International Journal for Parasitology 48 (2018) 473–481

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Phylogenetic and environmental DNA insights into emerging aquatic parasites: implications for risk management ☆

Salma Sana [a,*], Chris Williams [b], Emilie A. Hardouin [a], Adrian Blake [a], Phil Davison [a,c], Josie Pegg [a], Richard Paley [d], Tiantian Zhang [a], Demetra Andreou [a,*]

[a] Bournemouth University, Faculty of Science and Technology, Fern Barrow, Talbot Campus, Poole, Dorset BH12 5BB, UK
[b] Environment Agency, National Fisheries Laboratory, Bromholme Lane, Brampton, Cambridgeshire PE28 4NE, UK
[c] Centre for Environment, Fisheries and Aquaculture Science, Pakefield Road, Lowestoft, Suffolk NR33 0HT, UK
[d] Centre for Environment, Fisheries and Aquaculture Science, The Nothe, Barrack Road, Weymouth, Dorset DT4 8UB, UK

ARTICLE INFO

Article history:
Received 12 July 2017
Received in revised form 16 November 2017
Accepted 26 November 2017
Available online 10 February 2018

Keywords:
Emergence
Invasion history
Environmental DNA
Rosette agent
Topmouth gudgeon
Epidemiology

ABSTRACT

Species translocation leads to disease emergence in native species of considerable economic importance. Generalist parasites are more likely to be transported, become established and infect new hosts, thus their risk needs to be evaluated. Freshwater systems are particularly at risk from parasite introductions due to the frequency of fish movements, lack of international legislative controls for non-listed pathogens and inherent difficulties with monitoring disease introductions in wild fish populations. Here we used one of the world's most invasive freshwater fish, the topmouth gudgeon, *Pseudorasbora parva*, to demonstrate the risk posed by an emergent generalist parasite, *Sphaerothecum destruens*. *Pseudorasbora parva* has spread to 32 countries from its native range in China through the aquaculture trade and has introduced *S. destruens* to at least five of these. We systematically investigated the spread of *S. destruens* through Great Britain and its establishment in native fish communities through a combination of phylogenetic studies of the host and parasite and a novel environmental DNA detection assay. Molecular approaches confirmed that *S. destruens* is present in 50% of the *P. parva* communities tested and was also detected in resident native fish communities but in the absence of notable histopathological changes. We identified specific *P. parva* haplotypes associated with *S. destruens* and evaluated the risk of disease emergence from this cryptic fish parasite. We provide a framework that can be applied to any aquatic pathogen to enhance detection and help mitigate future disease risks in wild fish populations.

1. Introduction

Species translocation leads to disease emergence of considerable ecological and economic importance (Fisher et al., 2012). Generalist parasites are more likely to be transported, become established and infect new hosts, and pose a high risk to biodiversity across ecosystems. Freshwater systems are particularly at risk due to insufficient international legislation and system-inherent disease monitoring difficulties (Gozlan, 2012). This has resulted in the frequent introduction of non-native parasites to freshwater fisheries (Williams et al., 2013) with a risk of aquatic disease emergence and associated declines in wild fish populations (Peeler et al., 2011; Ercan et al., 2015). Non-native parasites with direct life-cycles, low host specificity, tolerant and long-lived environmental infectious propagules and a wide temperature tolerance are more likely to be translocated and become established in new environments (Andreou et al., 2009; Fisher et al., 2012).

The topmouth gudgeon, *Pseudorasbora parva*, is a small cyprinid fish that is naturally distributed in eastern Asia. It was introduced into Europe from China in the 1960s through a succession of accidental introductions into the area around the Black Sea through the trade of Chinese carp in aquaculture (Gozlan et al., 2010). It has now invaded at least 32 countries, including most of Europe, plus Turkey, Iran and Morocco, with their long-distance dispersal also occurring via aquaculture trade routes (Gozlan et al., 2010). In 2005, *P. parva* was identified as a healthy reservoir of the generalist parasite *Sphaerothecum destruens* which has been identified as a threat to freshwater fish biodiversity (Gozlan et al., 2005).

☆ Note: Nucleotide sequence data reported in this paper are available in the GenBank™ database under the accession numbers MG283239-MG283252, MG386173-MG386179.

* Corresponding authors.
E-mail addresses: ssana@bournemouth.ac.uk (S. Sana), dandreou@bournemouth.ac.uk (D. Andreou).

https://doi.org/10.1016/j.ijpara.2017.11.002
0020-7519/

The parasite has also been identified as non-native to Europe (Sana et al., 2017) having been introduced with the highly invasive fish *P. parva*. Great Britain (GB) is the first European country where *S. destruens* was identified in *P. parva* populations (Gozlan et al., 2005). *Pseudorasbora parva* was first recorded in the UK in an aquaculture facility in southern England in 1996 (Domaniewski and Wheeler, 1996) and has rapidly spread and colonized up to 23 UK water bodies (Britton et al., 2008). All *P. parva* populations in the UK have been associated with aquaculture or recreational fisheries with no recorded established populations in wild habitats such as streams, rivers or lakes. In response to the potential threats posed by *P. parva* (Britton et al., 2007), a national programme of eradication has been designed and administered (Britton et al., 2010). The programme aimed at complete eradication of *P. parva* from high risk sites (with high risk sites identified based on the conservation and fishery value of the adjacent water body) or containment in the case of medium risk sites (Britton et al., 2008). By 2014, 15 out of 23 confirmed *P. parva* sites had been eradicated, with a further six sites to be eradicated in England by 2017 (Britton et al., 2010; GBNNSS, 2015).

Despite *P. parva* having no wild populations in GB, several sites invaded by *P. parva* have water effluents which flow into wild freshwater habitats. This can have important implications for transmission of the parasite as epidemiological modelling has predicted that *S. destruens* can spread to and establish in connected downstream communities through environmental transmission of their infective spores and zoospores within 1 year post introduction of infected *P. parva* (Al-Shorbaji et al., 2016). The same work also predicted that *S. destruens* can establish in new hosts and maintain its transmission in the absence of the initial reservoir host - in this case *P. parva* (Al-Shorbaji et al., 2016). As *S. destruens* is a true generalist, it is highly probable that adjacent communities downstream from established *P. parva* populations, positive for *S. destruens*, have established *S. destruens* infections (Andreou and Gozlan, 2016). Despite an absence of disease detected in wild fish populations through existing monitoring activities, *S. destruens* has been proposed as a high risk parasite with the recommendation that its prevalence is closely monitored (Andreou and Gozlan, 2016). However, the cryptic nature of infections in fish can make the detection of *S. destruens* in asymptomatic fish problematic. Moreover, the sacrificial sampling of wild fish, in particular salmonids, is undesirable. As such, there is a need for a detailed epidemiological picture of *S. destruens* in GB, combining traditional methods of detection (e.g. DNA-based detection and microscopic examination of host tissue) with more novel approaches employing environmental DNA (eDNA) detection (due to its cost efficiency) (Andreou et al., 2011a). Here we used GB as a model country to determine how aquatic non-native parasites could spread through reservoir host translocation. Specifically, we (i) determined the distribution and presence of *S. destruens* in *P. parva* populations and deciphered the spread using the genetic diversity of the parasite and host and (ii) assessed the of risk of disease transfer to native fishes in water bodies with direct connection to *P. parva* holding waters, using a combination of in-tissue molecular detection, histopathology and a novel eDNA detection test for *S. destruens*.

2. Materials and methods

2.1. Detection of S. destruens in P. parva populations

Seven *P. parva* populations ($n = 210$ fish) were sampled from England and Wales prior to their eradication in 2013–2015 by the Environment Agency, National Fisheries Laboratory, Cambridgeshire, UK (Fig. 1; Table 1). Permission to sample these populations was granted by the Environment Agency, UK. Populations were sampled from six enclosed still water fisheries and two fisheries with outlets to streams. In two populations, roach (*Rutilus rutilus*) was also present and thus sampled ($n = 15$; Table 1). A number of native fish species were sampled from the stream adjacent to the proposed original site of *P. parva* introduction in 2015 and 2016 (Site 1; Table 1).

Fig. 1. Distribution of sampled *Pseudorasbora parva* populations across Great Britain (GB). Population 1a is the hypothesised first *P. parva* population in GB to have been introduced in the mid-1980s (Domaniewski and Wheeler, 1996). Details of each sampled population can be found in Table 1. The black and white numbering for each population represents the two genetically different metapopulations of the host *P. parva* in GB (Blake et al., unpublished data). The 18S rRNA haplotypes for *Sphaerothecum destruens* are represented in the left half of each circle, the cytochrome b (Cyt-b) gene haplotypes are in the right half of each circle. The different patterns and shadings represent different haplotypes.

All fish were euthanised through a lethal dose of benzocaine. From all *P. parva*, samples of liver and kidney tissues were divided with one half fixed in 100% ethanol for molecular detection and the remaining half fixed in 10% Neutral Buffered Formalin (NBF) for histopathology. From all native fish species, detailed post mortem examinations were performed to detect gross pathological changes and the presence of parasites. Liver and kidney samples were taken in 100% ethanol for molecular detection, with additional samples of liver, kidney, spleen, gut, heart and gill for histopathological assessment. Molecular detection using the 18S rRNA gene was performed in pooled kidney and liver samples as described in Andreou et al. (2012). All *S. destruens*-positive samples had their cytochrome b gene (Cyt-b) amplified as per Sana et al., (2017) and their tissues were histopathologically checked for the presence of *S. destruens* (Andreou et al., 2011a). For clarity, all *S. destruens* Cyt-b haplotypes will be named as *S. destruens*_Cytb. Samples for histopathology were trimmed, dehydrated in alcohol, embedded in paraffin wax, sectioned at 3 μm, stained using H&E and examined microscopically for pathological changes and the presence of *S. destruens*.

2.2. Deciphering invasion history through host and parasite phylogenetic relationships

In order to investigate any potential links between specific *P. parva* populations or genetic lineages and the spread of *S. destruens*, all *P. parva* had their Cyt-b gene amplified and sequenced as per Simon et al. (2011). For clarity, all *P. parva* Cyt-b haplotypes will be named as *P. parva*_Cytb. The obtained Cyt-b sequences for *P. parva* were then aligned with all the available *P. parva* Cyt-b

Table 1
Geographical location and *Sphaerothecum destruens* prevalence for all fish populations screened using two DNA markers – 18S rRNA and cytochrome b (Cyt-b).

Population	Water type	Sampling year	Geographical coordinates[a]	Sampled fish species[b]	Sample size	Positive fish species for *S. destruens*	Prevalence of *S. destruens* %	Genetic marker of *S. destruens*	
								18S rRNA	Cyt-b
1a	Disused aquaculture facility, online to river	2013	NGR: SU3822 S. England	*P. parva*	30	*P. parva*	3.33 (1/30)	✓ (Hap_1)	✓ (Hap_1)
1b	River adjacent to Site 1-part B (slower flowing section of river adjacent to Site 1a)			*S. trutta*	3	*S. trutta*	33.3 (1/3)	✓ (Hap_1)	–
				S. cephalus	4				
				R. rutilus	2	*S. cephalus*	50 (2/4)	✓ (Hap_4)	✓ (Hap_1)
				L. leuciscus	5				
						S. cephalus		✓ (Hap_1)	✓ (Hap_1)
						R. rutilus	100 (2/2)	✓ (Hap_1)	✓ (Hap_1)
						R. rutilus		✓ (Hap_1)	✓ (Hap_3)
						L. leuciscus	60 (3/5)	✓ (Hap_1)	–
						L. leuciscus		✓ (Hap_6)	–
						L. leuciscus		✓ (Hap_5)	–
1c	River adjacent to Site 1-part A (high flowing stream directly linked to site 1a			*S. trutta*	30	–	0		
2	Enclosed still water fishery	2013	NGR: SO7657 Midlands	*P. parva*	30	–	0		
				R. rutilus	5	*R. rutilus*	20 (1/5)	✓ (Hap_1)	
3	Ornamental pond with outlet to stream	2013	NGR: SY0786 SE England	*R. rutilus*	10	–	0		
4	Enclosed still water fishery	2014	NGR: SK7425 Midlands	*P. parva*	30	*P. parva*	6.66 (2/30)	✓ (Hap_3)	✓ (Hap_1)
								✓ (Hap_1)	✓ (Hap_2)
5	Reservoir	2014	NGR: SN5104 S. Wales	*P. parva*	30	–	0		
6	Enclosed still water fishery-1	2014	NGR: SJ2487	*P. parva*	30	*P. parva*	6.66 (2/30)	✓ (Hap_1)	–
								✓ (Hap_1)	–
	Enclosed still water fishery-2	2015		*P. parva*	30	–	0		
7	Enclosed still water fishery	2014	NGR: SU3922	*P. parva*	30	–	0		

[a] Fish were sampled from these localities, South England, South East England, South Wales, Midlands together with their National Grid reference (NGR) coordinates.
[b] Fish species sampled were *Pseudorasbora parva*, *Salmo trutta*, *Squalius cephalus*, *Rutilus rutilus* and *Leuciscus leuciscus*.

sequences on GenBank (JF489575-JF489887, KR074432-KR074994, MG432498-MG432659) from its native range (China) and invasive range (Europe). The sequences were aligned by Clustal W in BioEdit (Hall, 1999). Haplotype diversity was calculated in DnaSP version 5.10 (Librado and Rozas, 2009). Two phylogenetic trees were constructed to identify the *P. parva* haplotypes associated with the presence of *S. destruens* at the world and the GB levels. The phylogenetic analysis was performed using Mr Bayes (Ronquist et al., 2012) and posterior probabilities were obtained after 2,500,000 generations with a burn-in of 25%. The tree was calculated using the Hasegawa-Kishino-Yano model with a Gamma distribution (HKY+G) model determined with jModeltest v 2.1.4 (Darriba et al., 2011). The Cyt-b gene sequences from *Ictiobus bubalus* (JF799443.1), *Hypentelium nigricans* (JF799441.1), and *Danio rerio* (JN234356.1) were used as outgroups.

In order to investigate the genetic variation and potential phylogenetic relationships of *S. destruens* populations, phylogenetic networks were generated for the 18S rRNA and Cyt-b sequences using DnaSP version 5.10 (Librado and Rozas, 2009) and Network Publisher ((Bandelt et al., 1999); available at http://www.fluxus-engineering.com) using sequences generated in the present study and GenBank accession sequences: AY267344.1, AY267345.1, AY267346.1, FN996945.1, as well as MF062546-MF062560 for the 18S rRNA gene (Arkush et al., 2003; Paley et al., 2012). For the Cyt-b marker, we used the seven sequences generated in this study and GenBank accession sequences MF101749-MF101755 (Sana et al., 2017).

2.3. eDNA detection of S. destruens

A real-time PCR (RT-PCR) detection tool was designed. Primers and a probe specific to the *S. destruens* 18S rRNA gene segment (Supplementary Table S1) were created with Primer Express 2.0 (Applied Biosystems, UK) using an alignment of fish and *S. destruens* sequences (GenBank sequences used: *S. destruens*: AY267344.1, AY267345.1, AY267346.1, FN996945.1, and MF062546-MF062560; and fish species: *Salmo trutta* (DQ009482.1), *R. rutilus* (AY770580.1), *Oncorhynchus mykiss* (FJ710874.1) and *Cyprinus carpio* (FJ710827.1)). The Taqman Minor Groove Binder (MGB) probe was labelled with the fluorescent reporter dye Fluorescein amidite (FAM) at the 5′-end and a non-fluorescent quencher MGBNFQ at the 3′-end. The unlabelled PCR primers and probe were purchased from Applied Biosystems.

DNA amplification utilised the TaqMan® Gene Expression Master Mix-UDG (Invitrogen, UK) with 20 μl reaction volumes containing 10 μl of TaqMan® Gene Expression Master Mix-UDG, 1 μl of assay mix (primers and probe) and 2 μl of DNA template (undiluted). All reactions were performed in a StepOne real-time PCR machine (Applied Biosystem) and analysed by StepOne software v 2.0. In all the analyses, the software-defined baseline was auto-

matically set to 0.02, and 6-carboxyl-X-Rhodamine (ROX) was selected as a passive reference. Cycling conditions consisted of a holding stage at 50 °C for 2 min to allow Uracil DNA Glycosylase (UDG) enzymatic activity and initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. Blank extraction controls from the DNA extraction were included together with the no DNA template PCR controls.

2.3.1. eDNA assay – detection limits, specificity and validation

Detection limits were determined using a serial dilution of pure *S. destruens* DNA extracted from pure *S. destruens* spores (Paley et al., 2012) using the Qiagen DNeasy blood and tissue kit (Germany). A calibration curve was generated using a 10-fold serial dilution of *S. destruens* genomic DNA to give a range of template concentrations from 10 ng/μl to 1 fg/μl. The standards were run in triplicate to test the repeatability of the quantification using RT-PCR assay. Negative PCR controls consisted of sterile water. The detection limit was defined as the lowest genomic *S. destruens* DNA concentration detected at least 95% of the time by the assay.

The specificity of the assay was determined through testing for cross-reactivity with pure fish DNA and the only close relative to *S. destruens* for which we had access to genomic DNA - *Dermocystidium salmonis* 18S rDNA section inserted pGEM® –T vector (Promega, UK). The fish species tested for cross-reactivity included carp (*C. carpio*), roach (*R. rutilus*), minnow (*Phoxinus phoxinus*), common bream (*Abramis brama*), chub (*Squalius cephalus*), barbel (*Barbus barbus*) and *P. parva*. The assay was tested on a total genomic DNA range of 10 ng to 0.01 ng for each species.

The efficacy of the assay in detecting *S. destruens* eDNA was first tested in a laboratory experiment where *S. destruens* spores were incubated in water at 18 °C for 20 days. Specifically, two water types were used to incubate spores – distilled and turbid water. Turbid water was created by adding 10 g of un-autoclaved soil, 200 ml of aquarium water and 1800 ml of distilled water to represent a closer approximation to river water. Spores were incubated in 3 L aquariums which were filled with 2 L of water (distilled or turbid) and were spiked with three *S. destruens* spore concentrations; 1500 spores/ml (high), 150 spores/ml (medium) and 50 spores/ml (low). Each water and spore combination was repeated three times. One hundred ml of water were sampled on days 6, 12, and 20.

2.3.2. Application of the eDNA assay to environmental samples

The eDNA assay was further tested on water samples from the first recorded site for *P. parva* introduction, Site 1 (Table 1, Fig. 1) which also had *S. destruens*-positive *P. parva*. Water sampling took place in 2013 whilst the site still maintained *P. parva* populations and in 2016 (following *P. parva* eradication by the Environment Agency in 2015). The 2013 sampling focused on sampling ponds and water channels of the disused fishery at Site 1 (Section 1a in Supplementary Fig. S1). In 2016, sampling was extended to include the stream flowing into the disused fishery (Section 1d in Supplementary Fig. S1). the fishery (Section 1a in Supplementary Fig. S1) and the adjacent Tadburn Lake stream (Sections 1b and 1c in Supplementary Fig. S1) which flows after the fishery. Two water sites that had no previous record of *S. destruens* and its reservoir host *P. parva* presence were sampled to act as negative controls. These were the Bourne stream ($n = 3$) and the River Teme ($n = 2$).

Environmental water samples were collected in 1 L sterile plastic bottles attached to rods and the bottles were submerged vertically, ensuring that a vertical column of water was sampled. The sampling equipment was changed between each sampling point and disposable gloves were used for every site. Samples were stored on ice and filtered within 24 h. In the laboratory, the water was pre-filtered using a 200 μm filter to remove coarse material. An 80–500 ml subsample was further filtered using a 0.45 μm cellulose nitrate filter membrane (Whatman™, UK). Negative field controls were included at the start and end of sample collection in the field. Negative field controls consisted of 1 L sterile plastic bottles filled with sterile water, which were treated in the field in the exact manner as sample collection bottles. The DNA was extracted from filter papers using a Power Water DNA Isolation Kit (MO BIO, Inc, UK). The extracted DNA was then screened for the presence of *S. destruens* DNA using the developed eDNA assay.

3. Results

3.1. Sphaerothecum destruens prevalence

Sphaerothecum destruens was detected using molecular tools in 14 individuals out of the 269 fish (all species) sampled across seven sites (Table 1, Fig. 1). Fifty percent of the *P. parva* sites tested positive for *S. destruens* with prevalence ranging from 3 to 6% (Table 1). In Site 2, both *P. parva* and *R. rutilus* were sampled, with *S. destruens* being present only in *R. rutilus* with a prevalence of 20%. Two of the three *S. destruens*-positive *P. parva* populations were from enclosed still water fisheries (Populations 4 and 6, with 6.7% prevalence; Fig. 1). The third *S. destruens*-positive *P. parva* population was from Site 1a which represented the first accidental *P. parva* introduction into GB in the 1980s and tested positive for *S. destruens* with a prevalence of 3% (Fig. 1). This population was from a disused fishery from which effluents are discharged in the adjacent Tadburn Lake stream (Sites 1b, c; Table 1) which connects with the River Test in Hampshire. A number of native species were sampled from the Tadburn lake stream and tested positive for *S. destruens*. These included chub (*S. cephalus*), dace (*Leuciscus leuciscus*), brown trout (*S. trutta*) and roach (*R. rutilus*) (Population 1b) with an overall prevalence across all species of 57%. All fish which tested positive by molecular detection did not show any signs of clinical disease and all organs were considered to be histopathologically normal.

3.2. Phylogenetic analysis of S. destruens using the 18S rRNA and Cyt-b sequences

Eight *S. destruens* haplotypes were detected for the 18S rRNA gene in 14 individuals from five GB water bodies (Table 1). Site 1 had the highest 18S rRNA haplotype diversity with three new haplotypes being found (Haplotypes 6–8) which were detected in *S. cephalus* and *L. leuciscus* (Fig. 2A). The unique 18S rRNA Haplotype 5 was found in Site 4 which also had the unique *S. destruens*_Cytb Haplotype 2 (Fig. 2B). *Sphaerothecum destruens* Cyt-b was amplified from seven fish individuals at Sites 1a, 1b and 4. Only three haplotypes were found in our samples. Sites 1a and 1b had *S. destruens*_Cytb Haplotypes 1 and 3 (Fig. 2B).

3.3. Phylogenetic analysis of P. parva

A total of 91 haplotypes from 957 *P. parva* individuals were identified in the dataset of Cyt-b sequences of *P. parva* populations across the world. Four *P.* parva_Cytb haplotypes were identified in GB, *P. parva*_Cytb Haplotype 1, 3, 4 and 6 (Figs. 3 and 4). *Pseudorasbora parva* Cytb Haplotypes 4 and 6 were positive for *S. destruens* (Fig. 3) with the highest proportion of infected fish belonging to *P. parva*_Cytb Haplotype 4. This haplotype was positive for *S. destruens* in samples across two European countries - GB and Spain (Sana et al., 2017) and was also associated with the unique *S. destruens*_Cytb Haplotype 2 from Site 4 (Table 1). Across native and invasive ranges of *P. parva*, the *P. parva*_Cytb haplotypes associated with the presence of *S. destruens* are: *P. parva*_Cytb Haplotypes 1, 4, 6, 7 and 12 (Fig. 4).

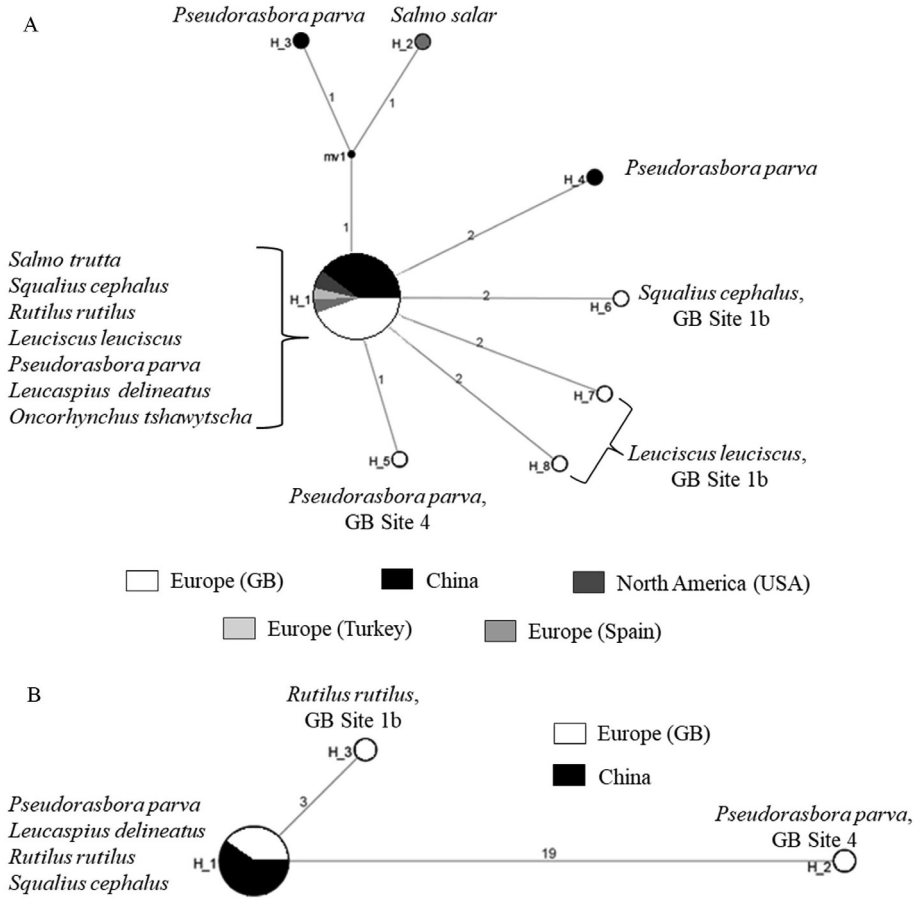

Fig. 2. Minimum spanning network based on (A) 18S rRNA (397 bp) and (B) mitochondrial cytochrome b (Cyt-b, 700 bp) sequences of *Sphaerothecum destruens* isolated from fish hosts. The sizes of the different circles represent the frequencies of each respective haplotype. The numbers on the branches indicate the number of mutations between the nodes. Black circles indicate branch splits. The colour code indicates *S. destruens* individuals from different localities: black, China; medium grey, Spain; light grey, Turkey; white, Great Britain (GB); and dark grey, USA. (A) Haplotypes H_1, H_2, H_3, H_4, H_5, H_6, H_7 and H_8 represent *S. destruens* 18S rRNA haplotypes. (B) Haplotypes H_1, H_2 and H_3 are *S. destruens*_Cytb haplotypes.

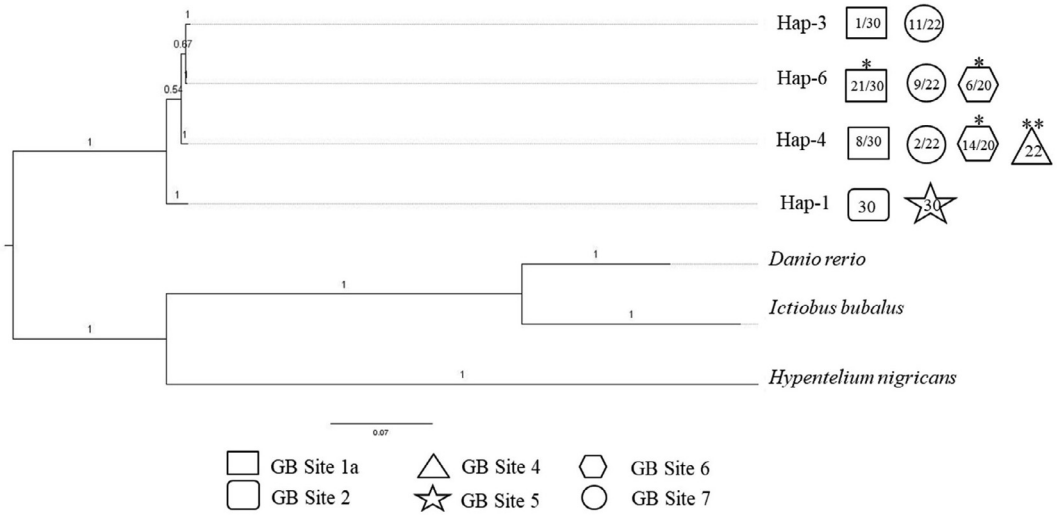

Fig. 3. Cytochrome b (Cyt-b) haplotypes of *Pseudorasbora parva* across the sampled Great Britain (GB) sites. Within the shapes, the proportions of each haplotype are indicated for each population. The number of asterisks on each site specifies the number of *P. parva* individuals positive for *Sphaerothecum destruens*. All *S. destruens*-positive *P. parva* had *P. parva*_Cytb haplotypes 4 and 6. The tree was built using the Bayesian inference method based on the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985) with Gamma distribution analysis of mitochondrial cyt-b gene of *P. parva* in Mr Bayes (Ronquist et al., 2012).

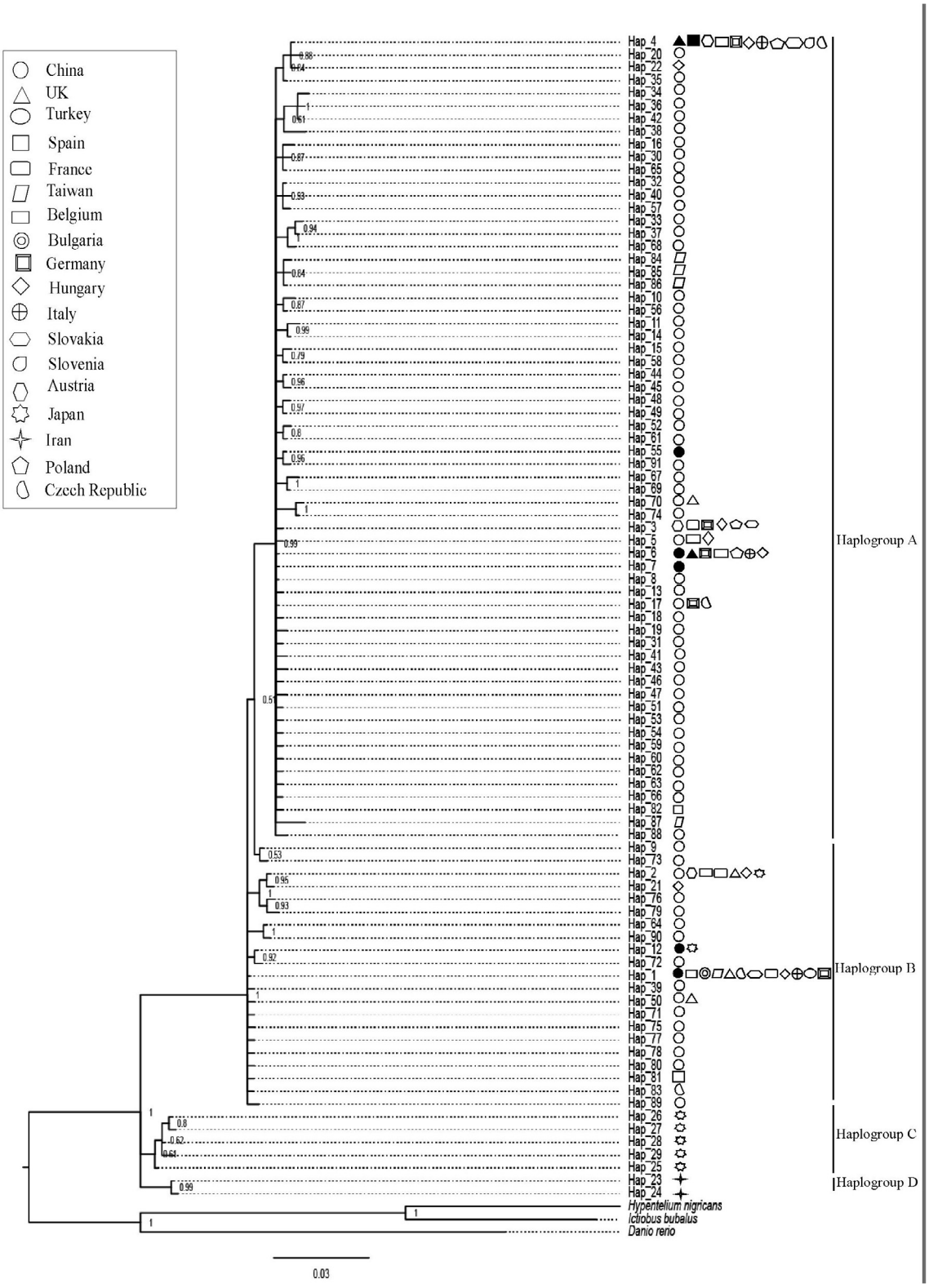

Fig. 4. Molecular phylogenetic analysis of Cytochrome b (Cyt-b) haplotypes of *Pseudorasbora parva* populations across its native and non-native range. The tree was built using the Bayesian inference method based on the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985) with Gamma distribution in Mr Bayes (Ronquist et al., 2012). The different shapes indicate the countries that each haplotype has been found in and the black coloured shapes indicate *Sphaerothecum destruens*-positive haplotypes in those countries.

Table 2
Field sampling for the validation of the environmental DNA (eDNA) technique for *Sphaerothecum destruens* at Site 1 where *S. destruens* was detected in fish samples and at two expected *S. destruens* negative sites. Mean C_t values per site are provided with the S.D. The number of samples with undetected C_t values are indicated. Geographical coordinates of sampled locations were: Site 1a and 1b: SU3862; Site 1c: SU3842; Site 1d: SU3932; Bourne Stream Site 1 and 2: SZ0689, Bourne Stream site 3: SZ0679; River Teme Site 1: SO8335; River Teme Site 2: SO7237.

Site	Ponds/stream	Sampling points	Volume of water filtered (ml)	Mean C_t value (S.D.)	Fish sampled[a] (number)	*S. destruens* positive fish (prevalence)
GB Site 1						
(i) Pre-eradication of *P. parva* (2013)	Section 1a Pond 12 (52 m × 7 m)	Six 1 L samples (12-1–12-6) around the pond edge app. 15 m apart		Undetected		
	Section 1a Pond 14 (52 m × 7 m)	Six 1 L samples (14-7–14-12) around the pond edge app. 15 m apart	80	30.87 (±0.88)	*P. parva* (30)	*P. parva* (3.33%)
	Section 1a Over Flow Pond (pond running east–west, to south of fishery pond row). (65 m × 15 m)	Five 1 L samples (OF-13-OF17) around the pond edge app. 20 m apart		34.12 (±0.77)	*S. trutta* (3) *S. cephalus* (4) *R. rutilus* (2) *L. leuciscus* (5)	*S. trutta* (33.3%) *S. cephalus* (50%) *R. rutilus* (100%) *L. leuciscus* (60%)
(ii) post-eradication of *P. parva* (2016)	Section 1d (60 m stretch to inlet to fishery)	Three 1 L samples app. 20 m apart	500	35.48 (±1.03)	–	
	Section 1a Pond 1 (52 m × 7 m)	Two 1 L samples (1a-1b) from two extremes of the pond.		Undetected	–	–
	Section 1b- (122 m downstream of fishery)SU3862	Three 1 L samples (1b1- 1b3 along the stream stretch app. 40 m apart		36.2 (±0.78)		
	Section 1b (360 m downstream of fishery)SU3848	Three 1 L samples (1b4-1b6) along the stretch app. 50 m apart		33.33 (±0.20)	Stone loach *N. barbatulus* (3), bullhead *C. gobio* (5), stickleback *G. aculeatus* (9)	
	Section 1c (500 m downstream of fishery) SU3842	Three 1 L samples (1c1-1c3)		35.35 (±2.76)	Bullhead *C. gobio* (2)	
Bourne stream	Site 1 (stagnant water) Usually Rudd, Carp, Minnow, Sticklebacks and Chub	1 L water samples at each site	1000		–	
	Site 2 (Fast flowing water)				–	
	Site 3 (this site was further downstream from site 1 & site 2)			37.26 (±0.12)	–	
River Teme	Site 1SO8335	1 L water samples	1000	Undetected	–	
	Site 2				–	

GB, Great Britain.
[a] Fish species sampled were *Pseudorasbora parva*, *Salmo trutta*, *Squalius cephalus*, *Rutilus rutilus*, *Leuciscus leuciscus*, *Noemacheilus barbatulus*, *Cottus gobio* and *Gasterosteus aculeatus.*

3.4. eDNA assay – detection limits, specificity and validation

Using a 10-fold serial dilution of *S. destruens* genomic DNA, the limit of detection of the Taqman assay was 1 pg/μl (Supplementary Table S2). The cycle threshold (C_t) values with standard genomic DNA dilutions in the late cycle (>36) which corresponded to 0.1 pg/μl were unreliable as the probability of detection was <95% (Burns and Valdivia, 2008). Therefore, the C_t values >36.55 were scored as negative or below the detection limit, in line with other studies in the development of the eDNA method for parasite detection (Kirshtein et al., 2007; Huver et al., 2015). In the assay, PCR negatives had no C_t readings. The RT-PCR was also highly specific to *S. destruens* with all tested fishes and *D. salmonis* yielding no C_t values following amplification. In the experimental validation set-up, *S. destruens*-specific DNA was detected by RT-PCR in both natural and turbid water conditions until day 20 across all spore concentrations (Supplementary Table S3).

3.4.1. Detecting S. destruens in environmental samples

Applying the minimum detection threshold ($C_t = 36.55$), *S. destruens* eDNA was identified in GB Site 1 both pre-eradication of *P. parva* and post-eradication (Table 2). *Sphaerothecum destruens* was detected in tissues of *P. parva*, *S. trutta*, *S. cephalus* and *L. leuciscus* samples pre-eradication of *P. parva* (Table 1), confirming a source of spores in the environment. We were not successful in catching any *S. cephalus* or *L. leuciscus* during our post-eradication fish sampling and were unable to sample *S. trutta*, preventing a check for the presence of *S. destruens* in these species post *P. parva* eradication. We were able to sample a number of other fish species (Table 2) which all tested negative for *S. destruens*. DNA from *S. destruens* was not detected in the two negative control sites - the Bourne Stream and River Teme.

4. Discussion

Generalist parasites are more likely to be transported and establish in new communities, and here we show that the non-native parasite *S. destruens* has been transported to multiple locations in GB and has potentially spread to adjacent native fish communities. The persistence of *S. destruens* DNA in water bodies from which the reservoir host has been eradicated corroborates model predictions that the parasite is able to spread and establish in native fish communities within a year of its introduction (Al-Shorbaji et al., 2015, 2016). Phylogenetic analysis has identified a small number of lineages of the reservoir host, *P. parva*, associated with the parasite's presence in native and invasive ranges of *P. parva*. These lineages are more often detected in the invasive range *P. parva*, increasing the possibility that the parasite is more widely prevalent throughout continental Europe than previously thought.

The high genetic diversity of *S. destruens* in the hypothesised first site of introduction indicates that *S. destruens* was first introduced in southwestern England. The parasite has since spread to other locations through the accidental translocation of its reservoir host, as exemplified by the reduced genetic diversity in subsequently invaded sites (Fig. 1). Additionally, we have identified two new mitochondrial haplotypes of *S. destruens* which coincide with specific *P. parva* haplotypes, supporting the view that the parasite was introduced to GB through at least two independent introductions of its host. The high genetic diversity in the south-west is mainly observed as the presence of different *S. destruens* haplotypes in new fish hosts. Although we were unable to test whether this has been driven by host-switching, this would be an important avenue for future work. More importantly, however, we propose that whenever *P. parva* are identified as *S. destruens*-positive, native fish populations that share those waters may also harbour the parasite and could be moved to other waters with fish stocking activity.

We evaluated the distribution of the parasite through molecular detection and used histopathology to evaluate the disease risk associated with this parasite. We did not find evidence of clinical or histopathological disease in any of the fishes that tested positive for *S. destruens*, indicating that although the parasite is present in both *P. parva* and native fishes, it is not causing pathological changes. This suggests that *S. destruens* may not be as pathogenic as recent literature suggests; that the triggers for disease were absent in the waters examined; or that infections were so light that this cryptic parasite evaded detection during these examinations. Notwithstanding, due to the number of waters examined, the prevalence of infection within these populations and the range of species infected, the present study suggests that *S. destruens* poses a low risk of disease to native freshwater diversity. However, we recommend an ongoing awareness of *S. destruens* and that disease surveillance continues to take account of emerging pathogens in a range of species and environments. This is important where infections could impact pressured host species (e.g. wild salmonids), and where disease severity can be influenced by varied environmental and host factors such as reproductive state (Andreou et al., 2011b). Furthermore, single point sampling is insufficient to detect population level changes and we thus recommend that native species in affected locations are subject to monitoring using varied measures of change (Williams et al., 2013) including catch per unit effort measurements to monitor population level changes (Ercan et al., 2015).

The invasive *P. parva* has now spread to 32 countries worldwide with a high probability that *S. destruens* has also been introduced and established in these waters. We provide a framework and tool set through which these waters can be screened for the presence of this parasite and its risk evaluated through a combination of molecular detection and routine pathological assessments. Although this approach and tools have been applied to a freshwater environment, they are transferrable to the marine environment and can be useful to monitor *S. destruens* in marine fish industries (e.g. Ercan et al., 2015 identified *S. destruens* in sea bass fisheries). The eDNA assay can also be used to screen fish consignments prior to fish movements, reducing the risk of introduction to new sites. This work has also, to our knowledge, provided the first data suggesting that host specialisation could be occurring in this true generalist as most new genetic variants of *S. destruens* were detected in new hosts. Species translocation has led to disease spread across ecosystems (Fisher et al., 2012), warranting heightened awareness and detailed detection methods to better understand disease risk. Here, we have provided new insights of *S. destruens* infections in new localities and provide further evidence of the potential for parasite invasion and the need for monitoring to understand disease risk and protect native aquatic resources.

Acknowledgements

The authors would like to thank staff of the Environment Agency, UK for assistance with fish sampling. Also Dr S. Feist (Centre for Environment, Fisheries and Aquaculture Science, (Cefas), Weymouth, UK) and Prof. Randolph Richards (Stirling University, Scotland, UK) for assistance with interpretation of histology slides and Dr David Stone (Cefas, Weymouth, UK) for his help. The views expressed in this article are the authors' own and do not reflect the view of Government bodies.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijpara.2017.11.002.

References

Al-Shorbaji, F., Roche, B., Gozlan, R., Britton, R., Andreou, D., 2016. The consequences of reservoir host eradication on disease epidemiology in animal communities. Emerg. Microbes Infect. 5, e46.

Al-Shorbaji, F.N., Gozlan, R.E., Roche, B., Britton, J.R., Andreou, D., 2015. The alternate role of direct and environmental transmission in fungal infectious disease in wildlife: threats for biodiversity conservation. Sci. Rep. 5.

Andreou, D., Arkush, K.D., Guégan, J.-F., Gozlan, R.E., 2012. Introduced pathogens and native freshwater biodiversity: a case study of *Sphaerothecum destruens*. PLoS One 7, e36998.

Andreou, D., Gozlan, R., Paley, R., 2009. Temperature influence on production and longevity of *Sphaerothecum destruens* zoospores. J. Parasitol. 95, 1539–1541.

Andreou, D., Gozlan, R.E., 2016. Associated disease risk from the introduced generalist pathogen *Sphaerothecum destruens*: Management and policy implications. Parasitology 143, 1204–1210.

Andreou, D., Gozlan, R.E., Stone, D., Martin, P., Bateman, K., Feist, S.W., 2011a. *Sphaerothecum destruens* pathology in cyprinids. Dis. Aquat. Organ. 95, 145.

Andreou, D., Hussey, M., Griffiths, S.W., Gozlan, R., 2011b. Influence of host reproductive state on *Sphaerothecum destruens* prevalence and infection level. Parasitology 138, 26–34.

Arkush, K.D., Mendoza, L., Adkison, M.A., Hedrick, R.P., 2003. Observations on the life stages of *Sphaerothecum destruens* ng, n. sp., a mesomycetozoean fish pathogen formally referred to as the rosette agent. J. Eukaryot. Microbiol. 50, 430–438.

Bandelt, H.-J., Forster, P., Röhl, A., 1999. Median-joining networks for inferring intraspecific phylogenies. Mol. Biol. Evol. 16, 37–48.

Britton, J., Brazier, M., Davies, G., Chare, S., 2008. Case studies on eradicating the Asiatic cyprinid *Pseudorasbora parva* from fishing lakes in England to prevent their riverine dispersal. Aquat. Conserv. Mar. Freshw. Ecosyst. 18, 867–876.

Britton, J., Davies, G., Brazier, M., Pinder, A., 2007. A case study on the population ecology of a topmouth gudgeon (*Pseudorasbora parva*) population in the UK and the implications for native fish communities. Aquat. Conserv. Mar. Freshw. Ecosyst. 17, 749–759.

Britton, J.R., Davies, G.D., Brazier, M., 2010. Towards the successful control of the invasive *Pseudorasbora parva* in the UK. Biol. Invasions 12, 125–131.

Burns, M., Valdivia, H., 2008. Modelling the limit oqf detection in real-time quantitative PCR. Eur. Food Res. Technol. 226, 1513–1524.

Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2011. ProtTest 3: fast selection of best-fit models of protein evolution. Bioinformatics 27, 1164–1165.

Domaniewski, J., Wheeler, A., 1996. The topmouth gudgeon has arrived. Fish 43, 40.

Ercan, D., Andreou, D., Sana, S., Öntaş, C., Baba, E., Top, N., Karakuş, U., Tarkan, A.S., Gozlan, R.E., 2015. Evidence of threat to European economy and biodiversity following the introduction of an alien pathogen on the fungal–animal boundary. Emerg. Microbes Infect. 4, e52.

Fisher, M.C., Henk, D.A., Briggs, C.J., Brownstein, J.S., Madoff, L.C., McCraw, S.L., Gurr, S.J., 2012. Emerging fungal threats to animal, plant and ecosystem health. Nature 484, 186–194.

GBNNSS, 2015. The Great Britain Invasive Non-native Species Strategy August 2015. The Great Britain Non-native Species Secretariat, York. http://www.gov.uk/government/uploads/system/uploads/attachment_data/file/455526/gb-non-native-species-strategy-pb14324.pdf. Accessed 15 August 2016

Gozlan, R., 2012. Pathogens: Monitoring fungal infections in fish. Nature 485. 446 446.

Gozlan, R., Andreou, D., Asaeda, T., Beyer, K., Bouhadad, R., Burnard, D., Caiola, N., Cakic, P., Djikanovic, V., Esmaeili, H.R., 2010. Pan-continental invasion of *Pseudorasbora parva*: towards a better understanding of freshwater fish invasions. Fish Fish 11, 315–340.

Gozlan, R.E., St-Hilaire, S., Feist, S.W., Martin, P., Kent, M.L., 2005. Biodiversity: disease threat to European fish. Nature 435. 1046 1046.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser., 95–98

Hasegawa, M., Kishino, H., Yano, T.-A., 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22, 160–174.

Huver, J., Koprivnikar, J., Johnson, P., Whyard, S., 2015. Development and application of an eDNA method to detect and quantify a pathogenic parasite in aquatic ecosystems. Ecol. Appl. 25, 991–1002.

Kirshtein, J.D., Anderson, C.W., Wood, J.S., Longcore, J.E., Voytek, M.A., 2007. Quantitative PCR detection of *Batrachochytrium dendrobatidis* DNA from sediments and water. Dis. Aquat. Organ. 77, 11–15.

Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452.

Paley, R., Andreou, D., Bateman, K., Feist, S., 2012. Isolation and culture of *Sphaerothecum destruens* from Sunbleak (*Leucaspius delineatus*) in the UK and pathogenicity experiments in Atlantic salmon (*Salmo salar*). Parasitology 139, 904.

Peeler, E.J., Oidtmann, B.C., Midtlyng, P.J., Miossec, L., Gozlan, R.E., 2011. Non-native aquatic animals introductions have driven disease emergence in Europe. Biol. Invasions 13, 1291–1303.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539–542.

Sana, S., Hardouin, E.A., Gozlan, R.E., Ercan, D., Tarkan, A.S., Zhang, T., Andreou, D., 2017. Origin and invasion of the emerging infectious pathogen *Sphaerothecum destruens*. Emerg. Microbes Infect. 6, e76.

Simon, A., Britton, R., Gozlan, R., Van Oosterhout, C., Volckaert, F.A., Hänfling, B., 2011. Invasive cyprinid fish in Europe originate from the single introduction of an admixed source population followed by a complex pattern of spread. PLoS One 6, e18560.

Williams, C.F., Britton, J.R., Turnbull, J.F., 2013. A risk assessment for managing non-native parasites. Biol. Invasions 15, 1273–1286.