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Neoparamoeba perurans is a cosmopolitan aetiological agent of amoebic gill disease

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ABSTRACT: Previously we described a new member of the *Neoparamoeba* genus, *N. perurans*, and showed that it is an agent of amoebic gill disease (AGD) of Atlantic salmon *Salmo salar* cultured in southeast Tasmania, Australia. Given the broad distribution of cases of AGD, we were interested in extending our studies to epizootics in farmed fish from other sites around the world. Oligonucleotide probes that hybridise with the 18S rRNA of *N. perurans*, *N. branchiphila* or *N. pemaquidensis* were used to examine archival samples of AGD in Tasmania as well as samples obtained from 4 host fish species cultured across 6 countries. In archival samples, *N. perurans* was the only detectable amoeba, confirming that it has been the predominant aetiological agent of AGD in Tasmania since epizootics were first reported. *N. perurans* was also the exclusive agent of AGD in 4 host species across 6 countries. Together, these data show that *N. perurans* is a cosmopolitan agent of AGD and, therefore, of significance to the global mariculture industry.

KEY WORDS: Amoebic gill disease · *Neoparamoeba perurans* · *In situ* hybridisation · Aquaculture

INTRODUCTION

Amoebic gill disease (AGD) is an ectoparasitic condition of some farm-reared marine fish (Munday 1986, Kent et al. 1988, Dyková et al. 1995). The first AGD epizootic was reported by Munday (1986) and affected both rainbow trout *Oncorhynchus mykiss* and Atlantic salmon *Salmo salar* cultured in southeast Tasmania, Australia. At that time the pathogen was not identified, but later, by means of morphological evidence, the aetiological agent was described as *Paramoeba* sp. (Roubal et al. 1989). In the interim, Kent et al. (1988) described *P. pemaquidensis* (now *Neoparamoeba pemaquidensis*) as the agent of AGD of coho salmon *O. kisutch* farmed in the state of Washington, USA.

Many authors assumed that *Neoparamoeba pemaquidensis* was the sole aetiological agent of AGD of Atlantic salmon (Douglas-Helders et al. 2000, 2001a,b, 2002, 2003a,b,c, 2005, Adams & Nowak 2001, Munday et al. 2001, Tan et al. 2002, Clark et al. 2003, Powell & Clark 2003, 2004, Bowman & Nowak 2004, Wong et al. 2004, Zilberg 2005). However, the isolation of *N. branchiphila* from the gills of AGD-affected fish (Dyková et al. 2005) suggested that AGD may be a condition of mixed aetiology. While *N. pemaquidensis* and *N. branchiphila* were the predominant species of amoebae cultured from the gills of AGD-affected fish, neither have been shown to induce AGD in fish that were experimentally inoculated with clonal cultured strains (Kent et al. 1988, Howard & Carson 1993a, Morrison et al. 2005, Vincent et al. 2007). Recently, Young et al. (2007) discovered *N. perurans* and showed it to be an agent of AGD in Atlantic salmon cultured in southeast Tasmania. In this instance, neither *N. pemaquidensis* nor *N. branchiphila* were detected in sections of gill tissue affected by AGD. In

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cases of AGD reported elsewhere, it is not known what role *N. perurans*, *N. pemaquidensis* and/or *N. branchiphila* play. Therefore, our objective was to use species-specific molecular probes to determine the aetiological agent or agents of AGD in 4 host species in 6 countries.

MATERIALS AND METHODS

Paraffin-embedded gill tissues were obtained from 4 fish species predominantly during or following epizootics at commercial fish farming operations in 6 countries (Table 1). An epizootic was not reported from Chinook salmon *Oncorhynchus tshawytscha* cultured in New Zealand; however, the smallest fish (runts) within the healthy population were observed to have gill lesions that corresponded with AGD and these fish were used in this study. Gill tissues were sectioned (3 to 7 µm), stained with haematoxylin and eosin (H&E) and examined with light microscopy. Alternatively, sections (7 µm) of gill tissues were placed onto Polysine glass slides (Menzel-Gläser) and dried overnight at 37°C. Sections were hybridised with a digoxigenin (DIG)-labelled 'universal' 18S rRNA oligonucleotide probe to verify the integrity of rRNA as previously described (Young et al. 2007). All gill tissues with suitable host and amoeba rRNA were serially sectioned, placed onto Polysine glass slides and incubated with *Neoparamoeba perurans*, *N. branchiphila* and *N. pemaquidensis* DIG-labelled oligonucleotide probes as previously described (Young et al. 2007). Positive and negative (no probe) controls were run in parallel with each *in situ* hybridisation experiment by hybridising each probe with a section containing representative strains of each *Neoparamoeba* species termed an 'amoebae array' as previously described (Young et al. 2007). Tissue sections were incubated for up to 1 h with in a premixed solution of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT) (Sigma-Aldrich) for colour development. The hybridisation procedure for the gill tissue sections from AGD-affected turbot *Scophthalmus maximus* (= *Psetta maxima*) was modified as gill tissue was provided pre-sectioned (5 µm) on non-coated slides. Preliminary hybridisation experiments with these sections resulted in tissues detaching from the slides. Therefore, the prehybridisation procedure was limited to dewaxing, rehydration and sequential washes of sections for 2 × 5 min with diethyl pyrocarbonate-treated phosphate buffered saline (PBS) at room temperature. Sections were then directly probed with *N. perurans*, *N. branchiphila* and *N. pemaquidensis* oligonucleotides as previously described (Young et al. 2007). Positive and no-probe controls were included with each *in situ* hybridisation experiment as described previously. An extended incubation (18 h) with premixed BCIP/NBT solution was required for colour development.

RESULTS AND DISCUSSION

When Dyková et al. (2000) described several strains of *Neoparamoeba* from AGD- affected European bass *Dicentrarchus labrax* and turbot, the authors suggested that the agents of AGD should only be assigned to *Neoparamoeba*, since members of the genus, *N. pemaquidensis* and *N. aestuarina*, were morphologically identical. Since then, another morphologically indistinct species, *N. branchiphila*, from the same genus was cultured from AGD-affected fish (Dyková et al. 2005) and, therefore, up

Table 1. Gill tissue samples obtained from farm-reared populations of fish with presumptive cases of amoebic gill disease. Year: year gill tissues were originally sampled. n: number of fish sampled at each time point. Fixative used to prepare gill tissues for histology: seawater Davidson's fixative (SWD), 10% formalin prepared in seawater (SWF) or 10% neutral buffered formalin (NBF)

Species	Location	Year	n	Fixative
Atlantic salmon	Bruny Island, southeast Tasmania, Australia[a]	1987	1	SWD
	Brabazon Point, southeast Tasmania, Australia[a]	1987	1	SWD
	Tamar River, north Tasmania, Australia[a]	2006	3	NBF
		2007	1	SWD
	Galway, Ireland	2004	3	NBF
	Puget Sound, Washington, USA	2006	3	NBF
	North Uist, Western Isles, Scotland, UK	2006	4	SWF
Rainbow trout	Recherche Bay, southeast Tasmania, Australia[a]	1988	1	SWD
Chinook salmon	Picton, Queen Charlotte Sound, New Zealand	2006	3	NBF
Turbot	Northwest Spain	2001	1	SWD

[a]The geographical location of samples obtained from Australia is specified to denote the division between north and south Tasmania

to 3 *Neoparamoeba* species were potential aetiological agents of AGD. Recently, we described a new species, *N. perurans*, and showed that it is an agent of AGD in Atlantic salmon from southeast Tasmania (Young et al. 2007). This resolved AGD aetiology in recent cases of AGD in Atlantic salmon cultured in southeast Tasmania, but not in historical cases of AGD from southeast Tasmania, nor in cases described elsewhere.

In this study, cases of AGD were verified by histological examination (Dyková & Novoa 2001). All histological sections revealed structural changes consistent with the pathology described in cases of AGD (Kent et al. 1988, Roubal et al. 1989, Munday et al. 1990, Dyková et al. 1995, Rodger & McArdle 1996). The most significant change was the development of gill lesions due to the hyperplasia of epithelial-like cells (Figs. 1, 2 & 3). This resulted in extensive secondary lamellar fusion and the formation of interlamellar vesicles (Figs. 2 & 3). Amoebae were located between the secondary lamellae or at the distal ends of fused lamellae (Figs. 1, 2 & 3). The presence of a perinuclear eosinophilic body was representative of the endosymbiotic *Perkinsiella amoebae*-like organism (PLO) (Dyková et al. 2003) (Fig. 1) and was identified in amoebae in all sections examined.

Retrospective studies applying *in situ* hybridisation probes to tissue samples have been used to determine

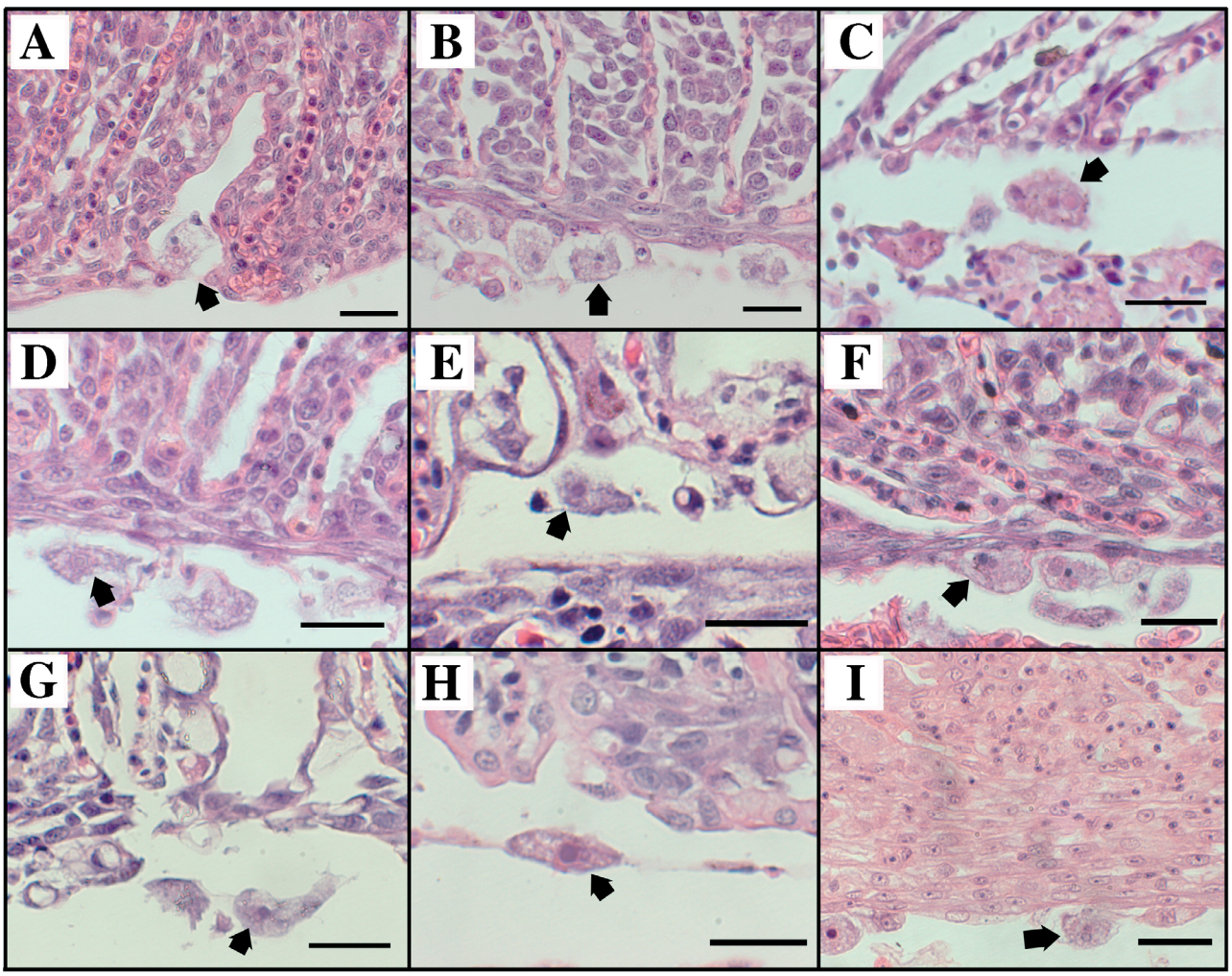

Fig. 1. Confirmation of the presence of amoebae (arrows) containing endosymbiotic *Perkinsiella amoebae*-like organisms in H&E stained, 3 to 7 µm thick sections from fish examined in this study. Images are representative of the pathology observed in specimens from each species and location. (A) Atlantic salmon, Tamar River, north Tasmania, Australia (n = 4). (B) Atlantic salmon, Bruny Island, southeast Tasmania, Australia, 1987 (n = 1). (C) Atlantic salmon, Brabazon Point, southeast Tasmania, Australia, 1987 (n = 1). (D) Rainbow trout, Recherche Bay, southeast Tasmania, Australia, 1988 (n = 1). (E) Atlantic salmon, Galway, Ireland (n = 3). (F) Atlantic salmon, Western Isles, Scotland, UK (n = 4). (G) Atlantic salmon, Washington, USA (n = 3). (H) Rainbow trout, Picton, New Zealand (n = 3). (I) Turbot, northwest Spain (n = 1). Scale bars = 25 µm

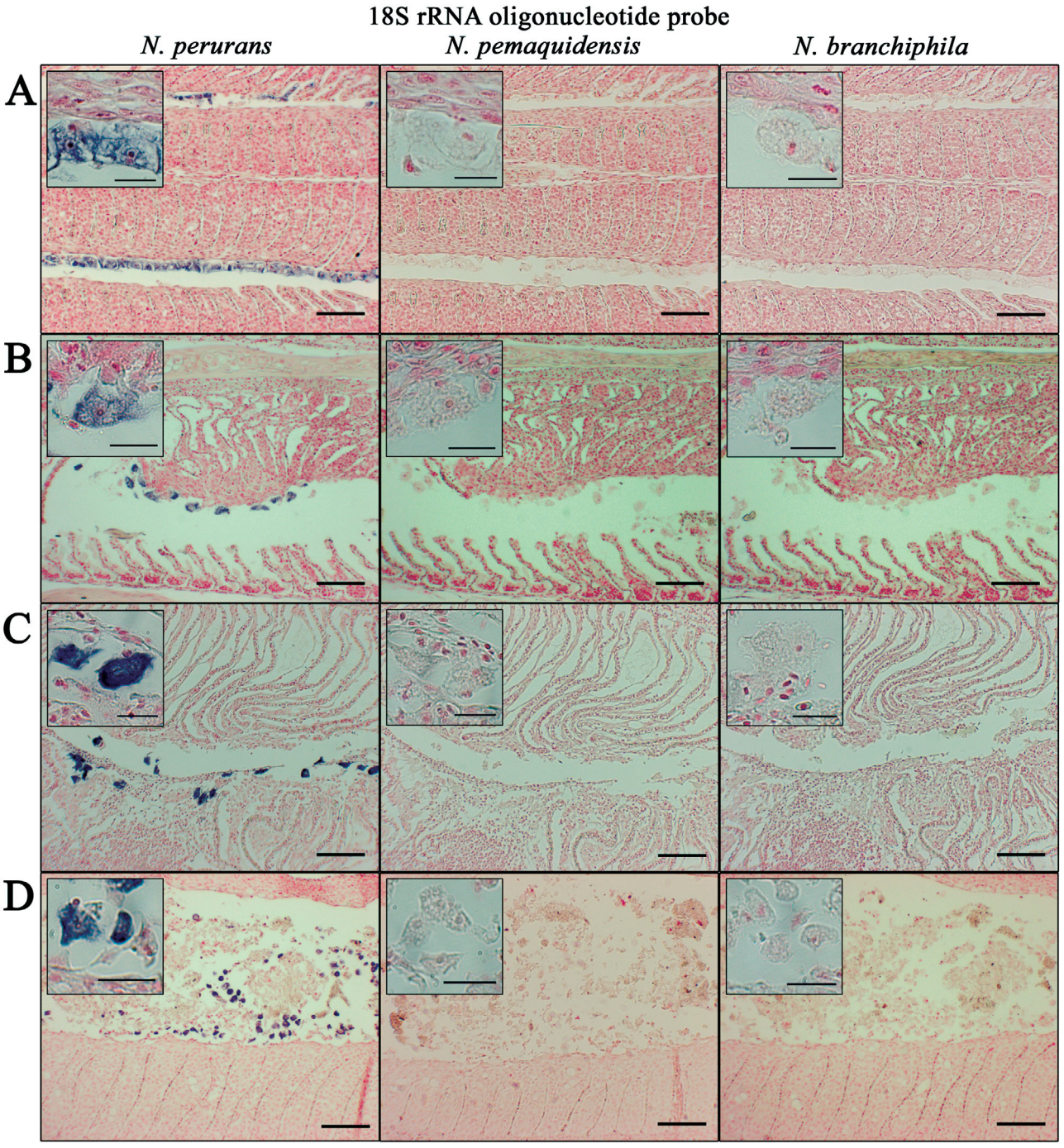

Fig. 2. Results of *in situ* hybridisation experiments based on species-specific probes that hybridise to the 18S rRNA of either *Neoparamoeba perurans*, *N. pemaquidensis* or *N. branchiphila* in gill samples from fish cultured in Australia. Images are representative of the pathology observed in specimens from each species and location. (A) Atlantic salmon, Bruny Island, southeast Tasmania, 1987 (n = 1). (B) Atlantic salmon, Brabazon Point, southeast Tasmania, 1987 (n = 1). (C) Rainbow trout, Recherche Bay, southeast Tasmania, 1988 (n = 1). (D) Atlantic salmon, Tamar River, north Tasmania, 2006 (n = 4). Probe positive and probe negative amoebae are magnified within the insets. Scale bars = 100 µm (main image) or 20 µm (inset)

the aetiological agents of disease and the geographical and temporal distribution of marine diseases (Hasson et al. 1999, Friedman et al. 2005, Yee et al. 2005). In this study, archival tissues from cultured Atlantic salmon and rainbow trout in southeast Tasmania, Australia, were probed for *Neoparamoeba perurans*, *N. bran-*

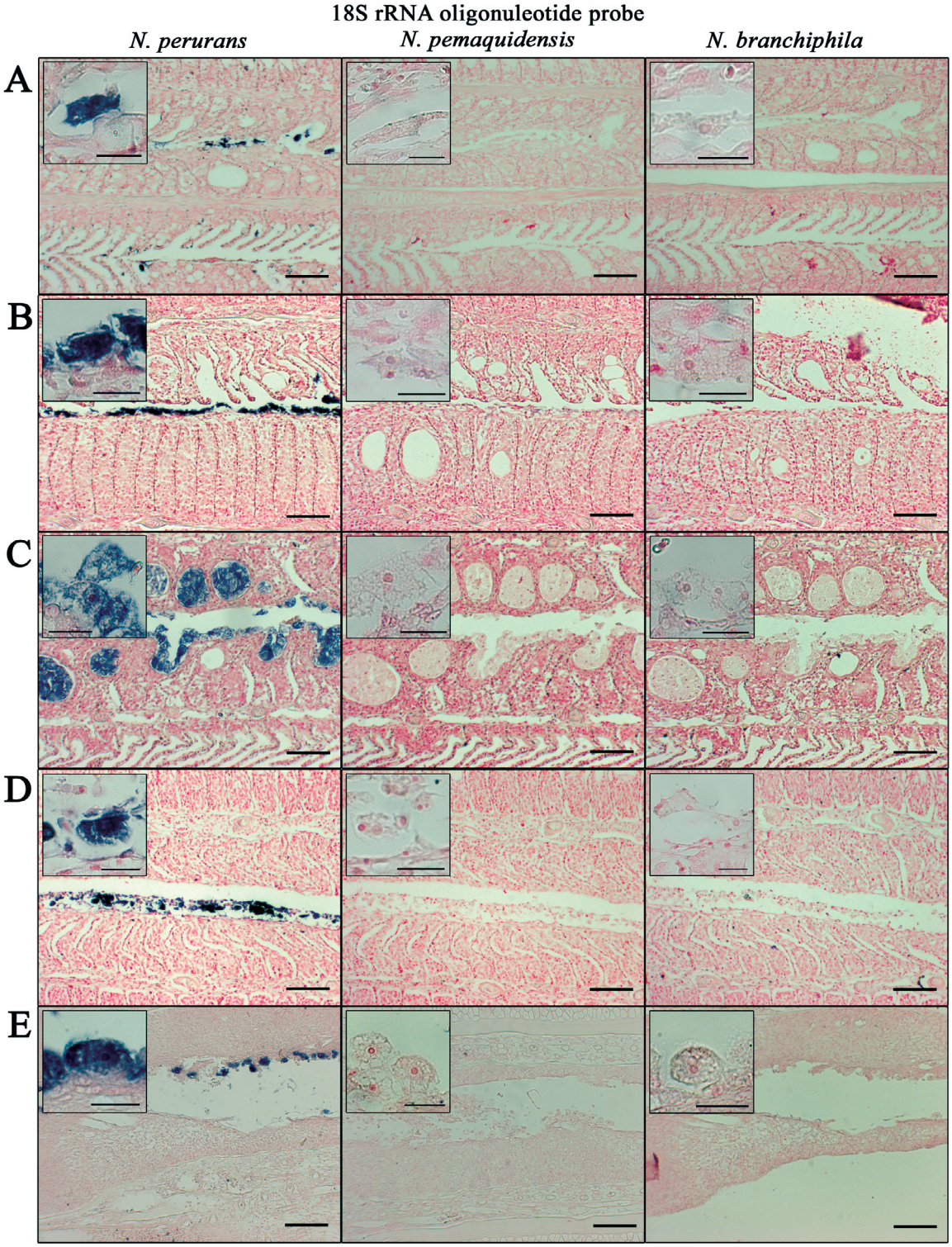

Fig. 3. Results of *in situ* hybridisation experiments based on species-specific probes that hybridise to the 18S rRNA of either *Neoparamoeba perurans*, *N. pemaquidensis* or *N. branchiphila* in gill samples from fish sampled worldwide. Images are representative of the pathology observed in specimens from each species and location. (A) Chinook salmon, Picton, New Zealand (n = 3). (B) Atlantic salmon, Galway, Ireland (n = 3). (C) Atlantic salmon, Western Isles, Scotland, UK (n = 4). (D) Atlantic salmon, Washington, USA (n = 3). (E) Turbot, northwest Spain (n = 1). Images are representative of *in situ* hybridisation experiments using 1 to 4 amoebic gill disease (AGD)-affected fish from each location (see Table 1). Probe positive and probe negative amoebae are magnified within the insets. Scale bars = 100 µm (main image) or 20 µm (inset)

chiphila and *N. pemaquidensis*. These samples were obtained in 1987 and 1988, coinciding with the first confirmed records of AGD epizootics (Munday 1986, Roubal et al. 1989). In southeast Tasmania, recurrent AGD epizootics occur (Roubal et al. 1989, Munday et al. 1990, 1993) and fish are repeatedly treated sometimes more often than monthly during summer. Therefore, we sought to clarify whether there was temporal change in *Neoparamoeba* species associated with AGD in southeast Tasmania. The *N. perurans* specific probe hybridised with all trophozoites in all sections examined (Fig. 2A–C) consistent with samples obtained in recent cases of AGD in this region (Young et al. 2007). In serially sectioned gill tissues neither the *N. pemaquidensis* nor the *N. branchiphila* specific probes hybridised with any trophozoites (Fig. 2A–C). *Neoparamoeba* species-specific probes hybridised with the corresponding *Neoparamoeba* species on the amoebae array while no signal was detected in trophozoites on the amoebae array when the probes were omitted from the hybridisation procedure. This occurred in all hybridisation experiments. In northern Tasmania, there is a single Atlantic salmon farm located on the Tamar River. During the 2006–07 summer, an AGD epizootic occurred, and in all samples obtained from affected fish *N. perurans* was the only detectable amoeba (Fig. 2D). Together, these data suggest that *N. perurans* has been and remains the predominant aetiological agent of AGD throughout Tasmania.

There have been numerous published and unpublished reports of AGD in cultured fish (Nowak et al. 2002). Several hosts are susceptible and there is a worldwide distribution of cases (see review by Munday et al. 2001). Many salmonids are susceptible to AGD, and in this analysis, cases of AGD in Atlantic salmon, rainbow trout and Chinook salmon were verified and the sole agent confirmed as *N. perurans* (Figs. 2 & 3). The locations of these cases were Tasmania (Atlantic salmon and rainbow trout), Galway, Ireland (Atlantic salmon) and Washington (Atlantic salmon). In addition, the first formal case of AGD in Atlantic salmon from Scotland, UK, is presented together with a case of AGD in runt Chinook salmon from New Zealand, confirming the observations of AGD-like gill lesions in fish from this location (Munday et al. 1990, Howard & Carson 1993b). Other than salmonids, AGD also affects turbot (Dyková et al. 1995, 1998) and the agent in 1 case from northwest Spain is confirmed here as *N. perurans*. Some background signal was detected in sections of turbot gill tissue. This was due to the extended colour development (18 h) that resulted in a non-specific pale blue colouration of cartilaginous tissues. These tissues were not associated with primary or secondary gill lamellae or trophozoites (data not shown).

In summary, we used a molecular-based procedure to probe histological sections obtained from 4 host fish species cultured across 6 countries. In all 21 specimens, *N. perurans* was the only species of *Neoparamoeba* shown to elicit AGD, confirming that this species is a cosmopolitan protozoan parasite of the temperate marine fish examined. Confirmation of *N. perurans* in regions of significant finfish production indicates that AGD is of global significance to the mariculture industry.

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