in one microsporidian species may not be ideal for other species.

Future studies of this kind should probably focus on divergent single copy orthologues between strains of interest. Primer design for more variable genes is more challenging, but the advent of single-cell sequencing will remove this issue completely. As microsporidian genomes become increasingly available, we envisage a switch from phylogenetics to phylogenomics as the latter presents a more holistic approach to understanding close phylogenetic relationships and providing information for more robust taxonomic assignments.

Taxonomy of Hepatospora

Despite the caveats exposed in the preceding section, it is useful to consider the taxonomic placement of the parasites infecting the three host crabs. Here, it is useful to look to other genera within the phylum. Since already established subspecies of *E. cuniculi, E. hellem* and *Nematocida* (also included within our analysis) have nucleotide similarity for the same genes used in this study ranging between 92·13 and 99·95%, assigning different species names to the host-specific isolates of *Hepatospora* (98·53–98·88% nucleotide similarity) is not supported (see highlighted boxes in Table 4). On the bases of their high nucleotide similarity and their consequent grouping with minimal branching distances on our multi-gene phylogenetic tree (shown in bold in Fig. 4), we propose that *Hepatospora* parasites investigated in the current study should therefore be regarded as the same species, *H. eriocheir*, or potentially very closely related microvariants thereof. However, in consideration of the differences in morphological features that would have placed these microsporidians in completely different

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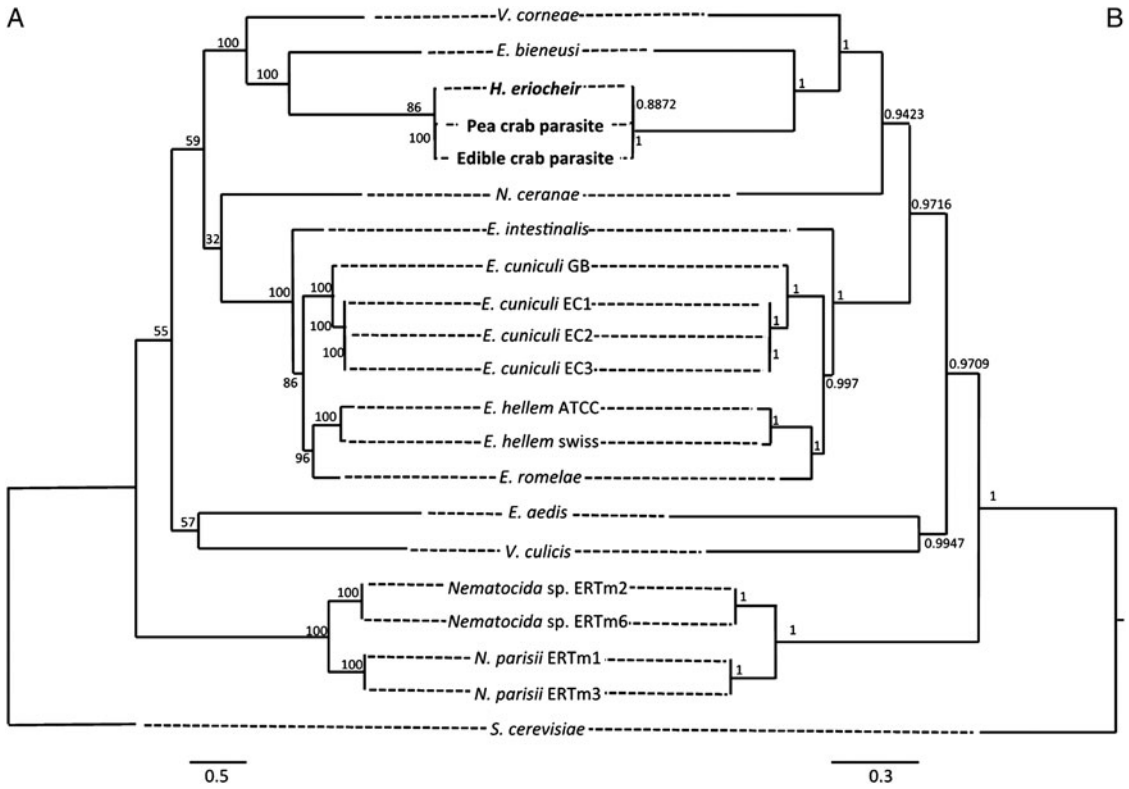

Fig. 4. Grouping of three *Hepatospora*/*Hepatospora*-like species suggests they are closely related. Phylogenetic trees based on (A.) maximum likelihood, (B.) Bayesian inference of 20 microsporidians for six concatenated genes rooted with *S. cerevisiae*. Numbers on nodes are (A.) Bootstrap confidence levels from 100 replicates, (B.) Bayesian posterior probability values. Both trees displaying identical topologies and grouping of *Hepatospora*/*Hepatospora*-like clade are shown in bold. The scale bars represent nucleotide substitutions per site.

Table 4. High nucleotide sequence similarity of the six marker genes used in this study between the parasites isolated from three different crab hosts

	arginyl tRNA synthetase	prolyl tRNA synthetase	beta-tubulin	chitin synthase	HSP70	RNA polymerase II
H. *eriocheir vs* Pea crab parasite	**99** *21/669*	**98** *8/482*	***99*** *1/643*	**100** *0/228*	**100** *0/305*	**99** *14/1341*
H. eriocheir vs Edible crab parasite	**99** *23/1064*	**99** *29/1401*	**99** *6/1200*	**99** *15/2211*	**99** *6/744*	**99** *22/3303*
Edible crab parasite *vs* Pea crab parasite	**99** *20/669*	**99** *1/482*	**99** *1/643*	**100** *0/228*	**99** *4/305*	**99** *15/1341*

Numbers in bold are percentage identity comparison of sites (i.e. nucleotides + gaps) resulting from the alignment of each of the six marker genes of three *Hepatospora*/*Hepatospora*-like microsporidian species. Italicized numbers represent number of variable nucleotides in the pairwise alignment of two species without taking gaps into account (number given of variable nucleotides/total number of aligned nucleotides).

taxonomic ranks using traditional approaches (and considering that taxonomy of the phylum abides by rules laid down by the ICZN), it is perhaps appropriate to consider these microvariants as subspecies. We therefore propose the assignment of *H. eriocheir pinnotheres* and *H. eriocheir canceri* as subspecies of *H. eriocheir* infecting the pea crab and edible crab, respectively. Regardless of use of particular nomenclature, it is noteworthy that the genus *Hepatospora* (and perhaps specifically its type taxon *H. eriocheir*) may represent an example of a parasite cline, infecting the guts of one of the most abundant host groups in our oceans, the crustaceans. The minor differences in rDNA-based and even concatenated phylogenies for *Hepatospora* may underlie a subtly shifting genome required for survival in hosts from different habitats. As more subspecies are discovered from variant hosts in different habitats, the concept of the parasite cline can be better studied.

An alternative hypothesis would reflect the potential that a previously host-specific parasite, *H. eriocheir* has been inadvertently introduced to European waters by its invasive host, the Chinese mitten crab; following

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which it has subsequently switched to hosts which at least have niche overlap at some point in their life cycle (Ingle, 1980; Lawton, 1989; Clark *et al*. 1998; Becker and Türkay, 2010). The description of gut infecting microsporidian taxa from locations where such niche overlap are absent will address this key question.

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