Validating the identity of *Paramoeba invadens*, the causative agent of recurrent mass mortality of sea urchins in Nova Scotia, Canada

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ABSTRACT: Green sea urchins *Strongylocentrotus droebachiensis* along the coast of Nova Scotia, Canada, suffer mass mortalities from infection by the pathogenic amoeba *Paramoeba invadens* Jones, 1985. It has been speculated that *P. invadens* could be a form of *Neoparamoeba pemaquidensis*, a species associated with disease in *S. droebachiensis* and lobsters in the northeast USA. During a disease outbreak in fall 2011, we isolated amoebae from moribund urchins collected from 4 locations along ~200 km of coastline. In laboratory infection trials, we found that timing and rate of morbidity corresponded to that of similar experiments conducted in the early 1980s, when *P. invadens* was first identified. All isolates had a similar size and morphology to the original description, including an absence of microscapes. Sequences of nuclear SSU rDNA show that disease was caused by one 'species' of amoeba across the range sampled. Phylogenetic analyses prove that *P. invadens* is not conspecific with *N. pemaquidensis*, but is a distinct species most closely related to *N. branchiphila*, a suspected pathogen of sea urchins *Diadema* aff. *antillarum* in the Canary Islands, Spain. Morphology and closest phylogenetic affinities suggest that *P. invadens* would be assignable to the genus *Neoparamoeba*; however, nuclear SSU rDNA trees show that *Neoparamoeba* and *Paramoeba* are phylogenetically inseparable. Therefore, we treat *Neoparamoeba* as a junior synonym of *Paramoeba*, with *P. invadens* retaining that name, and *N. pemaquidensis* and *N. aestuarina* reverting to their original names (*P. pemaquidensis* and *P. aestuarina*), and with new combinations for *N. branchiphila* Dykova et al., 2005, and *N. perurans* Young et al., 2007, namely *P. branchiphila* comb. nov. and *P. perurans* comb. nov.

KEY WORDS: *Paramoeba invadens* · *Neoparamoeba* · Paramoebiasis · Sea urchin · Parasite · SSU rDNA · Disease

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INTRODUCTION

In marine ecosystems, the identity and source of the causative agent(s) of epizootics are often unresolved, due in part to difficulties in detecting potential pathogens in host tissues or the surrounding environment. For example, the pathogenic agent that decimated the sea urchin *Diadema antillarum* throughout its geographic range in the Caribbean in 1983, causing a phase shift from corals to fleshy

macroalgae on many reefs, remains unidentified (Lessios 1988). Similarly, causal agents have only been identified for 6 of 19 common coral diseases, which also have had important ecosystem-level effects (Harvell et al. 2007). Even when agents are identified, difficulties with reliably identifying pathogens to the 'species' level can result in incorrect assumptions about the host-specificity or generality of the agent (Young et al. 2008, Crosbie et al. 2012). In recent years, genetic tools have become a reliable

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means of precisely identifying pathogens associated with diseases of marine organisms (e.g. Senapin et al. 2007, Young et al. 2007, Dyková et al. 2011).

Along the Atlantic Coast of Nova Scotia, Canada, recurrent mass mortalities of the green sea urchin *Strongylocentrotus droebachiensis* drive transitions between the alternative community states in the rocky subtidal zone, viz. kelp beds and sea urchin barrens (Miller 1985, Scheibling 1986). These disease outbreaks are associated with tropical storm and hurricane activity and warm sea temperatures, and have increased in frequency over the past 3 decades (Scheibling & Hennigar 1997, Scheibling & Lauzon-Guay 2010). The pathogenic agent, an amoeba isolated from moribund urchins following a disease outbreak in 1983, was described as a new species, *Paramoeba invadens* (Jones 1985). The pathology of paramoebiasis in sea urchins has been characterized and Koch's postulates confirmed (Jones et al. 1985, Jones & Scheibling 1985). Paramoebiasis is highly host-specific and temperature-dependent (Scheibling & Stephenson 1984, Jellett et al. 1988, Jellett & Scheibling 1988a), and only affects sea urchins in shallow water (<25 m depth) in the summer or early fall when sea temperatures are above ~10°C, the thermal threshold for disease propagation (Scheibling 1984, 1988, Brady & Scheibling 2005, Feehan et al. 2012). However, the source population(s) of the pathogenic amoeba and mechanism of spread along the coast of Nova Scotia remain poorly understood.

Organisms assigned to the genera *Paramoeba* and *Neoparamoeba* are dactylopodid amoebozoan amoebae that carry a characteristic perinuclear body known as the parasome (Page 1987, Kudryavtsev et al. 2011). The parasome is actually a eukaryotic endosymbiont (specifically a kinetoplastid), leading to its alternative moniker of 'Perkinsella-like organism', or 'PLO'. The genera are nominally distinguished primarily by the presence in *Paramoeba* of surface microscscales (Page 1987), which are organic boat-shaped surface structures several hundred nanometers across that are readily observed by electron microscopy (Kudryavtsev et al. 2011). *Paramoeba* and *Neoparamoeba* are ubiquitous in temperate marine environments, and most species are free-living (Page 1970, Jones 1985, Dyková et al. 2005, Kudryavtsev et al. 2011). Nonetheless, they also act as facultative parasites in a variety of marine species aside from sea urchins, including Atlantic salmon *Salmo salar* in aquaculture, and decapod crustaceans along the eastern seaboard of the USA (Table 1). Analysis of small-subunit (SSU) ribosomal DNA (rDNA) is an effective

method used to unambiguously discriminate between closely related species of *Paramoeba* and *Neoparamoeba* (Table 1). No genetic information has been available on *P. invadens*, however, and in its absence it has been suggested that this species could be a form of *N. pemaquidensis* (basonym *P. pemaquidensis*), a morphologically similar amoeba associated with diseases of both lobsters *Homarus americanus* and sea urchins *Strongylocentrotus droebachiensis* in the northeastern USA (Mullen et al. 2005).

The issue of whether *Paramoeba invadens* exists as a distinct species has obvious implications for understanding the transmission of this disease within the environment, its geographic extent and the nature of sources for new outbreaks. The objectives of our study were (1) to confirm that the amoebae infecting sea urchins in Nova Scotia in fall 2011 were functionally and morphologically similar to the original *P. invadens* isolated from diseased sea urchins in the early 1980s, and (2) to test whether *P. invadens* is a distinct species using SSU rDNA data, and if so, determine its phylogenetic affinities and appropriate systematic designation. We find that *P. invadens* is a distinct *Neoparamoeba*-like species (i.e. without microscscales) most closely related to an organism originally described as *N. branchiphila*, but in the process confirm that *Paramoeba* and *Neoparamoeba* cannot be reconciled as distinct clades in nuclear SSU rDNA phylogenies. Rather than assign *P. invadens* to *Neoparamoeba*, we conclude that *Neoparamoeba* must be regarded as a synonym of *Paramoeba*, and that all *Neoparamoeba* species be treated (again) as species of *Paramoeba*.

MATERIALS AND METHODS

Collection of infected sea urchins

Following the passage of Hurricane Katia on 9 September 2011, SCUBA divers observed a mass mortality of sea urchins at 8 m depth in barrens at Splitnose Point, Nova Scotia, and in experimental cages in kelp beds within and immediately outside St. Margarets Bay, 40 km west-southwest of Splitnose Point (Fig. 1). Caged sea urchins had been transplanted to St. Margarets Bay from Splitnose Point on 6 August 2011 as part of an ongoing experiment investigating the association between hurricanes and disease outbreaks (Feehan et al. 2012). Moribund sea urchins, with overt signs of paramoebiasis, including loss of attachment to the substrate, disheveled spines, shriveled and non-functional tube feet and a gaping peri-

Table 1. *Paramoeba/Neoparamoeba* spp. identified from marine invertebrate and vertebrate hosts. 'Disease' refers to whether *Paramoeba/Neoparamoeba* spp. were associated with disease (morbidity or mortality) in the host (Yes) or not (No). 'Mode of ID' refers to the identification of the amoeba species from each host.

Amoeba sp./Host	Disease	Location	Mode of ID	Source
<i>Paramoeba invadens</i>				
Sea urchin <i>Strongylocentrotus droebachiensis</i> ^a	Yes	Nova Scotia, Canada	Morphology	Jones (1985), Jones & Scheibling (1985), Jones et al. (1985), Jelllett & Scheibling 1988b
Sea urchin <i>S. droebachiensis</i> ^a	Yes	Nova Scotia, Canada	SSU rDNA	This paper
<i>Paramoeba perniciosus</i>				
Blue crab <i>Callinectes sapidus</i>	Yes	Eastern seaboard USA	Morphology	Sprague et al. (1969), Johnson (1977), Messick (2002)
<i>Paramoeba/Neoparamoeba branchiphila</i>				
Atlantic salmon <i>Salmo salar</i> (aquaculture)	Yes ^b	Tasmania, Australia	SSU rDNA	Dyková et al. (2005)
Blue crab	No	Gulf of Mexico, USA	SSU rDNA	Dyková et al. (2007)
Sea urchin <i>Diadema</i> aff. <i>antillarum</i>	Yes	Canary Islands, Spain	SSU rDNA	Dyková et al. (2011)
Sea urchin <i>Heliocidaris erythrogramma</i>	No	Tasmania, Australia	SSU rDNA	Dyková et al. (2007)
Sea urchin <i>Paracentrotus lividus</i>	No	Cretan Sea, Greece	SSU rDNA	Dyková et al. (2007)
Southern bluefin tuna <i>Thunnus maccoyii</i> (carcass)	No	Port Lincoln, Australia	SSU rDNA	Dyková et al. (2007)
<i>Paramoeba/Neoparamoeba pemaquidensis</i>				
American lobster <i>Homarus americanus</i>	Yes	Long Island Sound, USA	SSU rDNA	Mullen et al. (2005)
Atlantic salmon (aquaculture)	Yes ^b	Tasmania, Australia	Morphology	Roubal et al. (1989)
Atlantic salmon (aquaculture)	Yes ^b	Tasmania, Australia	SSU rDNA	Wong et al. (2004), Dyková et al. (2007)
Atlantic salmon (aquaculture)	Yes ^b	Tasmania, Australia	Immuno-dot blot assay	Douglas-Helders et al. (2002)
Atlantic salmon (aquaculture)	Yes ^b	Ireland	SSU rDNA	Wong et al. (2004)
Coho salmon <i>Oncorhynchus kisutch</i> (aquaculture)	Yes	Washington State, USA	SSU rDNA	Caraguel et al. (2007)
Coho salmon (aquaculture)	Yes	Washington State and California, USA	Morphology	Kent et al. (1988)
Coho salmon (aquaculture)	Yes	Washington State and California, USA	SSU rDNA	Wong et al. (2004)
Sea urchin <i>S. droebachiensis</i>	Yes	Gulf of Maine, USA	SSU rDNA	Caraguel et al. (2007)
Southern bluefin tuna (carcass)	No	Port Lincoln, Australia	SSU rDNA	Dyková et al. (2007)
Turbot <i>Scophthalmus maximus</i> (aquaculture)	Yes	NW Spain	Morphology	Dyková et al. (1998)
Turbot (aquaculture)	No	NW Spain	SSU rDNA	Fiala & Dyková (2003)
<i>Paramoeba/Neoparamoeba perurans</i>				
Atlantic salmon (aquaculture)	Yes	Chiloe Island, Chile	SSU rDNA	Bustos et al. (2011)
Atlantic salmon (aquaculture)	Yes	Galway, Ireland	SSU rDNA	Young et al. (2008)
Atlantic salmon (aquaculture)	Yes	West coast of Norway	SSU cDNA	Steinum et al. (2008)
Atlantic salmon (aquaculture)	Yes	Western Isles, Scotland	SSU rDNA	Young et al. (2008)
Atlantic salmon (aquaculture) ^a	Yes	Tasmania, Australia	SSU rDNA	Young et al. (2007, 2008), Bridle et al. (2010), Crosbie et al. (2012)
Atlantic salmon (aquaculture)	Yes	Washington State, USA	SSU rDNA	Young et al. (2008), Nowak et al. (2010)
Ayu <i>Plecoglossus altivelis</i> (aquaculture)	Yes	Japan	SSU rDNA	Crosbie et al. (2010b)
Chinook salmon <i>Oncorhynchus tshawytscha</i>	Yes	Queen Charlotte Sound, New Zealand	SSU rDNA	Young et al. (2008)
Rainbow trout <i>O. mykiss</i> (aquaculture)	Yes	Tasmania, Australia	SSU rDNA	Young et al. (2008)
Turbot (aquaculture)	Yes	NW Spain	SSU rDNA	Young et al. (2008)
<i>Paramoeba/Neoparamoeba sp.</i>				
Atlantic salmon (aquaculture)	Yes	Galway and Cork, Ireland	Morphology	Rodger et al. (2011)
Blue warehou <i>Seriola lalandi</i>	Yes	Tasmania, Australia	Morphology	Adams et al. (2008)
Sea bass <i>Dicentrarchus labrax</i> (larvae, aquaculture)	Yes	Mediterranean Sea	Morphology	Dyková et al. (2000)
Turbot (aquaculture)	Yes	NW Spain	Morphology	Dyková et al. (1998, 2000), Dyková & Novoa (2001)
Rainbow trout (aquaculture)	Yes	Tasmania, Australia	Morphology	Munday et al. (1990)
Atlantic salmon (aquaculture)	Yes	Tasmania, Australia	Morphology	Munday et al. (1990), Clark & Nowak (1999), Zilberg et al. (2001)
Seabream <i>Diplodus puntazzo</i> (aquaculture)	Yes	NW Spain and Mediterranean Sea	Morphology	Dyková & Novoa (2001)
Sea bass (aquaculture)	Yes	NW Spain and Mediterranean Sea	Morphology	Dyková & Novoa (2001)
<i>Paramoeba sp. (probably P./N. pemaquidensis)</i>				
American lobster	Yes	Long Island Sound, USA	Morphology	Mullen et al. (2004)

^aKoch's postulates fulfilled; ^b*Paramoeba/Neoparamoeba* sp. likely misidentified as causative agent of disease (Young et al. 2008)

Fig. 1. Collection sites of *Strongylocentrotus droebachiensis* along the coast of Nova Scotia, Canada. The insets show 1 site near Shelburne, Nova Scotia (SPS: Sandy Point, Shelburne), 5 sea urchin caging sites within and immediately outside of St. Margarets Bay (MC: Mill Cove; TL: The Lodge; GI: Gravel Island; CC: Cranberry Cove; LI: Luke Island), 1 site at the mouth of St. Margarets Bay (OH: Owls Head) and 1 site along a headland near the mouth of Halifax Harbour (SP: Splitnose Point)

stome (Scheibling & Stephenson 1984), were collected by divers from cages in St. Margarets Bay and from barrens at Splitnose Point on 29 September and 9 November 2011, respectively.

To determine the extent of the disease outbreak, and to obtain additional isolates of amoebae, we acquired sea urchins from 2 other sites along the coast of Nova Scotia (Fig. 1). On 19 October 2011, we collected sea urchins in baited traps from a sedimentary basin at 60 m depth off Owl's Head, near the mouth of St. Margarets Bay. On 22 November 2011, we acquired sea urchins that had been collected by divers from barrens at Sandy Point, Shelburne County, ~200 km southwest of St. Margarets Bay (purchased from C. Hopkins, Barrington Passage, Nova Scotia). At both sites, bottom temperature at the time of collection was below the thermal threshold for paramoebiasis (~5°C at 60 m in St. Margarets Bay, K. Filbee-Dexter & R. E. Scheibling unpubl. data; ~9°C at 8 m depth at Sandy Point, C. Hopkins pers. comm.). Both groups of sea urchins developed signs of paramoebiasis after they were maintained in flowing seawater aquaria at 16°C in the laboratory, suggesting that the pathogenic agent was present at these sites but suppressed by low temperatures in the field. Observations made by local fishers of moribund urchins at Sandy Point, and mass mortality of sea urchins at nearby sites (Barrington Bay and Lockport, Shelburne County) in October and November 2011 (C. Hopkins pers. comm.) provide corroborating evidence that paramoebiasis was present at this site.

All sea urchins were transported in chilled plastic bins to the Aquatron at Dalhousie University, Halifax, Nova Scotia, within 6 h of collection. Sea urchins collected from Splitnose Point on 5 August 2011 (prior to disease outbreak), and maintained at 16°C in the laboratory from 19 September to 24 November did not exhibit signs of morbidity, indicating that a pathogen was not present in the seawater system of the Aquatron.

Polyxenic and monoxenic culturing of amoebae

Amoebae were isolated from the radial nerve of moribund sea urchins obtained either directly from the field (Splitnose Point at 8 m depth: isolate SP; St. Margarets Bay cages at 8 m: isolate SMB-8) or from thermal induction experiments (St. Margarets Bay at 60 m: isolate SMB-60; Sandy Point, Shelburne at 8 m: isolate SPS), using the methods of Jones & Scheibling (1985). Briefly, strips of radial nerve with associated radial water-vascular canal (0.5 cm length) were excised from the oral region of sea urchins and placed on 0.6% non-nutrient (NN) marine agar (salinity 35). After 1 wk, amoebae had moved out of the tissues and into the semi-solid agar. Amoebae were subcultured by removing a 1 cm² piece of 0.6% agar onto 1.2% NN agar with a 200 µl liquid overlay containing bacterial prey (*Escherichia coli* in sterile seawater). The liquid overlay was prepared by inoculating 3 ml of lyso-

geny broth (LB) with *E. coli* at 37°C for 24 h, centrifuging the liquid culture (3000 × *g*, 5 min), and replacing the supernatant and resuspending the pellet in 3 ml of seawater.

Monoxenic cultures of amoebae were obtained using the methods of Jellett & Scheibling (1988b). A 1 cm² piece of polyxenic culture of amoebae was removed to 1 ml of antibiotic solution (10 000 UI penicillin, 10 000 µg streptomycin) for 5 h, and then plated onto 1.2% NN agar with liquid overlay (as described above). Amoebae in both monoxenic and polyxenic culture were maintained at 18°C and sub-cultured onto 1.2% NN agar with liquid overlay at 1 to 2 wk intervals.

Laboratory experiments

We conducted 2 types of experiments (below) to test whether the dynamics of paramoebiasis caused by amoebae isolated from moribund sea urchins in fall 2011 were consistent with those observed in similar experiments since the early 1980s and attributed to *Paramoeba invadens* (Scheibling & Stephenson 1984, Jones & Scheibling 1985, Jellett & Scheibling 1988b, Scheibling et al. 2010, Feehan et al. 2012). All experiments were conducted in 47 l glass aquaria supplied with oxygenated temperature-controlled flowing seawater (~3 l min⁻¹) in a quarantine laboratory of the Aquatron at Dalhousie University.

Table 2. *Strongylocentrotus droebachiensis*. Injection experiments to measure rate of morbidity of sea urchins injected with *Paramoeba invadens*. 'Urchin source' is the collection site for healthy experimental sea urchins (SP: Splitnose Point, Nova Scotia; GSL: Gulf of St. Lawrence, Quebec; NS: unknown sites along the coast of Nova Scotia). 'Amoeba source' is the collection site (and depth in m) of moribund sea urchins from which amoebae were isolated (SMB: St. Margarets Bay, Nova Scotia; SPS: Sandy Point, Shelburne, Nova Scotia). 'Culture age' refers to the start of the experiment. Dose is the estimated mean (± SE) number of amoebae ml⁻¹ injected into sea urchins. n is the number of urchins per aquarium. ND: no data available

Expt.	Period	Urchin source	Amoeba source	Culture age (wk)	Dose (ml ⁻¹)	n
1	24 Nov–12 Dec	SP	SMB (8)	5	600 ± 90	3–4
2	12 Feb–14 Mar	GSL	SMB (8)	16.5	300 ± 82	10
			SMB (60)	12.5	300 ± 63	
			SP (8)	13.5	300 ± 45	
			SPS (8)	11.5	300 ± 51	
3	26 Mar–15 May	GSL	SMB (8)	22.5	333 ± 49	10
			SP (8)	19.5	333 ± 20	
Jones & Scheibling (1985)		NS	NS	ND	100–300	10
Jellett & Scheibling (1988)		NS	NS	5	300	5

Water-borne transmission experiment

To determine whether the temperature-dependent rate of transmission and progression of disease from moribund sea urchins to healthy conspecifics was consistent with paramoebiasis, we conducted a water-borne transmission experiment from 30 September to 16 October 2011, using the same methodology and apparatus as Scheibling & Stephenson (1984). We recorded time to ≥50% morbidity (*t*₅₀, d) of sea urchins after exposure to infected conspecifics, for comparison with values from previous water-borne transmission experiments at 16°C using sea urchins infected with *Paramoeba invadens* (Scheibling & Stephenson 1984, Jellett & Scheibling 1988b, Scheibling et al. 2010, Feehan et al. 2012). Morbidity was measured as time to loss of attachment, when sea urchins had detached from the sides or bottom of aquaria and could not right themselves within 20 min. Moribund sea urchins were recorded daily and removed from each aquarium. Groups of 20 healthy sea urchins (collected from Splitnose Point on 5 August 2011, prior to the disease outbreak and maintained in flow-through seawater tanks in the laboratory until use) were placed in 2 aquaria supplied with 16°C seawater. Sea urchins were visually inspected to ensure that they had no lesions or other abnormalities, and a subset was tested to ensure that they could right themselves within 5 min when inverted. Three moribund sea urchins collected from 8 m cages in St. Margarets Bay on 29 September (the same source as isolate SMB-8) were placed in a glass 'source' tube that was spliced into the incurrent seawater supply of one aquarium as the disease-exposed treatment. Three healthy sea urchins (from the same Splitnose Point collection as above) were placed in another tube that supplied the remaining aquarium as the control treatment.

Injection experiments

To determine whether the temperature-dependent rate of progression of disease in healthy sea urchins injected with cultured amoebae was consistent with paramoebiasis, we conducted an injection experiment using the methods of Jones & Scheibling (1985) and Jellett & Scheibling (1988b) (Table 2). As in the water-borne transmission

experiment, moribund sea urchins were recorded daily and removed from each aquarium. We measured t_{50} of sea urchins following injection with amoebae for comparison with similar experiments conducted in the 1980s (Jones & Scheibling 1985, Jellett & Scheibling 1988b, Table 2). Groups of 3 to 4 sea urchins (from the sample collected from Splitnose Point on 5 August) were placed in 6 replicate aquaria with seawater at 16°C. Sea urchins were visually inspected and a subset was tested to ensure that they could right themselves when inverted. Sea urchins in 3 randomly selected aquaria were injected with 1 ml of monoxenic culture of amoebae (isolate SMB-8) that was treated with antibiotics (10 000 IU penicillin, 10 000 µg streptomycin) 2 h prior to injection, as the infection treatment. The inoculum was diluted to a concentration of ~600 amoebae ml⁻¹, as measured using a haemocytometer. Sea urchins in the remaining 3 aquaria were injected with 1 ml of antibiotic-treated *Escherichia coli* as a control. The control inoculum was diluted to the same concentration of bacterial cells as the inoculum of the infection treatment. Streaks with the injection treatment and control inoculum on marine nutrient agar showed no growth after 24 h and individual colonies after 1 wk at 18°C. These colonies were demonstrated to be *E. coli* by replicated direct-sequencing of PCR-amplified partial SSU rDNA sequences (not shown).

To determine whether there are different infective strains of the amoeba along the coast of Nova Scotia, we conducted a second injection experiment to compare the virulence of the 4 isolates: SMB-8, SMB-60, SP and SPS (Table 2). We measured rates of sea urchin morbidity (t_{50}) following injection with amoebae from the respective sites using an experimental design similar to the first injection experiment. Because all healthy sea urchins from laboratory reserves (sea urchins collected from Splitnose Point on 5 August 2011) had been used in previous experiments, healthy sea urchins for the second injection experiment were obtained from the St. Lawrence estuary, where paramoebiasis has not been observed. Sea urchins were collected on 13 December 2011 by divers at Fisheries and Oceans Canada, Institut Maurice-Lamontagne, Mont Joli, Quebec, and ground-transported in chilled plastic bins to Dalhousie University within 24 h of collection. Groups of 10 sea urchins were placed in 10 replicate aquaria with 16°C seawater. Sea urchins within 2 randomly selected aquaria for each of the 4 sites were injected with 1 ml of antibiotic-treated monoxenic culture of amoebae isolated from moribund urchins from a given site, to generate 4 infection treatments based on site-specific amoeba cultures. Each in-

oculum was diluted to a concentration of ~300 amoebae ml⁻¹. Sea urchins in the remaining 2 aquaria were injected with 1 ml of antibiotic-treated *Escherichia coli* as a control. The control inoculum was diluted to the same concentration of bacterial cells as the infection treatment (isolate SMB-8) with the greatest concentration (smallest dilution factor), as a conservative measure. Streaks of inocula from the 4 infection treatments and the control on marine nutrient agar showed no growth after 24 h at 18°C. The effect of amoeba source (fixed factor, 4 levels: SMB-8, SMB-60, SP, SPS) on t_{50} of sea urchins was analysed using 1-way analysis of variance (ANOVA).

To determine whether the virulence of the amoeba is maintained in culture over time, we conducted a third injection experiment reusing 2 of the amoeba cultures used in the second experiment (SP, SMB-8; Table 2). The experimental design was identical to the second experiment, except that the cultures were ~6 wk older. The effect of amoeba source (fixed factor, 2 levels: SMB-8, SP) and culture age (fixed factor, 2 levels: ~15 wk, ~21 wk) on t_{50} of sea urchins was analysed using 2-way ANOVA.

For each injection experiment, we cultured the radial nerve of 3 to 5 moribund sea urchins in each infection treatment within 24 h of the onset of signs of paramoebiasis, and in controls at the end of the experiment, to determine whether amoebae were present, using the methods described above.

Light microscopy

Live cells from the 4 isolates, SMB-8, SMB-60, SPS and SP, were collected from plates using a loop or sterile scraper, and mixed with 5 to 10 µl of sterile seawater on a glass slide. Cells were observed using phase-contrast and differential interference contrast (DIC) light microscopy with 100× magnification objective lenses on a Zeiss Axiovert 200M microscope (Carl Zeiss Canada) fitted with a Zeiss Axio-Cam HRc camera. Length and width measurements were taken from samples prepared using the protocol outlined by Jones (1985). Briefly, amoebae were taken using the method above from cultures that were 6 to 8 d old and were then mounted on a Vaseline-supported slide preparation. Photographs were taken using a 40× objective lens with an additional 1.6× 'optivar' lens in the lightpath, under bright-field optics. The length and width were measured from 30 randomly selected cells per isolate using the Axio-Vision-V4.6.1.0 software (Carl Zeiss). Differences in length, width and length:width ratio were compared

using 1-way ANOVA. Pairwise comparisons were conducted using Tukey's test ($\alpha = 0.05$). All statistical tests were run with Statistica 8 (StatSoft). Data for each test satisfied the assumption of homoscedasticity according to Cochran's *C*-test ($\alpha = 0.05$).

Scanning electron microscopy

The surfaces of week-old plate cultures of all 4 isolates were scraped using sterile scrapers, and for each, a 50 μ l drop of the resulting cell suspension was transferred to the center of a 6 mm diameter coverslip and left to stand for 20 min to allow amoebae to attach to the glass. Coverslips were then prepared following a slightly modified version of the protocol of Kudryavtsev et al. (2011). Briefly, the coverslips were fixed by suspension on 125 μ l of 1% osmium tetroxide and 2.5% glutaraldehyde in seawater (thus the final concentrations of osmium tetroxide and glutaraldehyde were 0.7 and 1.8% due to dilution by the 50 μ l of fluid already present on the coverslips). The coverslips were rinsed and then dehydrated using washes of 100% seawater, 50% seawater and 2 washes of distilled water followed by approximately 10 min in each of the following ethanol mixes: 35, 50, 70, 90 and 95% and then 3 washes in 100% ethanol. The coverslips were critical-point-dried, mounted on stubs, and sputter-coated with a mixture of gold and palladium. The finished coverslips were observed using a Hitachi S-4700 scanning electron microscope (SEM), and photographs were taken of the amoebae, paying attention to the cell surface.

Molecular sequencing

Genomic DNA was extracted from isolates SMB-8, SMB-60, SPS and SP, using the DNeasy Blood & Tissue Kit (Qiagen). The nuclear SSU rDNA sequences were amplified by PCR using primers Euk A and Euk B (Medlin et al. 1988) in a 20 μ l reaction containing the following final reagent concentrations: 1 $\times$ PCR buffer (Life Technologies), 2 mM MgCl₂, 1 μ M each primer, 0.2 mM dNTPs and 0.05 U μ l⁻¹ *Taq* polymerase. The thermocycling protocol was as follows: 94°C for 5 min, then 35 cycles of 94°C for 20 s, 55°C for 2 min, 72°C for 3 min, then 72°C for 10 min. The PCR products were gel-purified using the QIAquick Gel Extraction Kit (Qiagen). The products were directly sequenced in both directions using the amplification primers Euk A and Euk B, plus internal primers 514F (5'-GGT GCC AGC ASC CGC GGT

AA-3'), 536R (5'-GGA ATT ACC GCG GCT GCT GGC A-3'), 1055R (5'-CGG CCA TGC ACC ACC-3') and 1498R (5'-CAC CTA CGG AAA CCT TGT TA-3').

Subsequently, the nuclear SSU rDNA amplicons from 2 isolates, SP and SMB-8, were cloned using the pGEM-T Easy vector system (Promega). Six randomly chosen positive clones from each isolate were grown up overnight in 3 ml LB medium with 3 μ l of 100 mg ml⁻¹ ampicillin, and the plasmid DNA was purified using the Qiagen MiniPrep Kit (Qiagen). Following sequencing of all clones using 514F (~800 bases determined), 2 representative clones from each of the 2 isolates were selected to be completely sequenced in both directions using vector primers and internal sequencing primers 514F, 536R, 1055R and 1055F (5'-GGT GGT GCA TGG CCG-3'). The clones selected to be completely sequenced differed in length at a region at which length heterogeneity was suspected, based on direct sequencing of PCR products (see previous paragraph).

To allow an independent examination of the phylogenetic position of *Paramoeba invadens* and genetic variability amongst strains, parasome SSU rDNA sequences for the SP and SMB-8 isolates were obtained. These were PCR-amplified from the same genomic DNA preparations, using the kinetoplastid-specific primers 'Kineto 14F' and 'Kineto 2026R' (Von der Heyden & Cavalier-Smith 2005). Other than the primers, the reaction mix was as reported above. The following thermocycling protocol was used: 94°C for 5 min, 35 cycles of 94°C for 20 s, 60°C for 2 min and 72°C for 3 min, then 72°C for 10 min. The PCR products were cleaned using the QIAquick Gel Extraction Kit and directly sequenced in both directions using Kineto 14F, Kineto 2026R, 514F, 536R, 1055R and 1055F. The new sequences from *P. invadens* were deposited in GenBank under accession numbers KC790384–KC790389.

Sequence analysis

The 4 complete nuclear SSU rDNA clone sequences from *Paramoeba invadens*, plus the SSU rDNA sequence from *P. atlantica* (Kudryavtsev et al. 2011) were added to the alignment from Dyková et al. (2011), by eye or using MAFFT v6 (Katoh & Toh 2008), with the resulting alignment then corrected by eye. The alignment contained 61 sequences, including 53 *Paramoeba*/*Neoparamoeba* sequences and 8 other Paramoebidae as outgroups. Three datasets were created from the master alignment; '1N' (61 sequences; 1597 well-aligned sites) included the out-

groups, '2N' (53 sequences; 1860 sites) included only *Paramoeba/Neoparamoeba*, and '3N' (52 sequences; 1986 sites) included all *Paramoeba/Neoparamoeba* except *P. atlantica*.

A similar process was followed to align the parasite SSU rDNA sequences, with the 2 new sequences from *Paramoeba invadens* being added to the alignment from Kudryavtsev et al. (2011), using MAFFT v6 with subsequent correction by eye. The final alignment contained 56 sequences including 39 *Paramoeba/Neoparamoeba* parasite sequences, and 18 other Prokaryotoplastida sequences as outgroups. Three datasets were again created: '1P' (56 sequences; 1363 well-aligned sites) included outgroups; '2P' (39 sequences; 1502 sites) included only the parasite sequences, and '3P' (38 sequences; 1576 sites) contained only parasites, with the *P. atlantica* parasite excluded. Alignments are available from the authors upon request.

Maximum likelihood (ML) trees were estimated for all 6 datasets using RAxML v7.2.8 (Stamatakis 2006). All analyses used the GTR model of nucleotide substitution, with among-site rate variation modelled by a gamma distribution (α was estimated from the data in each case; 1N $\alpha = 0.38$; 2N $\alpha = 0.21$; 3N $\alpha = 0.20$; 1P $\alpha = 0.28$; 2P $\alpha = 0.30$; 3P $\alpha = 0.24$). In each case, 20 independent searches were run from different random-taxon-addition parsimony starting trees. Support for bipartitions was assessed from 500 bootstrap replicates.

RESULTS

Laboratory experiments

In the water-borne transmission experiment, healthy sea urchins exposed to moribund conspecifics from cages at 8 m depth in St. Margarets Bay were all moribund by Day 12, and t_{50} was reached after 9 d (Table 3, Fig. 2). This t_{50} was within the ranges observed in previous water-borne transmission experiments conducted since the early 1980s (Table 3).

In the first injection experiment, which examined whether the temperature-dependent rate of progression of disease in healthy sea urchins injected with cultured amoebae was consistent with paramoebiasis, all but one of the healthy sea urchins injected with amoebae isolated from moribund conspecifics in cages in St. Margarets Bay (isolate SMB-8) were also moribund by Day 15, with a mean t_{50} of 12 d (Table 3, Fig. 3). Mean t_{50} conformed closely to that observed in similar experiments conducted in the early to mid-1980s.

Table 3. *Strongylocentrotus droebachiensis*. Waterborne transmission and injection experiments conducted following disease outbreaks since 1980. t_{50} is the time to $\geq 50\%$ morbidity (exhibiting signs of paramoebiasis, in days) following exposure to 3 to 5 moribund conspecifics. N is the number of experiments conducted in each year. For all experiments, survival was 100% in controls. In disease-exposed treatments, survival was 0% in waterborne transmission experiments and 10% in injection experiments. For all experiments, water temperature was 16°C

Year(s)	t_{50} (d)	N	Source
Waterborne			
2011	9	1	This study
2010	8–10	3	Feehan et al. (2012)
2009	11	1	Scheibling et al. (2010)
1982	12–13.5	7	Jellett & Scheibling (1988b)
1982–83	8.5–11.5	7	Scheibling & Stephenson (1984)
Injection			
2011	12	1	This study
1982	13.5	1	Jellett & Scheibling (1988b)
1980–83	13	1	Jones & Scheibling (1985)

Fig. 2. *Strongylocentrotus droebachiensis*. Cumulative frequency of morbidity of sea urchins exposed to moribund conspecifics (from cages in St. Margarets Bay) in a water-borne transmission experiment. Dashed line indicates 50% morbidity. There was no morbidity in controls (not shown). Temperature = 16°C

In the second injection experiment, which compared the virulence of 4 isolates of amoebae (cultures of *Paramoeba invadens* from moribund sea urchins collected at different sites: SMB-8, SMB-60, SP and SPS), 1-way ANOVA showed significant differences in t_{50} among the 4 isolate treatments ($F_{3,4} = 7.73$, $p = 0.039$; Fig. 4A). In one replicate of the SP treatment, sea urchins only reached 40% morbidity after 31 d, which was used as a conservative estimate of t_{50} for that replicate in the analysis. The

Fig. 3. *Strongylocentrotus droebachiensis*. Comparison of cumulative frequency of morbidity of sea urchins injected with amoebae isolated from moribund conspecifics collected at 8 m depth in St. Margarets Bay in 2011 and in a similar experiment conducted by Jones & Scheibling (1985). Dashed line indicates 50% morbidity. There was no mortality in controls (not shown) in either experiment. Temperature = 16°C. Data are mean $\pm$ SE for 3 replicate aquaria. Data from 1985 are offset by +0.25 d to avoid overlap of data points between years

Fig. 4. *Strongylocentrotus droebachiensis*. Cumulative frequency of morbidity of sea urchins injected with amoebae isolated from (A) moribund conspecifics from 4 sites along the coast of Nova Scotia: St. Margarets Bay at 8 m (SMB-8) and 60 m depth (SMB-60), Sandy Point, Shelburne (SPS) and Splitnose Point (SP); and (B) the same cultures for SMB-8 and SP that were 6 wk older. Dashed line indicates 50% morbidity. There was no morbidity in controls (not shown) in either experiment. Data are mean $\pm$ SE for 2 replicate aquaria. Data for SMB-8 are offset by ± 0.25 d for clarity

estimated mean t_{50} of sea urchins in the SP treatment (30 d) was greater than the mean t_{50} in SMB-8, SMB-60 and SPS treatments (17 to 19 d), meaning that SP was less virulent.

In the third injection experiment, sea urchins were injected with amoebae from 2 of the source treatments used in the second experiment (SMB-8, SP), but when these cultures were 6 wk older. In both cases, t_{50} was about 1.5 times greater than in the second experiment, but the difference between source treatments was consistent between experiments, with SP again producing a larger t_{50} than SMB-8 (Fig. 4A,B). The estimated mean t_{50} in the SP treatment was 44 d in the third experiment, while mean t_{50} in the SMB-8 treatment was 28 d (Fig. 4B; note that in 1 replicate of the SP treatment, sea urchins only reached 20% morbidity after 50 d, which we used as a highly conservative estimate of t_{50} for that replicate). A 2-way ANOVA showed significant effects of amoeba source/isolate ($F_{1,4} = 8.37$, $p = 0.044$) and culture age ($F_{1,4} = 11.32$, $p = 0.028$), but no interaction between these factors ($F_{1,4} = 0.09$, $p = 0.783$).

Paramoeba-like amoebae (Jones 1985) were observed in all cultures of radial nerves of moribund sea urchins taken from the infection treatments of injection experiments. No sea urchins died or showed signs of morbidity in any of the controls of both types of experiments, and amoebae were not observed in cultures of radial nerves of control sea urchins from injection experiments.

Microscopy

The cell dimensions for the 4 amoebae isolates in culture, estimated from 30 randomly selected cells, are given in Table 4. There was a significant difference in mean width among isolates ($F_{3,122} = 5.493$, $p = 0.001$), with SMB-60 having a smaller width than the other 3 isolates ($p < 0.044$). Mean length also was smallest for SMB-60, although length did not differ significantly among isolates ($F_{3,122} = 1.975$, $p = 0.121$). There was no significant difference in length:width ratio among the 4 isolates ($F_{3,122} = 1.353$, $p = 0.260$). Length and width of all 4

Table 4. *Paramoeba invadens*. Dimensions of amoebae. 'Source' is the collection site (in Nova Scotia) of moribund sea urchins from which amoebae were isolated (SMB-8: St. Margarets Bay at 8 m depth; SMB-60: St. Margarets Bay at 60 m depth; SPS: Sandy Point, Shelburne, 8 m depth; SP: Splitnose Point, 8 m depth) or data taken from Jones (1985). ND: no data available

Source	n	Length (μm)		Width (μm)		Length:Width	
		Mean	Range	Mean	Range	Mean	Range
SMB-8	30	23.7	9–49	9.3	3–19	2.6	1–6
SMB-60	30	21.8	11–31	6.7	3–12	3.3	2–7
SPS	30	27.0	10–46	10.4	3–23	2.6	1–6
SP	30	25.0	9–47	9.6	4–23	2.6	1–9
Jones (1985)	308	28.3	8–57	14.5	3–30	2.0	ND

isolates were within the ranges recorded by Jones (1985), and maximum lengths were similar (Table 4). The average width we recorded was somewhat smaller than that recorded by Jones (1985), and the length:width ratio correspondingly higher, but not profoundly so (3.3 for SMB-60, versus 2.0; Table 4). Locomotive forms were elongated in the direction of travel with a large variation in outline (Fig. 5A,E,I,M). Non-locomotive forms were broad and rounder. Both long, narrow pseudopodia and short, broad pseudopodia were regularly observed in all isolates (Fig. 5B,C,F,G,J,K,N,O). One parasome was observed inside each cell. The parasomes were small (approximately $5 \times 2 \mu\text{m}$), and pill-shaped, with a tripartite form, and were usually located near the nucleus (Fig. 5B,F,J,N). These observations were also in accordance with Jones (1985).

A class of small cells, typically $10 \mu\text{m}$ or less in length, were also observed occasionally in all 4 isolates (Fig. 5D,H,L,P). These small cells appeared to lack a parasome when viewed by phase contrast or DIC microscopy.

Cells prepared for SEM observation retained the basic morphology seen by light microscopy, with some shrinkage (Fig. 6A,C,E,G). High-resolution micrographs confirmed the lack of micro-scales on the surface of all 4 isolates (Fig. 6B,D,F,H). The minute particles visible on the surfaces of these amoebae are an order of magnitude smaller than the micro-scales of *Paramoeba* spp., and much more sparsely distributed (see Kudryavtsev et al. 2011).

Fig. 5. *Paramoeba invadens*. Phase contrast and DIC light microscopy, showing morphology of all 4 isolates: (A–D) SMB-8 (see Fig. 4 for site information), (E–H) SMB-60; (I–L) SPS; (M–P) SP. Panels A, E, I, M show the tripartite morphology of the parasome. B, F, J, N depict the broad, short form of pseudopodia. C, G, K, O show the long, narrow forms of pseudopodia. D, H, L, P show 'small cells'. Scale bar at bottom right represents $10 \mu\text{m}$ for all panels

Fig. 6. *Paramoeba invadens*. Scanning electron micrographs of the 4 studied isolates: (A,B) SMB-8 (see Fig. 4 for site information); (C,D) SMB-60; (E,F) SPS; (G,H) SP. Panels A, C, E, G illustrate the variety of general cell shape and pseudopodial forms. B, D, F, H show the cell surface and absence of microscales. Scale bar in G represents 10 μ m for A, C, E and G. Scale bars in B, D, F and H represent 1 μ m

Gene sequencing and phylogenetic analysis

No clear differences were seen in the completely sequenced PCR products of the nuclear SSU rDNA between isolates. Several ambiguities were observed that were shared amongst isolates, and there was one region in all 4 isolates that could not be sequenced directly in either direction, suggesting that there was

heterogeneity (including length heterogeneity) within isolates, probably among the SSU rDNA copies within a genome. Cloning was then performed from 2 isolates (SMB-8 and SP), and a large number of distinct sequence types observed by partial sequencing. Two representative clones from each isolate were then sequenced completely and used for the phylogenetic analysis.

Parasome SSU rDNA sequences from the same 2 isolates were sequenced directly from PCR products. No ambiguities were detected in these sequences, and the 2 isolates proved to be identical when compared.

Phylogenetic analysis of nuclear SSU rDNA sequences was initially conducted on dataset '1N', with outgroups included (Fig. 7A). Because of the proposed synonymisation of *Neoparamoeba* with *Paramoeba* (see 'Discussion'), all species typically assigned to *Neoparamoeba* are referred to here with both genus names. In the ML tree, *P. atlantica* falls as sister to all other *Paramoeba/Neoparamoeba* isolates, which form a maximally supported clade (bootstrap support, BS, 100%), as in a previous study (Kudryavtsev et al. 2011). *P./N. branchiphila* forms a distinct clade, as does *P./N. perurans*, with both receiving >90% BS. *P./N. pemaquidensis* and *P./N. aestuarina* isolates together form a strongly supported clade (BS 95%). Within this assemblage, *P./N. aestuarina* forms a distinct strongly supported clade (BS 83%) that emerges from within *P./N. pemaquidensis* as was also reported by Dyková et al. (2011); however, support for this internal placement is very weak (BS 56%). Our new sequences from *P. invadens* appear as a distinct clade that emerges as the sister group to *P./N. branchiphila*, with strong support (BS 100%). The *P./N. branchiphila* plus *P. invadens* clade branches with weak support (BS 51%) as the sister group to a very weakly supported clade of *P./N. perurans* plus the sole *P. eilhardi* sequence (BS 45%).

The recovered trees are broadly the same in both analyses where the outgroups are excluded (N2 and N3 analyses; Fig. 7B,C). In both analyses, *Paramoeba invadens* still emerges as the sister group to a robust *Paramoeba/Neoparamoeba branchiphila* clade, with strong support (BS 80%, 100%; Fig. 7B,C). When *P. atlantica* is retained in the analysis (N2 analysis, Fig. 7B) *P./N. perurans* moves to the 'next deepest' position within the tree, with very weak support (BS 38%). *P. eilhardi* moves to branch as a sister group to the *P./N. pemaquidensis* plus *P./N. aestuarina* clade, with very weak support (BS 34%), and thus remains separate from *P. atlantica* (i.e. not in a clan with *P. atlantica* on an unrooted tree). When *P. atlantica* is also excluded (N3 analysis, Fig. 7C), *P. eilhardi* is sister to the *P./N. branchiphila* plus *P. invadens* clade, with moderate support (BS 65%).

Phylogenetic analysis of the parasome SSU rDNA gene sequences gives broadly similar results to the analyses of nuclear SSU rDNA. In the tree containing all outgroups, *Paramoeba atlantica* falls as sister to all other *Paramoeba/Neoparamoeba* species, including

P. invadens (analysis 1P; Fig. 8A). *P. invadens* branches strongly as sister to the *P./N. branchiphila* clade (BS 81%). However, the single, quite divergent sequence from a *P./N. aestuarina* parasome branches separately from *P./N. pemaquidensis* and instead lies as sister to the *P./N. branchiphila* plus *P. invadens* clade, with 74% bootstrap support. In the ML tree, *P./N. perurans* is sister to all other *Paramoeba/Neoparamoeba* sequences other than *P. atlantica*, with maximal support. There were no reported sequences of *P. eilhardi* parasomes available for comparison.

The relationships amongst the *Paramoeba/Neoparamoeba* clades differ in both analyses where outgroups are excluded. When *P. atlantica* is retained (analysis 2P; Fig. 8B), the *P./N. aestuarina* sequence actually falls within the *P. invadens* plus *P./N. branchiphila* clade as sister to *P./N. branchiphila*, though with very weak support (BS 46%). The *P./N. perurans* clade falls as sister to these 3 species (BS 67%), and *P./N. pemaquidensis* is deeply diverging. When *P. atlantica* is excluded in addition to the outgroups (analysis 3P; Fig. 8C), a strongly supported relationship between *P. invadens* and *P./N. branchiphila* is again recovered (BS 83%), with *P./N. aestuarina* forming a clade with *P./N. pemaquidensis*, with moderate support (BS 67%), more similar to the results of the nuclear SSU rDNA analyses (see above).

DISCUSSION

Validating the identity of *Paramoeba invadens* as the pathogen of sea urchins in Nova Scotia

Water-borne disease transmission and injection experiments conducted from 1980 to 2011, culminating in the studies reported here, show a consistent rate of transmission and propagation of paramoebiasis in sea urchins *Strongylocentrotus droebachiensis*. We also confirm here that the amoebae infecting sea urchins along the coast of Nova Scotia in fall 2011 are morphologically similar to those associated with disease outbreaks in *S. droebachiensis* in the early 1980s, caused by *Paramoeba invadens*. Together this represents very strong evidence that the pathogen infecting sea urchins in 2011 is indeed *P. invadens*. Analyses of both nuclear and parasome SSU rDNA show clearly that *P. invadens* is a distinct species (not, for example, a variant of *Paramoeba/Neoparamoeba pemaquidensis*), that is almost certainly closely related to *P./N. branchiphila*, a suspected pathogen of sea urchins *Diadema* aff. *antillarum* in the Canary Islands, Spain. *P./N. branchiphila* also has been iso-

Fig. 7. *Paramoeba/Neoparamoeba* spp. Maximum likelihood trees (GTR+gamma model) of selected strains based on SSU rDNA sequences, showing the phylogenetic position of *P. invadens* based on (A) '1N' analysis: 1597 sites, and including all outgroups; (B) '2N' analysis: 1860 sites, and including only *Paramoeba/Neoparamoeba*; and (C) '3N' analysis: 1986 sites, also excluding *P. atlantica*. Numbers associated with internal branches depict bootstrap support >50%. Scale bars represent 0.02 expected substitutions per site. Branches depicted with dashed lines indicate a reduction in scale of 50%

lated from tissues of crabs in the US, sea urchins in Australia and Greece, and finfish in aquaculture throughout the world (Table 1).

Outbreaks of paramoebiasis in sea urchins along the Atlantic coast of Nova Scotia are associated with hurricanes and tropical storms, and warm sea temperatures (Scheibling & Lauzon-Guay 2010, Feehan et al. 2012); however, the source of the infective population of *Paramoeba invadens* remains unknown. The minimum thermal tolerance of *P. invadens* (~2°C) is above the winter minimum in ocean temperature (0 to -2°C) along this coast (Jellett & Scheibling 1988a). Therefore, it has been considered unlikely that *P. invadens* overwinters in sea urchins or in the surrounding environment (Jellett et al. 1989). Two mechanisms have been postulated to explain recurrent introductions of the pathogen to shallow coastal waters during severe storm events: (1) horizontal advection from distant source populations (Scheibling & Hennigar 1997) and (2) vertical mixing of amoebae residing in deep sedimentary basins along the Scotian shelf (Scheibling & Lauzon-Guay 2010), where temperatures rarely fall below ~5°C (K. Filbee-Dexter & R. E. Scheibling unpubl. data).

Our analysis of nuclear SSU rDNA showed no fixed genetic differences among *Paramoeba invadens* isolates derived from moribund sea urchins at 4 sites spanning ~200 km of coastline (linear distance) and across a depth range of ~50 m. However, injection experiments indicated differences in virulence among cultures of amoebae, with amoebae from one site (SP) having lower virulence than amoebae from the other 3 sites (SMB-8, SMB-60 and SPS). If there were indeed spatial variance in virulence, this would suggest that there is more than one infective subtype of *P. invadens* along the Nova Scotian coast and that the pathogen is introduced at local scales (10s of km), favouring the hypothesis that amoebae reside in nearshore sedimentary basins. It would be interesting to determine whether examining more variable molecular markers (e.g. ITS sequences) would reveal differences among isolates, and if so, whether this molecular difference corresponds to either geographical differences, or differences in virulence.

Global trends in *Paramoeba/Neoparamoeba*-associated disease events

Amoebae assigned to the genera *Paramoeba* and *Neoparamoeba* have been associated with disease in various marine organisms worldwide (Table 1). However, to our knowledge, Koch's postulates have only

been fulfilled for 2 species: *P. invadens* in sea urchins in Nova Scotia (Jones & Scheibling 1985, this study), and *Paramoeba/Neoparamoeba perurans* in Atlantic salmon in aquaculture (Crosbie et al. 2012, Table 1). This may reflect the difficulty of culturing these amoebae in the first place (Johnson 1977, Mullen et al. 2004, 2005, Crosbie et al. 2012) or the loss of virulence in culture over time (i.e. reduced infectivity or severity of disease; Jellett & Scheibling 1988b, Kent et al. 1988, Zilberg et al. 2001).

The results of our successive injection experiments confirm that *Paramoeba invadens* loses virulence in culture over the scale of weeks. We found a ca. 1.5-fold increase in t_{50} of infected *Strongylocentrotus droebachiensis* between ~15 and 21 wk old cultures. Jellett & Scheibling (1988b) also found that virulence of monoxenic cultures of *P. invadens* decreased in sea urchins after 15 wk. Other studies have documented changes in metabolism of other *Paramoeba/Neoparamoeba* spp. that are associated with a loss of virulence, including a decrease in the number of trophozoites with a fully developed parasome (Dyková et al. 2000), and changes in extracellular products (Kent et al. 1988, Dyková et al. 2000). Villavedra et al. (2007) found that cultured *P./N. pemaquidensis* has a different antigen profile than freshly isolated *P./N. pemaquidensis*.

There is evidence that some *Paramoeba/Neoparamoeba* spp. are opportunistic pathogens, infecting only stressed or diseased individuals. For example, an outbreak of *N. pemaquidensis* in lobster *Homarus americanus* in Long Island Sound, USA, in 1999 was associated with thermal stress and crowding (Mullen et al. 2004). In the Canary Islands, *P./N. branchiphila* was isolated from sea urchins *Diadema* aff. *antil-larum* infected with 'bald sea urchin disease' caused by the bacterium *Vibrio* sp. (Dyková et al. 2011). In amoebic gill disease (AGD)-infected Atlantic salmon in aquaculture, co-infections with sea lice, bacteria, gelatinous zooplankton, *Ichthyobodo* (marine costia) and other amoeba species have been observed (Bermingham & Mulcahy 2006, Nowak et al. 2010, Bustos et al. 2011, Rodger et al. 2011). In our laboratory infection experiments, there was no indication that sea urchins were physiologically stressed: water temperature (16°C) was well below the maximum thermal tolerance of *Strongylocentrotus droebachiensis* (22°C), and no mortality occurred in control sea urchins maintained under the same conditions for 9.5 wk. We found no evidence to indicate that a co-occurring pathogen is involved in disease outbreaks in sea urchins in Nova Scotia; however, we cannot disregard the possibility that an undetected and con-

sistently co-occurring microbe(s) was present in inocula of *P. invadens* used in injection experiments in our study or in previous ones (Jones & Scheibling 1985, Jellett & Scheibling 1988b).

It has been suggested that *Paramoeba/Neoparamoeba pemaquidensis* and *P./N. branchiphila* were misidentified as the causative agent of AGD in Atlantic salmon *Salmo salar* (Young et al. 2008). Retrospective analyses of Atlantic salmon gill tissue using *in situ* probes that hybridize with the SSU rDNA indicate that several cases of AGD previously attributed to *P./N. pemaquidensis* or *P./N. branchiphila* were actually caused by *P./N. perurans*, the proven causative agent of AGD (Young et al. 2008). Crosbie et al. (2012) suggested that current culturing procedures select for non-virulent amoebae species (*P./N. branchiphila* or *P./N. pemaquidensis*) instead of *P./N. perurans* when both are present, leading to misidentification of the actual disease agent and the artificial appearance of rapid loss of virulence. For example, investigators have found that naïve Atlantic salmon develop AGD following water-borne exposure to *P./N. pemaquidensis* or *P./N. branchiphila* freshly isolated from the gills of infected conspecifics (Douglas-Helders et al. 2003, Morrison et al. 2005, Vincent et al. 2007); however, AGD is not induced by exposure to cultured *P./N. pemaquidensis* or *P./N. branchiphila* (Douglas-Helders et al. 2003, Mullen et al. 2004, 2005, Crosbie et al. 2007, 2010a, Vincent et al. 2007). Similarly, Morrison et al. (2005) found that only fresh cultures of *P./N. pemaquidensis* (<72 h old) induced AGD in naïve Atlantic salmon.

Infections with *Paramoeba/Neoparamoeba* spp. typically exhibit strong temperature dependence with threshold dynamics. Paramoebiasis in sea urchins off Nova Scotia occurs only above a threshold temperature of ~10°C, and rates of morbidity of sea urchins increase up to ~20°C, corresponding to the thermal optimum for growth of *P. invadens* in culture (Scheibling & Stephenson 1984, Jellett & Scheibling 1988a). Similarly, infection by *Paramoeba/Neoparamoeba* of farmed salmonids in Tasmania, Australia (Munday et al. 1990, Clark & Nowak 1999, Douglas-Helders et al. 2001), and blue crab *Callinectes sapidus* in the northeastern US (Johnson 1977), occurs only above threshold temperatures of ~10 and 13°C, respectively. Furthermore, the only documented outbreak of AGD in Atlantic salmon in aquaculture in Norway occurred above a threshold of ~11°C and was associated with sea temperatures ~3.5°C above the seasonal average (Steinum et al. 2008). Given the temperature dependence of

Paramoeba/Neoparamoeba-associated disease outbreaks, continued ocean warming could have important implications for the resilience of susceptible host populations. Indeed, increases in the frequency and severity of outbreaks of sea urchin paramoebiasis and range of AGD are associated with increasing or unusually high sea temperatures in the affected regions (Steinum et al. 2008, Scheibling & Lauzon-Guay 2010).

Systematic and nomenclatural considerations

Prior to 1987, dactylopodial amoebae with paramoebae were assigned to one genus, *Paramoeba*. Page (1987), however, divided *Paramoeba* into 2 genera primarily on the presence or absence of surface microscscales. The scale-bearing *P. eilhardi* remained in *Paramoeba* as its type species, while a new genus, *Neoparamoeba*, was proposed for the species without scales. The organisms originally described as *P. pemaquidensis* and *P. aestuarina* were transferred to *Neoparamoeba*, as *N. pemaquidensis* and *N. aestuarina*, with *N. pemaquidensis* nominated as the type species (Page 1987). Two additional species of *Neoparamoeba* were described subsequently, viz. *N. branchiphila* (Dyková et al. 2005) and *N. perurans* (Young et al. 2007), while Kudryavtsev et al. (2011) recently described a new microscale-bearing amoeba as *P. atlantica*. *P. invadens* is anomalous, as it was described shortly before Page (1987) erected the genus *Neoparamoeba* but was not moved to *Neoparamoeba* despite positive evidence that it lacked scales (Jones 1985). Probably Page (1987) was unaware of the description.

Page (1987) assigned *Paramoeba* and *Neoparamoeba* to different families. Recent SSU rDNA phylogenies, however, show that they are very closely related. In fact *P. eilhardi*, represented by culture CCAP 1560/2, almost always branches in amongst the described *Neoparamoeba* species, including *N. pemaquidensis* (Dyková et al. 2007, 2008, 2011, Kudryavtsev et al. 2011, this study), while *P. atlantica* appears as the sister group to all other *Paramoeba/Neoparamoeba* (Kudryavtsev et al. 2011, this study). Kudryavtsev et al. (2011) confirmed that culture CCAP 1560/2 has microscscales, and that its reported SSU rDNA is correct and not from a contaminant (see Dyková et al. 2007). The most likely interpretation is that *Paramoeba* as currently circumscribed is paraphyletic, while *Neoparamoeba* is most likely polyphyletic and represents 2 or more lineages that independently lost microscscales (Kudryavtsev et al. 2011).

Most systematists hold that genera and other taxa should be monophyletic wherever possible. Given the phylogenetic placement of *Paramoeba* and *Neoparamoeba* species, in particular their type species, it is likely impossible to subdivide these organisms into 2 monophyletic genera, especially genera that meaningfully reflect the original morphological distinction between *Paramoeba* and *Neoparamoeba* (Kudryavtsev et al. 2011). The alternatives are either (1) to treat *Paramoeba* and *Neoparamoeba* as a single genus, which by priority would be *Paramoeba*, (2) to subdivide *Paramoeba* and probably *Neoparamoeba* into multiple genera each, or (3) to abandon phylogenetic taxonomy for these organisms. We believe the first option is the most conservative, and would be the least unpopular. Kudryavtsev et al. (2011) already discussed the likelihood that synonymisation of *Neoparamoeba* with *Paramoeba* will be required, and it is only because of their commendably very cautious approach to phylogenetic systematics that *Paramoeba* and *Neoparamoeba* have not been synonymised already.

Our phylogenetic analyses of nuclear SSU rDNA sequences, with the addition of *Paramoeba invadens*, return similar results to those of earlier studies (Young et al. 2007, Dyková et al. 2007, 2008, 2011, Kudryavtsev et al. 2011). These analyses do not provide positive evidence to support the existence of separate *Paramoeba* and *Neoparamoeba* clades, even (in our analyses) in the absence of outgroups which hypothetically could be affecting the topology recovered for the ingroup (see Fig. 7B), and irrespective of whether *P. invadens* is considered a member of *Paramoeba* or *Neoparamoeba*. Notwithstanding the desirability of additional confirming data (Kudryavtsev et al. 2011), to continue to distinguish *Paramoeba* and *Neoparamoeba* would simply be delaying the inevitable in our view. Resolving the genus-level taxonomy of *Paramoeba* and *Neoparamoeba* now avoids the problem of having to choose between (1) having *P. invadens* assigned to a genus to which it is now clear that it does not fit under rational criteria, or (2) transferring *P. invadens* to *Neoparamoeba*, thereby adding to the literature an extra name (a novel combination) that is likely to have only a very short active life.

Bearing this in mind, we formally propose that *Neoparamoeba* be considered a junior synonym of *Paramoeba*, with *Paramoeba* to include all dactylopodid amoebae with parasomes, both those with microscscales and those that lack them due to secondary loss. Species presently assigned to *Paramoeba* all remain in this genus, including *P. invadens* (see

below). *N. pemaquidensis* and *N. aestuarina* revert to their original names, *P. pemaquidensis* and *P. aestuarina*. Finally, new combinations are proposed for the 2 species originally described as members of *Neoparamoeba*: *N. branchiphila* Dykova et al. 2005 and *N. perurans* Young et al. 2007. These species become *P. branchiphila* comb. nov. and *P. perurans* comb. nov. (see below).

Taxonomic summary

Paramoeba Schaudinn, 1896

Emended diagnosis: Dactylopodial amoebae with one or more DNA-containing parasomes adjacent or near the nucleus. Surface may be covered with microscscales (if absent, these have presumably been lost secondarily).

Included species:

- *Paramoeba eilhardi* Schaudinn, 1896 (type species)
- *Paramoeba aestuarina* Page, 1970 [synonym *Neoparamoeba aestuarina* (Page, 1970) Page, 1987]
- *Paramoeba atlantica* Kudryavtsev et al., 2011
- *Paramoeba branchiphila* (Dyková et al., 2005) Feehan et al. comb. nov. [basonym *Neoparamoeba branchiphila* Dyková et al., 2005]
- *Paramoeba invadens* Jones, 1985
- *Paramoeba pemaquidensis* Page, 1970 [synonym *Neoparamoeba pemaquidensis* (Page, 1970) Page, 1987]
- *Paramoeba perniciosus* Sprague et al., 1969
- *Paramoeba perurans* (Young et al., 2007) Feehan et al. comb. nov. [basonym *Neoparamoeba perurans* Young et al., 2007]
- *Paramoeba schaudinni* de Faria et al., 1922

Acknowledgements. We thank C. Hopkins for providing sea urchins for experiments, J. Lindley, K. Krumhansl and K. Filbee-Dexter for field assistance, F. Francis for assistance with laboratory experiments and A. A. Heiss for assistance with SEM and phylogenetic analyses. We thank M. Kostka and A. Kudryavtsev for supplying SSU rDNA seed alignments. This research was funded by a Discovery Grant to R.E.S. from the Natural Sciences and Engineering Research Council (NSERC) of Canada, and by support from the Canadian Institute for Advanced Research (CIFAR) and an NSERC discovery grant to A.G.B.S. C.J.F. was supported by a Dalhousie Research Scholarship.

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