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Title

A new microsporidian parasite of the genus *Amblyospora* (Hazard and Oldacre, 1975) identified from the halophilic mosquito *Ochlerotatus detritus* (Haliday, 1833) (Diptera: Culicidae) through rDNA ITS sequencing

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ABSTRACT

Ochlerotatus detritus (Haliday, 1833) from Parkgate marshes, Wirral, U.K. are shown to be parasitised by a new species of *Amblyospora* (Hazard and Oldacre, 1975) microsporidian. Phylogenetic analysis shows that Internal Transcribed Spacer (ITS) sequences from this microsporidian are distinct from those of all known microsporidia identified to date, but form a clade with *Amblyospora weiseri* Lukeš and Vávra, 1990 and *A. stictici* Andreadis, 1994, microsporidia identified from *Ochlerotatus cantans* Meigen, 1818 and *O. sticticus* Meigen, 1838, respectively. Prevalence rates, from pooled samples (N = 5 per pool) were low (2.37%; lower limit 0.78%, upper limit 5.62%), which may be a consequence of these ephemeral brackish water pool habitats periodically drying out. There is increasing interest in the use of microsporidian parasites as novel vector control strategies and understanding the phenology of this microsporidian and its mosquito host may ultimately lead to new methods of control for this nuisance biting species.

KEYWORDS

Microsporidia; parasite; mosquito; *Ochlerotatus*; *Amblyospora*

Introduction

Microsporidia are a diverse group of obligate intracellular parasitic eukaryotes (Dunn and Smith 2001) for which possession of 70S ribosomes, primitive golgi apparatus and a lack of mitochondria suggest a primitive status (Curgy, Vavra, and Vivares 1980). Whilst molecular phylogenetics confirms the microsporidia as eukaryotes (Vossbrinck, Maddox, Friedman, Debrunner-Vossbrinck, and Woese 1987) and members of the protozoa (Franzen and Muller 1999), genomic studies, similarities in the process of cell division and the presence of a chitinous spore wall suggest that they are most closely related to fungi (Weiss and Vossbrinck 1998).

Initially observed as parasites of silkworms, there are currently an estimated 1400 species in over 200 genera recognised (Han and Weiss 2017) and microsporidia can be found in almost every environment. They are able to parasitise a wide variety of organisms including both vertebrates and invertebrates, and indeed some species of protist (Weiss and Becnel 2014). However, they are significantly pathogenic only in a small number of species including fish and insects in which they can have serious, destructive effects (Weiss and Becnel 2014). Infection is spread through spores which are most commonly found on the surface of stagnant water bodies (Izquierdo et al. 2011). These spores, when ingested by the future host, infect the surrounding cells of the gastrointestinal tract through a specialised infection apparatus known as the polar tube (Han and Weiss 2017) which extends, pierces the cytoplasm of the host cell, and allows for infection to begin (Keeling and Fast 2002). At this point, merogony (the proliferative stage) begins, and multiplication occurs by binary fission to give rise to sporoblasts which mature to become spores (sporogony). Mature spores are then released to infect further cells following rupture of infected cells (Han and Weiss 2017). Spore germination is facilitated by environmental triggers, a process which is poorly understood but thought to be associated with factors such as a change in pH or rehydration (Keeling and Fast 2002) and further infection is facilitated by the release of spores via rupturing vacuoles.

There is increasing interest in the role of microsporidia in the control of insects and the inhibition of development of vector-borne diseases since infections causes prolongation of larval stages, prevention of eclosion (Andreadis 2007; Becnel, Garcia, and Johnson 2000; Becnel and Johnson 2000; Koella, Lorenz, and Bargielowski 2009; Bjørnson and Oi 2014; Lacey, Frutos, Kaya, and Vail 2001; Lorenz and Koella 2011) and reduction of infection by other parasites (Duncan, Agnew, Noel, and Michalakis 2015). Indeed, recently a novel microsporidian symbiont has been shown to impair *Plasmodium falciparum* (Welch, 1897) transmission in

Anopheles arabiensis Patton, 1905 (Herren et al. 2020). Hence, knowledge of the range of microsporidian parasites in mosquitoes, and the extent of parasitisation is important.

The mosquito *Ochlerotatus* (= *Aedes*) *detritus* (Haliday, 1833) is a pernicious nuisance biter in some parts of the UK with the Dee estuary salt-marsh of south-west Wirral, and River Stour estuary at Sandwich in Kent being hotspots for complaints from residents about nuisance biting (Medlock, Hansford, Anderson, Mayho, and Snow 2012; Ramsdale and Snow 1995). As a Site of Special Scientific Interest, there are considerable restrictions on available controls for this species on the Parkgate Marshes of the Dee estuary and hence use of such biological controls may be particularly pertinent. In a recent transcriptomic (RNASeq) study of this mosquito from this site in which differential gene expression was measured following challenge by entomopathogenic nematodes (*Steinernema carpocapsae* (Weiser, 1955)), the most upregulated transcript had a microsporidian sequence as the closest match (Edmunds 2018), suggesting the presence of microsporidial DNA in *O. detritus* from this site. However, microsporidial infection of *O. detritus* has not been reported previously, although other members of the genus, including brackish water breeders can be infected by microsporidians of the genus *Amblyospora* (Hazard and Oldacre, 1975) (Baker, Vossbrinck, Becnel, and Andreadis 1998; Weiss and Vossbrink 1999; Vossbrinck, Andreadis, Vavra, and Becnel 2004). At present, identification of microsporidia is chiefly undertaken on the basis of ultrastructural characteristics including the appearance of the polar tube, spore morphology and the identity of their hosts (Andreadis, Simakova, Vossbrinck, Shepard, and Yurchenko 2012; Han and Weiss 2017), however, molecular phylogenetic studies have also been undertaken (Weiss and Vossbrinck 1999; Vossbrink et al. 2004) to examine phylogenetic relationships of microsporidia (Andreadis et al. 2012; Baker et al. 1998; Franzen and Müller 1999; Vossbrinck and Debrunner-Vossbrinck 2005; Weiss and Vossbrinck 1999) and co-evolution with host species (Andreadis et al. 2012).

Here, we report a new microsporidian species and investigate the prevalence of this new microsporidian in *Ochlerotatus detritus* mosquitoes collected from the Parkgate marshes, Wirral, UK using sequencing of parasite rDNA Internal Transcribed Spacer (ITS) sequences for identification. Herein, we do not describe this new species of *Amblyospora* because full classification of the new species will ultimately require full ultrastructural description – but this will be hampered by the low prevalence in the population and the need to culture.

Material and methods

Sample collection

Mosquito larvae were collected by dipping or using a net from five separate brackish water pools (labelled A, B, D, E, F) at Little Neston, Parkgate Marshes, Wirral in August 2019 (Figure 1). Larvae were maintained in the laboratory in the water in which they were collected and fed crushed cat biscuits.

DNA extraction

Estimation of infection rates can be conducted through screening of pooled samples (Walter et al. 1980). To facilitate PCR screening of a representative number of larvae, pools of five larvae were prepared and DNA extracted from 10–15 pooled samples from each location (50–75 total individuals per water body). DNA was extracted using the Thermo Scientific GeneJet Genomic DNA extraction kit following the manufacturer's recommended protocol.

PCR

Two separate PCRs were conducted on pooled DNA. To analyse the presence/absence of microsporidia within mosquito pools, samples were screened using primers 18f and 1492r of Ghosh and Weiss (2009) which amplify a region of the Internal Transcribed Spacer of the rDNA. PCRs were carried out using 1x GoTaq colourless Hot Start mastermix (Promega), 2µM each primer and 1µl DNA with a PCR profile of 95°C for 3 min then 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min with a final 5 min extension at 72°C. Confirmation of species identity of mosquito samples was established through mitochondrial DNA barcoding using the primers L1490 and H2198 of Folmer, Black, Hoeh, Lutz, and Vrijenhoek (1994) with a PCR mix of 1x GoTaq colourless Hot Start mastermix (Promega), 2µM each primer and 1µl DNA and a PCR profile of 95°C for 3 min then 35 cycles of 95°C for 1 min, 40°C for 1 min, and 72°C for 1.5 min with a final 5 min extension at 72°C. PCR products were checked by electrophoresis on 1.5% agarose gels then purified using a GeneJet PCR purification kit following the manufacturer's recommendations. Sequencing was performed by Eurofins Genomics (Konstanz, Germany). Samples from Pool D required dilution (1/10) prior to PCR due to co-extraction of a PCR inhibiting compound.

Analysis

Sequences were manually inspected and edited using FinchTV. ITS sequences of known *Amblyospora* from Vossbrink et al. (2004) were downloaded and aligned to ITS sequences from this study using ClustalX (Larkin et al. 2007). Phylogenetic trees were constructed in Mega X (Kumar, Stecher, Li, Knyaz, and Tamura 2018)

following evaluation of the most appropriate evolutionary model (using Model Test) and constructed using Maximum Likelihood with 500 bootstrap replicates. mtDNA sequences were identified through BLAST (Altschul, Gish, Miller, Myers, and Lipman 1990) analysis.

Prevalence was calculated from the number of positive PCRs across the 10–15 pooled samples using PooledInfRate v4.0 https://www.cdc.gov/westnile/resourcepages/mosqSurvSoft.html.

Results

Mosquito species present

Preliminary morphological examination of larvae suggested that one pool (E) contained only *Culex* larvae whilst the other pools contained *Ochlerotatus* spp. Across the five pools, screening of pooled DNA with mtDNA barcoding primers indicated that three different species were identified across the five pools (*O. detritus*, *O. caspius* (Pallas, 1771) and *C. pipiens* L., 1758) (Table 1). Over the 657bp of *CoI* sequenced, *O. detritus* and *O. caspius* differ by over fifty base pairs (e.g., *O. detritus* accession number MG242486.1 differs from *O. caspius* accession MK047313.1 at 55 of 657bp) thus determining the proportion of the two species in pooled samples is possible through assessing relative peak height at these variant bases in sequence chromatograms. We note that this cannot be done precisely due to unequal allele amplification and dye bias (Visscher and Le Hellard 2003) but does serve to give an estimate of species proportion in pooled samples. Here, pools A, B and F exhibited mixed species assemblages with pool A predominantly *O. caspius* and pools B and F predominantly *O. detritus* (Table 1). Pooled sequences from pool D (*O. detritus*) and E (*C. pipiens*) exhibited no mtDNA sequence variability indicative of the presence of single species.

Parasite prevalence

No pooled samples of pool E (*Culex pipiens*) or mosquitoes from pool A (predominantly *O. caspius*) tested positive with the microsporidian ITS primers, but across the 35 pooled samples of solely or predominantly *O. detritus*, positive PCRs were found for four pools. From these data, the infection rate with *Amblyospora* across all of the *O. detritus* pools was calculated as 2.37% with a lower limit of 0.78% and an upper limit of 5.62% (Table 1).

Phylogenetic analysis

From the four positive pools, just two different microsporidian ITS sequences were obtained. These were 98.35% identical and have been submitted to Genbank with accession numbers MT118721 and MT118722. All differences between the two sequences were biased towards the 3′ end of the sequence (Figure 2).

These two ITS sequences (B5 and B9) were aligned to ITS sequences from a range of microsporidia (Vossbrink et al. 2004) and used to construct a phylogenetic tree (Figure 3). The two ITS sequences obtained from *O. detritus* were different in sequence from all known *Amblyospora* sequenced to date but were positioned within

the *Ochlerotatus*/*Aedes* parasite group and formed a well-supported clade (78% bootstrap support) with *Amblyospora weiseri* Lukeš and Vávra, 1990 and *A. stictici* Andreadis, 1994.

Discussion

PCR screening of pooled samples of mosquito showed that *Ochlerotatus* from Parkgate marshes are infected by a new species of *Amblyospora* microsporidian. Just two distinct ITS sequences were obtained from the four microsporidia-positive PCRs and phylogenetic analysis showed that these sequences cluster within all known *Amblyospora* species but most closely to those of *Amblyospora stictici* (parasite of *Ochlerotatus sticticus* Meigen, 1838) and *A. weiseri* (*O. cantans* Meigen, 1818). Whilst there were two distinct ITS sequences observed, in our opinion these likely represent intraspecific variation as the two sequences cluster extremely closely in the phylogenetic tree and have 98.35% sequence identity across the 1335bp of aligned ITS sequence, a level of sequence identity seen previously in other intraspecific microsporidian sequencing (Rinder, Katzwinkel-Wladarsch, and Löscher 1997). The rate of infection for this *Amblyospora sp.* (2.37%) was low but is in line with that seen for other species e.g., natural prevalence of *Amblyospora khaliulini* Hazard and Oldacre, 1975 infections in *Aedes communis* (De Geer, 1776) was 1.6%–3.6% (Andreadis, Thomas, and Shepard 2018). However, there is substantial variation in the reported infection rate of microsporidia, with rates of up to 60% reported (Andreadis 2007). *Amblyospora* and *Edhazardia aedis* (Kudo, 1930) can be both vertically and horizontally transmitted (Agnew, Becnel, Ebert, and Michalakis 2003; Andreadis et al. 2018; Zilio, Thiévent, and Koella 2018) and thus whilst we might expect the infection rate to be higher, the ephemeral nature of the brackish water pools at Parkgate Marshes may impact upon infection and spore survival. Due to the seasonal nature of the pools at Parkgate, infection rate may vary throughout the year and therefore additional time-course screening of *O. detritus* is recommended to examine how infection varies seasonally.

Ochlerotatus detritus is locally abundant at Parkgate and data from adult traps and larval collections indicate that it is the predominant mosquito at this site (Blagrove et al. 2016; Chapman, Archer, Torr, Solomon, and Baylis 2017; Currie-Jordan 2019). In recent work examining insecticide resistance in this mosquito, it was the only species found (Brown, Logan, and Wilding 2019), however, small numbers of *O. caspius* were detected as contaminating samples in a recent study of the effect of entomopathogenic nematode exposure on *Ochlerotatus* (Edmunds 2018). At the time of collection, three species of mosquito were present in the pools from which collections were made; *Ochlerotatus detritus*, *Ochlerotatus caspius* and *Culex pipiens* which are all species common in the area (Clarkson and Setzkorn 2011; Medlock et al. 2012). The habitat at Parkgate consists of a

number of semi-permanent pools, which dry up completely only at the height of summer, and a range of smaller, more temporary pools, which fill up after high spring tides or intense periods of rain. *Ochlerotatus caspius* was found in a temporary pool which had been filled with rainwater from a recent period of heavy rainfall and in smaller numbers in other pools. It was not surprising to see *O. caspius* larvae inhabiting these temporary pools as these mosquitoes lay their eggs in mud along the perimeter of receding pools which will then hatch under favourable temperatures and flooding (Milankov, Petric, Vujic, and Vapa 2009). It is therefore likely that these larvae hatched following the rainfall which created the temporary pool. Microsporidia were detected only in *O. detritus* with no positive samples from *C. pipiens* or *O. caspius* though more extensive sampling will be required to determine if these other mosquito species are definitively free of *Amblyospora* at this collection site.

Thus, molecular analysis indicates the presence of a species-specific *Amblyospora* parasite in larval samples of *O. detritus*. Microsporidia have been suggested as a species-specific method of control for mosquitoes (Andreadis 2007; Becnel et al. 2000; Becnel and Johnson 2000; Bjørnson and Oi 2014; Lacey et al. 2001; Lorenz and Koella 2011) and the possibility of developing this newly identified species as a biological control agent requires further investigation. *Ochlerotatus detritus* is a pernicious biting nuisance at this site (Davies 1995; Clarkson and Setzkorn 2011). Since the locality is a Site of Special Scientific Interest (SSSI), chemical control of mosquitoes is not permitted and the only recent attempts at insecticidal control involved the use of *Bacillus thuringiensis* Berliner, 1915, subsp. *israelensis* (Davies 1995; Clarkson and Setzkorn 2011). Thus, knowledge of the microsporidial parasites of *O. detritus*, which may impact host development, is therefore highly pertinent and deserving of further study. Further field-based research is particularly needed to understand the parasite-host dynamics at this site.

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Figure captions

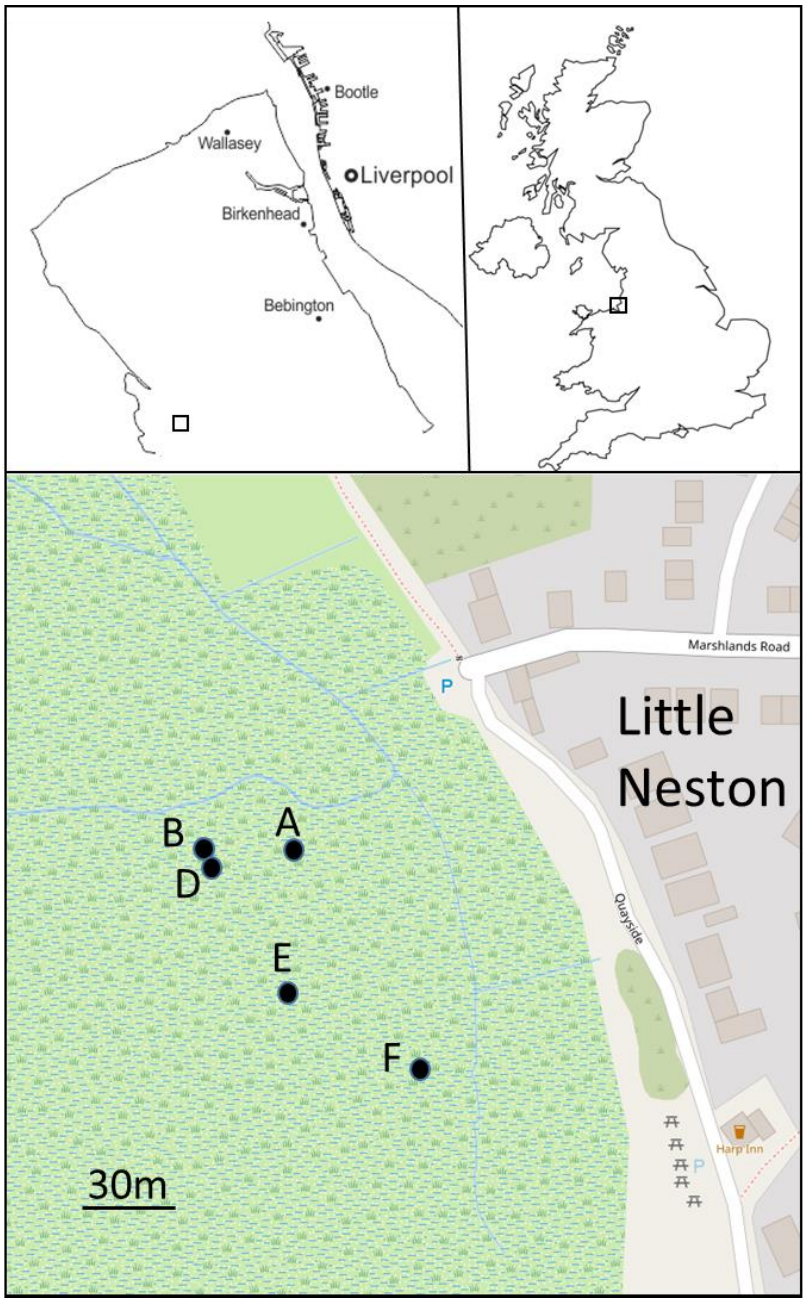
Figure 1. Location of pools sampled (labelled A, B, D, E, F) for mosquito larvae at Little Neston, Wirral, UK. Map produced in https://www.openstreetmap.org.

Figure 2. Alignment of the two *Amblyospora* ITS sequences from *Ochlerotatus* mosquitoes collected from Parkgate Marshes, Wirral UK. Samples B5 and B9 have been submitted to Genbank with accession numbers MT118721 and MT118722 respectively.

Figure 3. Phylogenetic analysis of *Ochlerotatus detritus* parasite sequences B5 and B9 (boxed) alongside other microsporidian sequences (from Vossbrinck et al. 2004). The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model. The tree with the highest log likelihood (-23880.01) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the

branches. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There was a total of 935 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018). The tree is rooted with the sequence from *Tritrichomonas foetus* (Riedmuller, 1928).

361


Bootle
Wallasey
Liverpool
Birkenhead
Bebington
Marshlands Road
P
Little
Neston
B
A
D
E
F
Quayside
Harp Inn
P
30m

362

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B5     1     CATGCAAGTCTGTGAATATGTTTATAGAAACAGTGTACGGCTCAGTATAACATGTCTATC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B9     1     CATGCAAGTCTGTGAATATGTTTATAGAAACAGTGTACGGCTCAGTATAACATGTCTATC  60

B5     61    TACCCATTTATATATAATAACCGTGGTAAACTATGGCTAATATAATGGATGAGGATGTGA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B9     61    TACCCATTTATATATAATAACCGTGGTAAACTATGGCTAATATAATGGATGAGGATGTGA  120

B5     121   CCTATCAGCTTGTCGGTACGGTAAGTGCGTACCGAGGCTATAACGGGTAACGGGGAATAT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B9     121   CCTATCAGCTTGTCGGTACGGTAAGTGCGTACCGAGGCTATAACGGGTAACGGGGAATAT  180

B5     181   GGGTTTTATTCCGGAGAGGGAGCCTGAGAGATGGCTGCCACGTCCAAGGACGGCAGCAGG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B9     181   GGGTTTTATTCCGGAGAGGGAGCCTGAGAGATGGCTGCCACGTCCAAGGACGGCAGCAGG  240

B5     241   CGCGAAACTTACCCAATGAACATTGAGGTAGTTACGAGGCGTATAGGGTTGTTTTGTATT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B9     241   CGCGAAACTTACCCAATGAACATTGAGGTAGTTACGAGGCGTATAGGGTTGTTTTGTATT  300

B5     301   CGGGATGTGTAAGTAGCATCCCCAAAGACTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B9     301   CGGGATGTGTAAGTAGCATCCCCAAAGACTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGG  360

B5     361   TAATACCAGCTCCAGTAGCGTCTGTGTTTATTGCTGCGGTTAAAATGTGCGTAGTCTGGT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B9     361   TAATACCAGCTCCAGTAGCGTCTGTGTTTATTGCTGCGGTTAAAATGTGCGTAGTCTGGT  420

B5     421   AATATGGCTTGAGTTTAATATACATTTTCATAGTGTAAAGACTCTCAGGAACTTATACCT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B9     421   AATATGGCTTGAGTTTAATATACATTTTCATAGTGTAAAGACTCTCAGGAACTTATACCT  480

B5     481   TGAGACAGGGAAGAGGTGATGTTATTTGGTAGCGAGAGGTGAAAATCGATGACCTACTGA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B9     481   TGAGACAGGGAAGAGGTGATGTTATTTGGTAGCGAGAGGTGAAAATCGATGACCTACTGA  540

B5     541   GGAGCGACAGAGGCGAAAGCGATCACCAAGAACTGTTCTGACGATCAAGCGCGTGAGCAG  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B9     541   GGAGCGACAGAGGCGAAAGCGATCACCAAGAACTGTTCTGACGATCAAGCGCGTGAGCAG  600

B5     601   GAGTATCGAAGAGGATTAGAGACCCACGTAGTTCCTAGCAGTCAACAATGCCAACACTGT  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B9     601   GAGTATCGAAGAGGATTAGAGACCCACGTAGTTCCTAGCAGTCAACAATGCCAACACTGT  660

B5     661   GGTGCTACTTTGCATTGCGGAAGCGAAAGCTAGTGTATGGGCTCCGGGGATAGTACGGAC  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B9     661   GGTGCTACTTTGCATTGCGGAAGCGAAAGCTAGTGTATGGGCTCCGGGGATAGTACGGAC  720

B5     721   GCAAGTTTGAAACTTGAAGAAATTGACGGAAGGACACCACAAGGAGTGGAGTGTGCGGGT  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B9     721   GCAAGTTTGAAACTTGAAGAAATTGACGGAAGGACACCACAAGGAGTGGAGTGTGCGGGT  780

B5     781   TAATTTGACTCAACGCGGGAAAACTTACCCGGGCAGGCAGTTATCGTGAGAAGTTA--TT  838
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||  ||
B9     781   TAATTTGACTCAACGCGGGAAAACTTACCCGGGCAGGCAGTTATCGTGAGAAGTTATTTT  840

B5     839   AAGTGTAACTGATGATACTGCGCGTGGTGCATGGCCGTTCTTAACACGTGGAGTGATCTG  898
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B9     841   AAGTGTAACTGATGATACTGCGCGTGGTGCATGGCCGTTCTTAACACGTGGAGTGATCTG  900

B5     899   TCTGGTCAAATCTGATAACGCGTGAGAGGTGAGTGTTTATGCATTAGCATGAGCAGACGA  958
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B9     901   TCTGGTCAAATCTGATAACGCGTGAGAGGTGAGTGTTTATGCATTAGCATGAGCAGACGA  960

B5     959   TGTATGTAAGTACAAGGAAGTAGCACCCGATAACAGGTCTGTGATGCCCGTAGATGTCCG  1018
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B9     961   TGTATGTAAGTACAAGGAAGTAGCACCCGATAACAGGTCTGTGATGCCCGTAGATGTCCG  1020

B5     1019  GGGCTCCACGCGCACTACAATGGATGGTAGTAT--TAGTAGTGTGTAACCAATTCGTAGT  1076
             ||||||||||||||||||||||||||||||||  ||||||||||||||||||||||||||
B9     1021  GGGCTCCACGCGCACTACAATGGATGGTAGTATTATAGTAGTGTGTAACCAATTCGTAGT  1080

B5     1077  TGGGATTGACATATGTAATTATGTCATGAACTTGGAATTCCTAGTAGTTGGTTGTCATTA  1136
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B9     1081  TGGGATTGACATATGTAATTATGTCATGAACTTGGAATTCCTAGTAGTTGGTTGTCATTA  1140

B5     1137  ACGACTGACGAATGCGTCCCTGTTCTTTGTACACACCGCCCGTCGTTATCTAAGATGGAA  1196
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                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B9      1141    ACGACTGACGAATGCGTCCCTGTTCTTTGTACACACCGCCCGTCGTTATCTAAGATGGAA  1200

B5      1197    GTGCGGGTGAAGATGTGAGTATAAACCATTAGGGTAATGATGAATATTTGTATATGCGTG  1256
                |||||||||||||||||||||||||||||||||||||||| ||||| || |||  |||
B9      1201    GTGCGGGTGAAGATGTGAGTATAAACCATTAGGGTAATGATGCATATTGGTGTATCTGTG  1260

B5      1257    TGAGTGT--TGG-AC-TTGTG-TTGT-----ATATATTAGTATGAATCTGACTGATGTTA  1306
                |||||||  ||  |  || || ||||     ||||||||||||||||||||||||||||||
B9      1261    TGAGTGTAATGTTATGTTATGCTTGTAGGGAATATATTAGTATGAATCTGACTGATGTTA  1320

B5      1307    GGTATAAGCATAAGA  1321
                |||||||||||||||
B9      1321    GGTATAAGCATAAGA  1335
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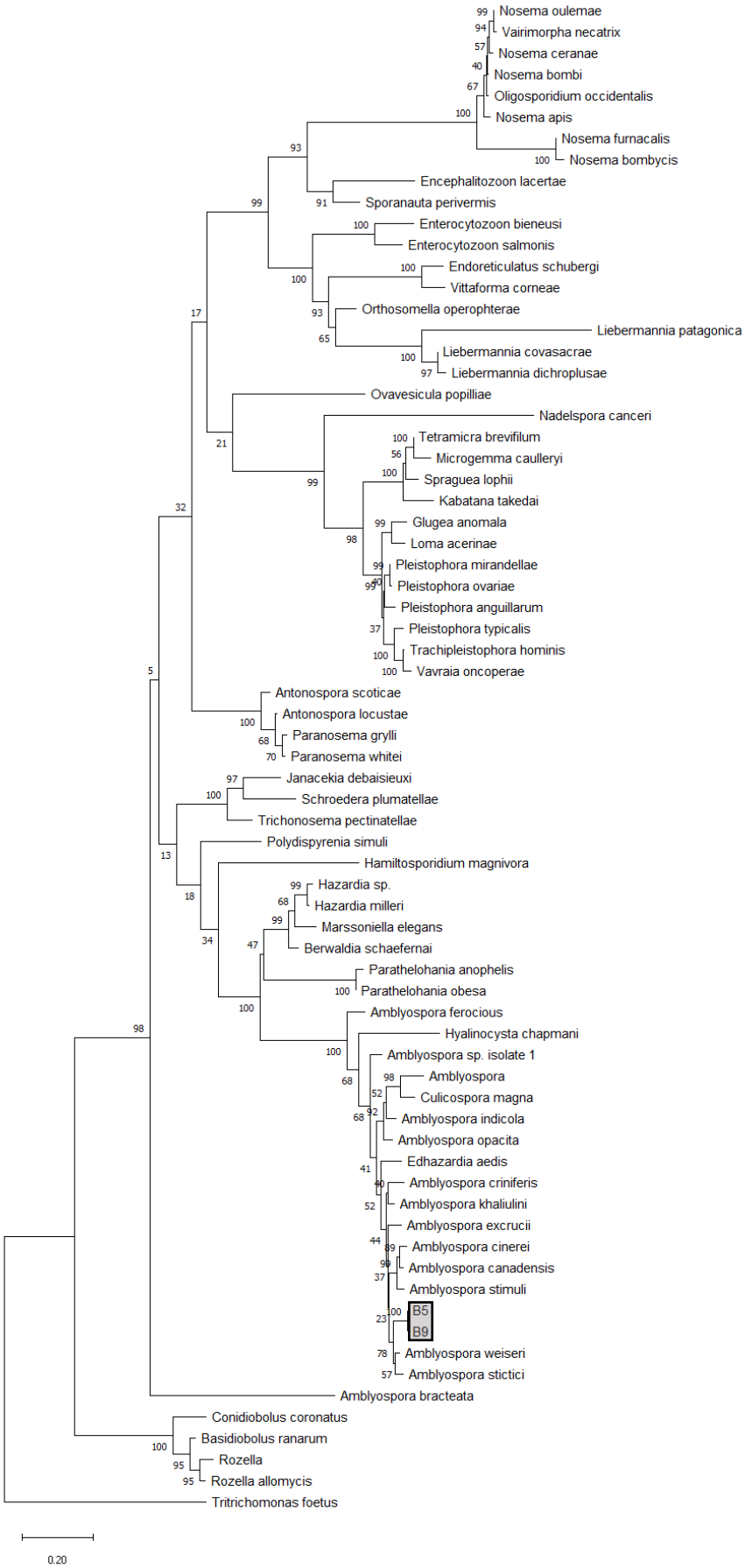
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Vairimorpha necatrix
Nosema ceranae
Nosema bombi
Oligosporidium occidentalis
Nosema apis
Nosema furnacalis
Nosema bombycis
Encephalitozoon lacertae
Sporanauta perivermis
Enterocytozoon bieneusi
Enterocytozoon salmonis
Endoreticulatus schubergi
Vittaforma corneae
Orthosomella operophterae
Liebermannia patagonica
Liebermannia covasacrae
Liebermannia dichroplusae
Ovavesicula popilliae
Nadelspora canceri
Tetramicra brevifilum
Microgemma caulleryi
Spraguea lophii
Kabatana takedai
Glugea anomala
Loma acerinae
Pleistophora mirandellae
Pleistophora ovariae
Pleistophora anguillarum
Pleistophora typicalis
Trachipleistophora hominis
Vavraia oncoperae
Antonospora scoticae
Antonospora locustae
Paranosema grylli
Paranosema whitei
Janacekia debaisieuxi
Schroedera plumatellae
Trichonosema pectinatellae
Polydispyrenia simuli
Hamiltosporidium magnivora
Hazardia sp.
Hazardia milleri
Marssoniella elegans
Berwaldia schaefernai
Parathelohania anophelis
Parathelohania obesa
Amblyospora ferocious
Hyalinocysta chapmani
Amblyospora sp. isolate 1
Amblyospora
Culicospora magna
Amblyospora indicola
Amblyospora opacita
Edhazardia aedis
Amblyospora criniferis
Amblyospora khaliulini
Amblyospora excrucii
Amblyospora cinerei
Amblyospora canadensis
Amblyospora stimuli
B5
B9
Amblyospora weiseri
Amblyospora stictici
Amblyospora bracteata
Conidiobolus coronatus
Basidiobolus ranarum
Rozella
Rozella allomycis
Tritrichomonas foetus
0.20

454

455 Table 1. Mosquito species identified, and *Amblyospora* (Hazard and Oldacre, 1975) infection rate across five
456 brackish water pools sampled at Parkgate Marshes, Wirral, UK. * Either solely, or predominantly *O. detritus*
457 (Haliday, 1833) (pools B/D/F).
458

Pool	Species ID	Infection (%)	Lower Limit	Upper Limit
A	80:20 *O. caspius*/*O. detritus*	0	0	0
B	80:20 *O. detritus*/*O. caspius*	6.57	1.79	17.28
D	*O. detritus*	1.33	0.08	6.36
E	*C. pipiens*	0	0	0
F	90:10 *O. detritus/O. caspius*	0		00
All *O. detritus combined**	*O. detritus*	2.37	0.78	5.62

459