NOTE

Detection of *Paramoeba perurans* in Scottish marine wild fish populations

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Abstract
Presence of *Paramoeba perurans,* causative agent of amoebic gill disease was determined by qPCR in wild fish from Scottish coastal waters (n = 2,348). Overall, the apparent prevalence was low. A single fish, a horse mackerel *Trachurus trachurus*, tested positive. This is the first report of detection of *P. perurans* in horse mackerel.

Paramoeba perurans is an amoeba parasite and the causative agent of amoebic gill disease (AGD) (Young et al., 2007, Crosbie et al., 2012). The disease was first reported in Scotland in 2006 with additional outbreaks occurring since 2011 and in recent years AGD has caused significant problems in the Scottish aquaculture industry (Marine Scotland Science unpublished data). It has also caused significant economic losses (Shinn et al., 2014) especially in the Australian salmon farming industry through mortalities or treatment costs since its first occurrence in 1984 (Munday, 1986). The disease has also been reported in the USA (Kent et al., 1988), Ireland (Rodger and McArdle, 1996), the Mediterranean (Dykova et al., 1998), New Zealand (Young et al., 2008b), Norway (Steinum et al., 2008), Japan (Crosbie et al., 2010), Chile (Bustos et al., 2011) and in South Africa (Mouton et al., 2014). Susceptible species to AGD include: Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss* (Munday et al., 1990); coho salmon *O. kisutch* (Kent et al., 1988); turbot *Scophthalmus maximus* (Dykova et al., 1998); sea bass *Dicentrarchus labrax* (Dykova et al., 2000); chinook salmon *O. tshawytscha* (Young et al., 2008b); ayu *Plecoglossus altivelis* (Crosbie et al., 2010); ballan *wrasse Labrus bergylta* (Karlsbakk et al., 2013); blue warehou *Seriolella brama* (Adams et al., 2008); and sea bream *Diplodus puntazzo* (Dykova and Novoa, 2001).

The potential for wild fish to act as carriers of *P. perurans* is an important factor to consider in the epidemiology of the disease and the transfer of the agent between geographical areas and to farmed fish. At present, *P. perurans* has only been detected in fish from aquaculture environments. There is one report of early stage AGD infection with *Paramoeba* sp. in wild fish

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(Adams et al., 2008) where a blue warehou was captured in the vicinity of a commercial Atlantic salmon farm in Tasmania and tested using histological and immunohistochemical techniques however, the amoeba species was not determined due to lack of material so the presence of *P. perurans* here is not confirmed. A previous survey in Tasmania found that wild fish are not a significant reservoir of *P. perurans* (Douglas-Helders et al., 2002), finding no indication of the presence of amoebae nor AGD in 325 wild fish using histological examination of gill material. The fish were caught in the vicinity of Atlantic salmon aquaculture facilities where AGD was present. This study was conducted when *Neoparamoeba pemaquidensis* was thought to be the causative agent of AGD, so although the presence of *P. perurans* was not determined, no paramoeba, or AGD lesions were observed in any of the gills sampled.

This study aimed to ascertain if *P. perurans* was present in the wild marine fish populations of Scotland and if so, to determine the background prevalence and potential for wild fish to act as reservoir species. Determining this background level would provide a useful baseline for detecting any future prevalence increase, therefore, areas in the vicinity of aquaculture facilities, where an elevated prevalence of *P. perurans* may be expected during an AGD outbreak in farmed Atlantic salmon, were not specifically targeted in this survey.

In this study, fish were captured by demersal trawling (depth range 46 – 192 m) from locations around the Scottish coastal zone (Figure 1) during the second week of February and the first week of March 2013. Demersal trawling methods have previously been reported as a successful approach to catch a variety of species of wild marine fish for disease surveys (King et al., 2001). Fifty fish were sampled within each haul based on the approximate proportion of each species present in the haul, ensuring results were representative of the population of catchable fish. This sampling strategy was selected to sample a wide range of species as the host range of *P. perurans* for wild marine fish species is unknown. Total number of each species sampled is shown in Table 1. A section of lamellae from the first gill arch, on the left side for round fish and the dorsal side for flatfish, was aseptically sampled into 100% ethanol (Sigma) and stored at 4 °C prior to processing.

Fish tissue was homogenised in ATL buffer (Qiagen) with 7 mm stainless steel beads using the Qiagen TissueLyser system (Qiagen) at a frequency of 25 Hz for a minimum of 4 min. The DNA was extracted from 5 mg homogenate using the Qiasymphony SP platform with the QIAsymphony DNA DSP kit (Qiagen) using the Tissue LC 200 DSP protocol with an elution volume of 200 µl.

Real-time polymerase chain reaction (qPCR) was performed in duplicate on a RocheLightcyler 480 I platform using Quanta Custom Toughmix (Quanta BioScience) with primers and probes as described by Fringuellli et al. (2012) and detailed in Table 2. A single universal fish endogenous control reaction, targeting the 18S gene, was also ran for each sample to ensure DNA quality and quantity. Samples were considered positive if both replicates had a Cp value and true amplification was observed. Samples positive by qPCR, and samples generating ambiguous results, where only one replicate had a late Cp value, were subjected to nested PCR

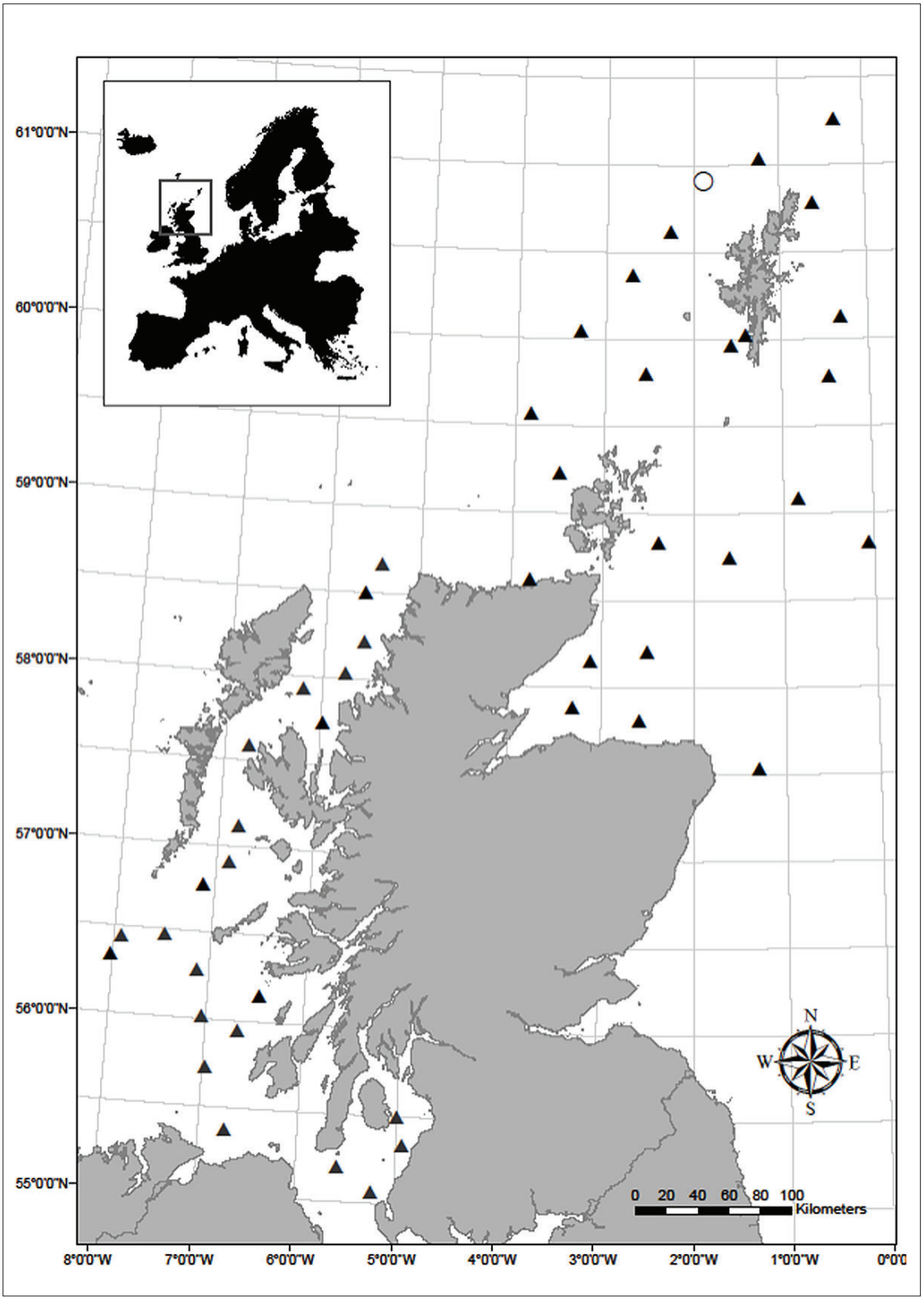

Figure 1. Specific trawl locations in Scottish coastal waters. Black triangle (▲) indicates sampling location were no fish were positive for *Paramoeba perurans*. White circle (○) indicates sampling location where one fish was positive for *P. perurans*.

Table 1. Total number of fish sampled and total number of positive results, ordered by species.

Fish Species		Number Sampled	Number Positive
Common Name	**Scientific Name**		
Angler (monkfish)	*Lophius piscatorius*	3	0
Blue whiting	*Micromesistius poutassou*	163	0
Common dab	*Limanda limanda*	95	0
Cod	*Gadus morhua*	45	0
Greater forkbeard	*Phycis blennoides*	5	0
Grey gurnard	*Eutrigla gurnardus*	45	0
Goldsinny wrasse	*Ctenolabrus rupestris*	1	0
Haddock	*Melanogrammus aeglefinus*	298	0
Hake	*Merluccius merluccius*	57	0
Herring	*Clupea harengus*	259	0
Horse mackerel (scad)	*Trachurus trachurus*	2	1
Lesser argentine	*Argentina sphyraena*	16	0
Ling	*Molva molva*	2	0
Long rough dab	*Hippoglossoides platessoides*	28	0
Lemon sole	*Microstomus kitt*	12	0
Lumpsucker	*Cyclopterus lumpus*	1	0
Common dragonet	*Callionymus lyra*	7	0
Mackerel	*Scomber scombrus*	12	0
Megrim	*Lepidorhombus whiffiagonis*	4	0
Norway pout	*Trisopterus esmarkii*	597	0
Poor cod	*Trisopterus minutus*	147	0
Plaice	*Pleuronectes platessa*	62	0
Saithe	*Pollachius virens*	54	0
Red gurnard	*Aspitrigla cuculus*	22	0
Silvery pout	*Gadiculus argenteus*	1	0
Sprat	*Sprattus sprattus*	134	0
Sea trout	*Salmo trutta*	1	0
Whiting	*Merlangius merlangus*	275	0

Table 2. Primer and probe sequences used in this study. The *Paramoeba perurans* qPCR assay has been published by Fringuelli et al. (2012), the endogenous control assay is an in house assay targeting the 18S gene designed to be suitable for use for all fish species. The first round nested PCR was developed using forward primer ERB1 (Barta et al., 1997) and reverse primer Np1045r (Steinum et al., 2008), the second round of the nested PCR used primers as described by Young et al. (2008a).

Assay	Primer/probe name	Primer/probe sequence
Paramoeba perurans QPCR	Peru For	5′-GTTCTTTCGGGAGCTGGGAG-3′
	Peru Rev	5′-GAACTATCGCCGGCACAAAAG-3′
	Peru probe	6FAM CAATGCCATTCTTTTCGGA-MGB
Endogenous control QPCR	UNIFISHFOR	5′-CCTGCGGCTTAATTTGACTCA-3′
	UNIFISHREV	5′-AAAGAGCTATCAATCTGTCAATCCTTT-3′
	UNIFISHPROBE	6FAM-CTCACCCGGCCCGGACACG-MGB
Nested PCR first round	ERB1	5′-ACCTGGTTGATCCTGCCAG-3′
	Np1045r	5′-CTGTCCCTTTTAATCATTACACTTC-3′
Nested PCR second round	YOUNG F	5′-ATCTTGACYGGTTCTTTCGRGA-3′
	YOUNG R	5′-ATAGGTCTGCTTATCACTYATTCT-3′

to obtain material for sequencing, or to confirm negative results by an alternative method, respectively. In analytical sensitivity testing of the assays, the nested PCR assay was found to have similar sensitivity to that of the qPCR assay (data not shown).

Nested PCR was carried out in triplicate for qPCR positive samples, and in duplicate for ambiguous result samples, in a 25 µl reaction as previously described (Snow et al., 2004) generating a 636 bp product using primer sets as described in Table 2. The first round nested PCR was developed using forward primer ERB1 (Barta et al., 1997) and reverse primer Np1045r (Steinum et al., 2008) with an annealing temperature of 47 °C. The second round of the nested PCR used primers and conditions as described by Young et al. (2008a). Second round PCR products were visualised by gel electrophoresis and purified using QIAquick PCR purification kit (Qiagen).

Negative controls consisting of molecular grade water were included in extraction, qPCR and nested PCR. Positive controls consisting of diluted DNA extracted from in house *P. perurans* culture (species confirmed by qPCR, Fringuelli et al., 2012) were ran in both qPCR and PCR.

The DNA sequencing was performed by DNA Sequencing & Services (MRCPPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using the second round nested PCR primers. The sequences were analysed using SequencherTM 3.0 software (Gene Codes Corporation, Ann Arbor, MI, USA) and BLASTn searches in GenBank.

The proportion of sampled fish testing positive for *P. perurans* and the apparent prevalence, which is the proportion of positive fish corrected for the total number of fish captured during each haul, were estimated. Confidence intervals (CI) were estimated by logistic regres-

sion as Wald-type intervals on the log-odds scale using Taylor linearised standard errors and then transformed to the probability scale. Calculations were carried out within the R statistical environment 3.1.2 (R Core Team, 2014) utilising the supplementary R package survey 3.30-3 (Lumley, 2004).

A total of 2,348 fish of various marine species (detailed in Table 1) were tested for *P. perurans* by qPCR. A single sample, from the horse mackerel *Trachurus trachurus*, tested positive for *P. perurans.* Fifty six samples gave ambiguous qPCR results where only one replicate tested positive, however all of these were negative for *P. perurans* by nested PCR and are therefore reported as negative. All other samples were negative for *P. perurans* by qPCR. These results are summarised in Table 1. All negative controls had acceptable results. All endogenous controls had Cp values of less than 20.

A partial sequence (495 nts, GenBank accession number KT989880) of the 18S gene of *P. perurans* was obtained from the positive horse mackerel sample. BLAST results confirmed *P. perurans* and the sequence was most similar to *P. perurans* isolated from the gills of ballan wrasse in Norway. Specifically, the isolate was 100% identical to KF179520.1 (unpublished) and had 99.8% identity (one nucleotide difference) to KF146711.1, KF146712.1, KF146713.1 (Karlsbakk et al., 2013). The horse mackerel isolate also showed 99.8% identity (one nucleotide difference) to *P. perurans* (GU574794.1) isolated from a salmon louse, *Lepeophtheirus salmonis,* on Atlantic salmon in USA (Nowak et al., 2010). The single nucleotide difference in all these cases was a guanine at position 449 of the horse mackerel partial sequence, a nucleotide ambiguity (R) representing guanine or adenine, which therefore may not be true, in the Karlsbakk sequences and an adenine in the sequence from the salmon louse. The horse mackerel isolate also showed 99.8% identity, (one nucleotide difference) to *P. perurans* (EU326494.1) isolated from Atlantic salmon gill in Norway (Nylund et al., 2008), where the single nucleotide difference was at a different position – 438 of the horse mackerel isolate. When compared with Scottish generic *P. perurans* culture (KT989881), 99.6% similarity (2 nucleotide differences) was found, one nucleotide difference at the 449 position as previously discussed and a second nucleotide difference at position 440 of the horse mackerel isolate. At both positions there were ambiguity in the culture isolate so again, this may not be a true difference. As both extraction and qPCR negative controls showed no amplification, indicating that there was no contamination, and the positive result was confirmed by sequencing it can be assumed that this is a true positive result.

The proportion of *P. perurans* positive sampled fish was 0.043% with a 95% CI of 0.006–0.293%. The estimate of apparent prevalence is 0.050% (0.007-0.364%); this does not take into account the diagnostic sensitivity and specificity of the assay or gill sampling technique but does represent a baseline estimate of potential value for future investigations.

A previous study (Douglas-Helders et al., 2002) reported that wild fish are not a significant reservoir for *P. perurans* after sampling 325 wild fish from and around salmon farms in Tasmania. If a similar apparent prevalence is found in Tasmanian waters as was found in Scottish waters then *P. perurans* was unlikely to have been found in a sample size of 325.

Sampling of the second gill arch is standard protocol for AGD screening, in part due to it being traditionally processed for histology, avoiding the first gill arch which is thought more exposed to non-specific environmental insult. As the current samples were screened using molecular techniques and tissue integrity is not critical, the first gill arch was chosen here assuming exposure to external environment might increase the likelihood of *P. perurans* detection. However, such an assumption is not valid as intake and expulsion of water over the gills may result in other gill arches and regions within those gills being more disposed to capturing and retaining amoebae. Adams and Nowak (2001) analysed lesions on the second gill arch of salmon with AGD. They found significantly more lesions in the dorsal section compared to median and ventral sections and suggested that this might be due to water flow being retarded in this region due to the cranial lobes, and therefore amoebae having a better chance of attaching and remaining. It may have been useful to adopt a similar sampling approach in the current study. However, given the wide range of fish species sampled in this study, the same gill section may not have been optimal for all. Since this study, the potential that gill swabs are more sensitive than tissue sampling for detection of *P. perurans* has arisen (pers. comm. Jamie Downes) i.e. allowing for a larger surface area of the gills to be sampled and the presence of less competing DNA.

Median water temperature and salinity (with 95 percentiles) were 7.5 (6.1 – 8.9) °C and 34.6 (33.5 – 35.3) ppt respectively. AGD outbreaks at aquaculture facilities have been observed at temperatures between 6.4 – 13.1 °C in Scotland (Marine Scotland Science unpublished data) and as high as 20 °C in other countries (Munday et al., 1990). It has been reported that salinity is a more relevant contributing environmental factor with AGD outbreaks occurring at salinities ≥32 ppt (Munday et al., 2001). A seasonal effect in AGD outbreaks has been observed in Scotland with outbreaks typically occurring from summer to winter, peaking in the Autumn (Marine Scotland Science unpublished data), if sampling during this survey had occurred during autumn, a higher prevalence of *P. perurans* might be expected.

There were two horse mackerel sampled in this survey - it is curious that the one positive result obtained came from a species of which so few were sampled and further species specific testing is required to ascertain if horse mackerel are a significant reservoir of *P. perurans.* Both horse mackerel were sampled from the same location in the North West as shown in Figure 1. The origin of the horse mackerel stock is unknown, however, it would most likely be of the North Sea or Western stock (pers. comm. Cindy van Damme). The sampling location was located in the vicinity of the Shetland Islands (approximately 50 km) where there is an established aquaculture industry and AGD has previously been observed. This may contribute to the increased prevalence at this location, however *P. perurans* was not detected in other sampling locations in the vicinity of Shetland. As *P. perurans* was detected in only a single fish, it is not possible to infer if the species or location are the most important causative explanation.

As only qPCR was used to detect *P. perurans* there is no indication of pathogenic effects of infection. Further studies could sample for histological processing in addition to qPCR to allow

assessment of active infection through pathology, which would also address if the wild fish were either susceptible to *P. perurans* infection causing AGD; or in a carrier state acting a reservoir for the pathogen. The potential for wild fish to act as a reservoir for *P. perurans* in relation to the Scottish aquaculture industry would require further sampling and analysis. Further sampling could include: targeting inshore areas in the vicinity to fish farms as well as offshore locations; and sampling during different times of the year to determine if any trends found reflect or proceed that observed in infections from aquaculture facilities. Analysis to infer if transmission of *P. perurans* from wild to farmed fish occurs would involve an approach similar to that used by Garver et al. (2013).

In conclusion *P. perurans* was detected in the wild fish population around Scottish coastal waters and the apparent prevalence in the population, as a whole, was substantially less than 1%. This is the first report of detection of *P. perurans* in horse mackerel.

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