Vol. 95: 225–231, 2011
doi: 10.3354/dao02361

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published July 12

Neoparamoeba branchiphila infections in moribund sea urchins *Diadema* aff. *antillarum* in Tenerife, Canary Islands, Spain

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ABSTRACT: A total of 109 sea urchins from 3 species collected in 2 localities off the coast of Tenerife Island, Spain, were examined for the presence of free-living amoebae in their coelomic fluid. Amoeba trophozoites were isolated exclusively from moribund individuals of long-spined sea urchins *Diadema* aff. *antillarum* (Philippi) (Echinoidea, Echinodermata) that manifested lesions related to sea urchin bald disease on their tests (16 out of 56 examined). No amoebae were detected in *Arbacia lixula* (L.) and *Paracentrotus lividus* (Lamarck). From the former sea urchin species, 8 strains, established from 10 primary isolates, were identified as *Neoparamoeba branchiphila* Dyková et al., 2005 using morphological and molecular methods. Results of this study (limited to the screening for free-living amoebae) together with data on agents of sea urchin mortalities reported to date justify the hypothesis that free-living amoebae play an opportunistic role in *D.* aff. *antillarum* mortality. The enlargement of the dataset of SSU rDNA sequences brought new insight into the phylogeny of *Neoparamoeba* species.

KEY WORDS: *Neoparamoeba* · *Diadema* aff. *antillarum* · SSU rDNA phylogeny · Tenerife

INTRODUCTION

Amoebae of the genera *Paramoeba* Schaudin, 1896 and *Neoparamoeba* Page, 1987 are free-living organisms that are also known as pathogens of marine fish and invertebrates. Species of these 2 genera are very similar in terms of features such as the morphotype of trophozoites and ultrastructure, including their kinetoplastid endosymbionts. The microscales described on the cell surface of *Paramoeba eilhardi* Schaudin, 1896 by Grell & Benwitz (1966, 1970) distinguish *Paramoeba* from *Neoparamoeba* (Page 1987).

The amount of morphological and molecular data collected on *Neoparamoeba* species from extensive studies on aetiology of amoebic gill disease (AGD) of marine fish far exceeds our knowledge of *Paramoeba* spp. Except for *P. eilhardi* isolated by Grell in 1960 in Villefranche, France (Grell 1961), no strain of *Paramoeba* sensu Page 1987 is available in culture collections. Although several disease outbreaks in marine invertebrates have been attributed to *Paramoeba* spp., subsequent revisions of published data reveal that neither *P. perniciosa*, described as the agent of 'gray crab disease' (Sprague & Beckett 1968, Sprague et al. 1969, Perkins & Castagna 1971, Johnson 1977), nor *P. invadens*, considered to cause mortalities of green sea urchins (Jones 1985, Jones & Scheibling 1985), meets the definition given for *Paramoeba* by Page (1987).

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Similarly, Mullen et al. (2004), who originally described 'paramoebiasis' of American lobster, later assigned the amoebae considered the primary cause of the 1999 lobster mass mortality to *Neoparamoeba pemaquidensis* (see Mullen et al. 2005). Aetiological conclusions mentioned above, inconsistencies persisting in the literature on amoebic diseases of invertebrates (Scheibling et al. 2010), and the scarcity of reliable data on *Paramoeba* spp. stimulated our interest in isolation of species of this genus and subsequent phylogenetic analysis of *Paramoeba* and *Neoparamoeba* strains. We took the opportunity to combine our research interests with those of a research team from the Department of Marine Biology at the University of La Laguna, Tenerife, Canary Islands, Spain, involved in monitoring the population and health status variations of sea urchins in the island as well as the biodiversity and ecology of sea urchins habitats. Results based on examination for amoebae of both asymptomatic and diseased sea urchins collected along the coasts of Tenerife Island are presented in this study.

MATERIALS AND METHODS

Collection of sea urchins. In April 2010, a total of 109 sea urchins from 3 species, the long-spined sea urchin *Diadema* aff. *antillarum* (Philippi) (n = 56), the black sea urchin *Arbacia lixula* (L.) (n = 29), and the purple sea urchin *Paracentrotus lividus* (Lamarck) (n = 24) were collected and examined from 2 localities off the coast of Tenerife Island (Abades 28° 08′ 26″ N, 16° 26′ 04″ W; Boca Cangrejo 28° 24′ 22″ N, 16° 18′ 52″ W) in which sea urchins showed signs of disease. Sea urchins were picked up from the seafloor at low tide by divers, transported immediately to the laboratory, and kept for a short time in aquarium tanks supplied with running seawater sterilized by UV light. The set of collected sea urchins contained specimens with pronounced disease symptoms (i.e. loss of spines, eroded areas, or small holes on their tests), as well as specimens of healthy appearance.

Isolation and culture of amoebae. Screening for the amoeba infections was restricted to the coelomic fluid of sea urchins sampled after the seawater was drained away from their mouth area and spiny surface. An 18G × 1½″ needle was inserted through the peristomal membrane at an angle that avoided contact with the lantern area, and a small volume of coelomic fluid (up to 5 ml from big specimens) was withdrawn into a sterile, disposable syringe. A total of 3 to 4 drops of coelomic fluid was placed on the surface of non-nutrient seawater agar prepared from sterilized seawater of the same origin as the sea urchins. Inoculated agar plates (3 per sea urchin individual) were stored upside-down in an air-conditioned laboratory (20°C). In order to detect primary isolates of amoebae as soon as possible and to avoid overgrowth with bacteria, agar plates were checked several times over the first week post-sampling. The handling of primary isolates, culture methods, and harvesting for ultrastructural and molecular studies were the same as those previously described (Dyková et al. 2000, 2005). Within the set of 109 sea urchins examined, 15 individuals (12 *Diadema* aff. *antillarum* and 3 *Arbacia lixula*) were moribund, and their body surface showed eroded areas devoid of spines. In total, 10 primary isolates were obtained (all those from coelomic fluid of moribund specimens of *Diadema* aff. *antillarum*), but 2 were lost due to bacterial overgrowth. We established strains from the remaining 8 isolates, which are characterized in this study using morphological and molecular methods.

Microscopy. Both light and electron microscopy was carried out as described elsewhere (Dyková et al. 2000, 2005). Trophozoites of isolated strains were observed in hanging drop preparations and documented using an Olympus BX51 equipped with Nomarski optics and DP70 digital camera. For electron microscopy, trophozoites were fixed on the surface of agar plate with cacodylate-buffered 3% glutaraldehyde, pelleted by centrifugation, postfixed with 1% osmium tetroxide, and embedded in Spurr resin after dehydration with acetone. Transmission electron microscope observations of ultrathin sections were accomplished with a JEOL JEM 1010 electron microscope operating at 80 kV. Images were collected with Megaview II soft imaging system using analySIS software.

DNA extraction, amplification and sequencing. Genomic DNA was extracted from pelleted trophozoites on culture day 4 or 5 using the JETQUICK Tissue DNA Spin Kit (Genomed) according to the manufacturer's protocol. The PCR amplification of SSU rRNA genes of *Neoparamoeba* strains and *Perkinsela amoebae*-like organisms was performed as described previously (Fiala & Dyková 2003, Dyková et al. 2008). The amplified products were gel-purified and cloned into pDrive Cloning Vector using the QIAGEN PCR Cloning Kit (Qiagen). Sequencing was carried out on an automatic sequencer ABI 3130×1 using the ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Phylogenetic analyses. A total of 8 SSU rDNA sequences generated for this study and another 39 selected sequences of *Neoparamoeba* spp., *Paramoeba eilhardi*, and 8 outgroups were used to create an alignment by ClustalX version 2.0.6 (Larkin et al. 2007). The alignment comprising all these sequences was then manually checked in BioEdit (Hall 1999) for ambiguously aligned positions, which were deleted. The final alignment contained 1932 positions. The program

RAxML version 7.0.3 (Stamatakis 2006) was used to compute the maximum likelihood (ML) tree with the general time reversible (GTR) model with gamma-distributed rare heterogeneity and rapid bootstrapping (1000 replicates). Maximum parsimony (MP) trees and trees computed with the Fitch-Margoliash method with LogDet distances (LD) were computed in the program PAUP* version 4.0b10 (Swofford 2003). In both cases, heuristic searches were conducted with 1000 independent searches starting from trees constructed by random taxa addition. For tree arrangements the tree bisection and reconnection (TBR) algorithm was used. To check the robustness of the resulting MP and LD trees, 1000 bootstrap replicates were analyzed (with only 10 random starting trees). Bayesian analysis (BA) was performed with MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003). Four simultaneous Markov chains Monte Carlo were run for 10^6 generations with sampling frequency 100 generations and burnin 2500 (after checking for the plateau of –lnL by plotting it vs. generation).

To calculate % identities of *Neoparamoeba* SSU rDNA sequences, another alignment of 42 sequences was prepared in ClustalX. It contained all *Neoparamoeba* SSU rDNA sequences used for phylogenetic analyses, with the exception of EU331036, AY714364, AY686575, EF474478, and EF474480, which were shorter than the rest. The alignment was trimmed to 2135 bp, and the sequence identity matrix was computed in BioEdit.

RESULTS

Light microscopy and ultrastructure of isolated amoebae

Trophozoites of amoeba strains were isolated exclusively from moribund long-spined sea urchins. Although a total of 109 sea urchins were subjected to isolation attempts, we failed to isolate a strain of *Paramoeba* sensu Page 1987. Trophozoites of all strains exhibited the same light microscopical features regard-

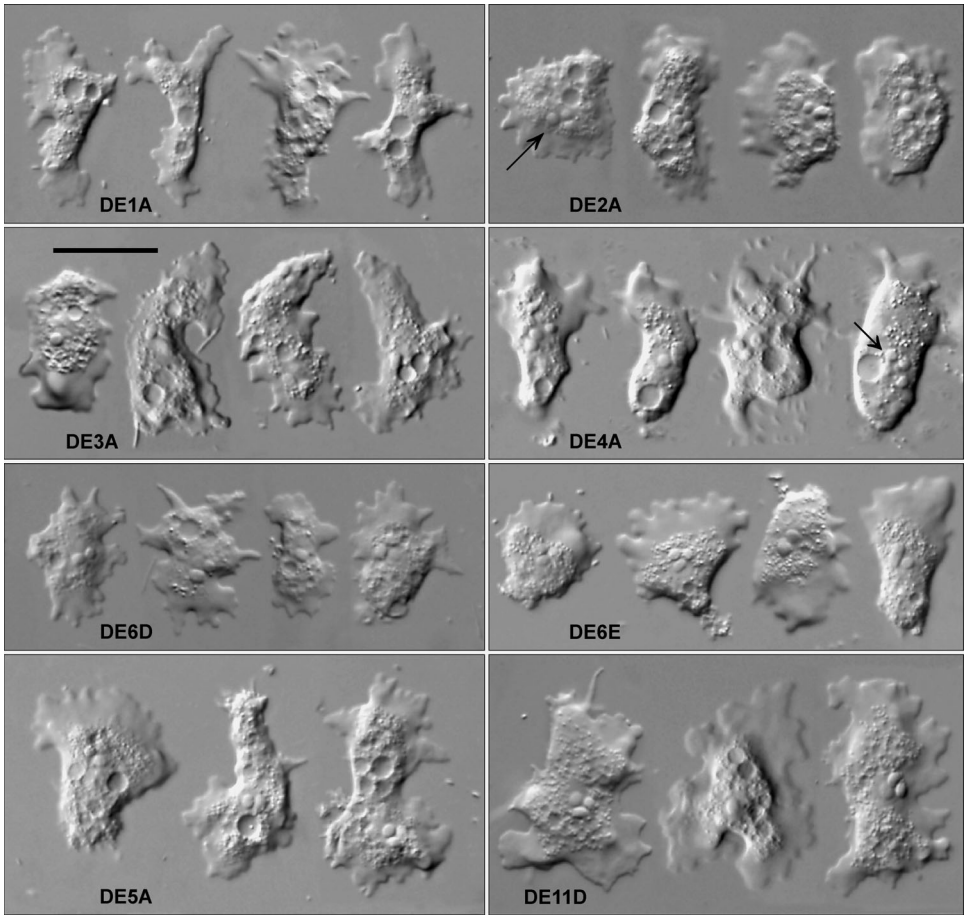


Fig. 1. *Neoparamoeba branchiphila* from *Diadema* aff. *antillarum*. Trophozoite representatives of *N. branchiphila* strains isolated from sea urchins *D.* aff. *antillarum* as seen in Nomarski differential interference contrast microscopy. Images are marked with strain codes also used in Fig. 7. Arrow marks 3 *Perkinsela amoebae*-like symbionts. Scale bar (for all panels) = 20 µm

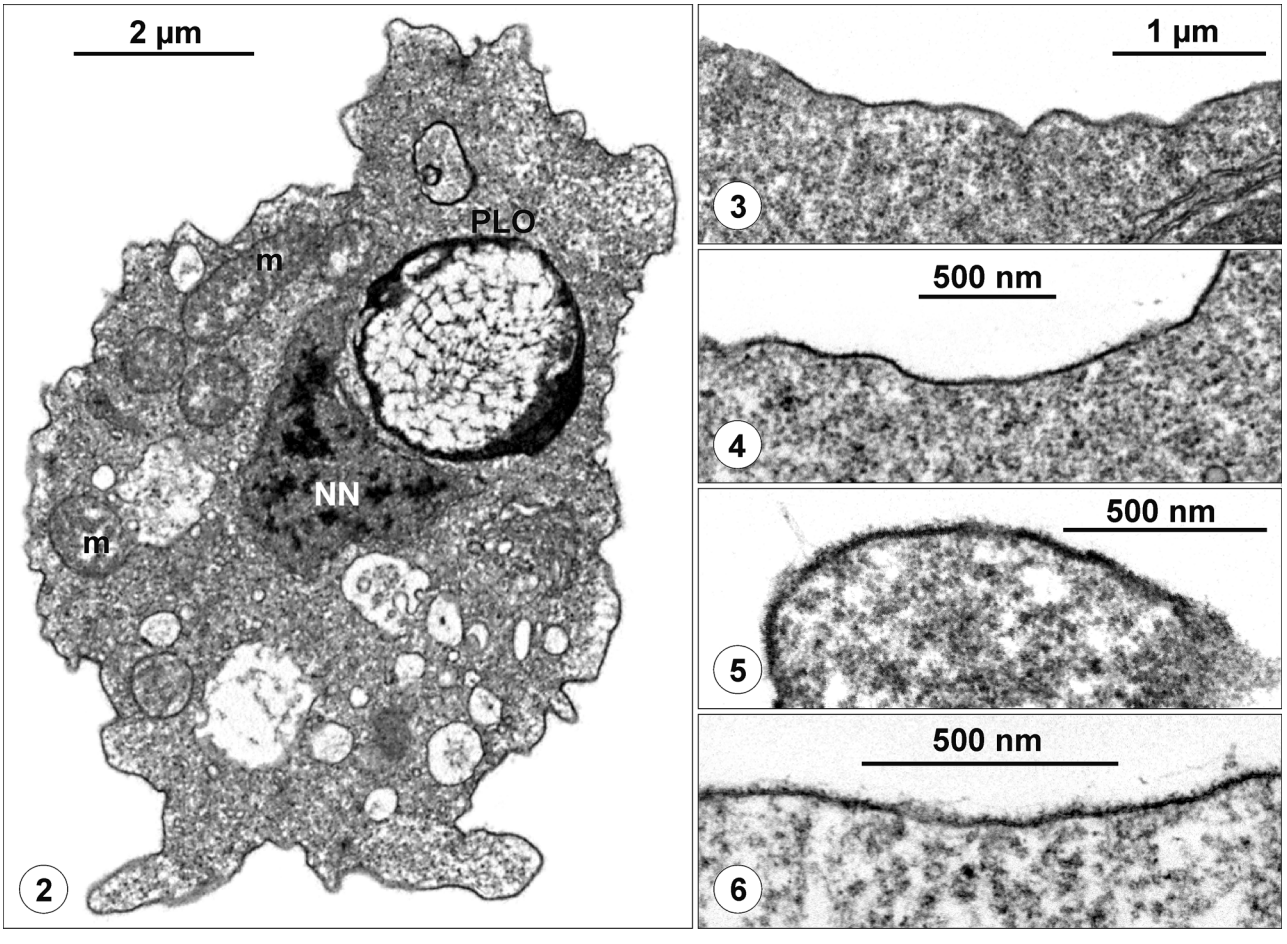
less of local origin of sea urchins (Fig. 1). They were flattened with a narrow zone of hyaloplasm and short blunt projections extending from it. One to three pronounced bodies (endosymbionts) joined the nucleus located in vacuolated granuloplasm. When measured in the same stage of subculturing, the average length of trophozoites reached 30 µm in all strains under study.

The fine structure of trophozoites observed in more than 100 sections fully corresponded to the diagnosis of *Neoparamoeba* given by Page (1987) and it was also absolutely consistent with the fine structure previously described for *Neoparamoeba* strains isolated from fish and invertebrates (Dyková et al. 2007). The overview of fine structure of the cell surface discriminating these newly isolated *Neoparamoeba* strains from *Paramoeba*, is presented in Figs. 2 to 6. Also comparisons of the ultrastructure of the kinetoplastid endosymbionts showed them to be identical to *Perkinsela amoebae*-like organisms (PLOs) as described in Hollande (1980) and Dyková et al. (2008).

Molecular characterization of isolated strains

The lengths of the 8 new SSU rDNA sequences of *Neoparamoeba* strains were 2008 to 2090 bp, with the exception of DE11D strain (2101 bp). These sequences as well as sequences of endosymbionts, i.e. PLOs of 2 *Neoparamoeba* strains, are deposited in GenBank database under accession numbers HQ132923–HQ132930 and HQ132931–HQ132932, respectively.

The 2 PLO SSU rDNA sequences isolated from DE11D and DE4A *Neoparamoeba* strains were blasted against the nucleotide collection of NCBI (http://www.ncbi.nlm.nih.gov/). The former sequence was most similar to SSU rDNA of PLO isolated from SM68 (EU331011; 99% identity, Expect value = 0.0), the latter to SSU rDNA of PLO isolated from AFSM3 strain (AY163355; 98% identity, Expect value = 0.0). Next best hits were, in both cases, SSU rDNAs of PLOs isolated from other strains belonging to the respective subclade (see Fig. 7) of *N. branchiphila*. The results are



Figs. 2 to 6. *Neoparamoeba branchiphila* and *Diadema* aff. *antillarum*. Trophozoites of *N. branchiphila* isolated from sea urchins *D.* aff. *antillarum* and details of their ultrastructure. Transmission electron microscopy. Fig. 2. Trophozoite of *N. branchiphila* with nucleus (NN), mitochondria (m), and *Perkinsela amoebae*-like symbiont (PLO) in close vicinity of the nucleus. Bipolarly symmetrical PLO was sectioned at the level of its kinetoplastid DNA network. Figs. 3 to 6. Examples of trophozoite cell surface with amorphous glycocalyx

thus in agreement with hypothesis of coevolution of amoebae and their symbionts (Dyková et al. 2008).

Phylogenetic analyses

Phylogenetic analyses assigned all strains isolated from coelomic fluid of moribund *Diadema* aff. *antillarum* to *Neoparamoeba branchiphila* (Fig. 7). The result was well supported by all 4 methods applied. The SSU rDNA sequences of *N. branchiphila* divided into 2 well-supported clusters. Seven of our new sequences grouped in the same cluster, within the strains of *N. branchiphila*; only strain DE11D was grouped in the other cluster. Phylogenetic affinities of the 2 PLO sequences (isolated from DE11D and DE4A) revealed by BLAST have clearly shown that these 2 PLOs are most closely related to PLOs isolated from other *Neoparamoeba* strains of the same *N. branchiphila* lineage.

Two notable features of the topology are: (1) the position of *Paramoeba eilhardi* well within *Neoparamoeba* as a sister taxon to *N. perurans* and (2) the possibility that *N. pemaquidensis* is paraphyletic (*N. aestuarina* being its ingroup). The latter result, namely the sister position of *N. aestuarina* and *N. pemaquidensis* AVG8194 strain, has rather high support from ML (88% bootstrap) and BA (posterior probability = 1), but LD strongly supports both species as monophyletic (more than 99% bootstrap in both cases). In any case, the 2 species are closely related, as further suggested by high % identities of their SSU rDNA sequences. Average sequence identities among and within *Neoparamoeba* species are given in Table 1.

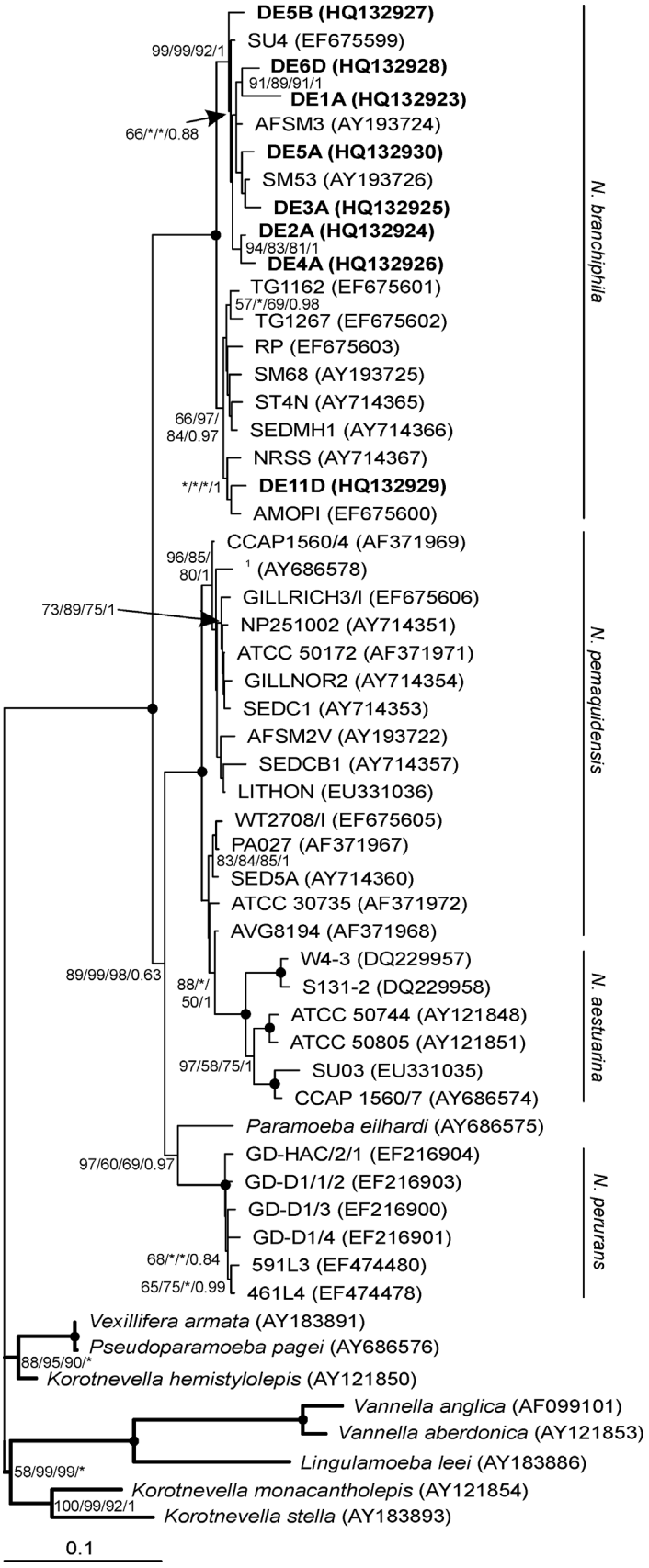


Fig. 7. Maximum likelihood tree based on SSU rDNA sequences of 47 *Neoparamoeba* + *Paramoeba* strains and 8 amoebozoa outgroups. Our new sequences of *Neoparamoeba branchiphila* are shown in **bold**. Bootstrap values for maximum likelihodd, LogDet distances, and maximum parsimony and Bayesian posterior probabilities are given at the nodes, respectively. Asterisks indicate bootstraps lower than 50%; nodes with black dots obtained bootstrap support of 99% or higher from all tree methods, and their posterior probability was 1.00; superscript 1 indicates amoeba infecting western Long Island Sound lobsters. Values for nodes that scored all bootstrap values lower that 60% and posterior probability <1.00 are not shown. Note that all branches leading to outgroups were shortened to one-third of their original length (these branches are in bold)

Table 1. Average sequence identities among and within *Neoparamoeba* species

	N. pemaquidensis	*N. aestuarina*	*N. perurans*	*N. branchiphila*
N. pemaquidensis	97.8	94.2	90.3	88.9
N. aestuarina	–	95.9	88.6	88.0
N. perurans	–	–	96.6	87.5
N. branchiphila	–	–	–	96.5

DISCUSSION

Trophozoites of *Neoparamoeba* strains isolated from *Diadema* aff. *antillarum* do not differ in their morphology from environmental or fish- and invertebrate-isolated *Neoparamoeba* strains (Dyková et al. 2003, 2005, 2007). Also, their fine structure is absolutely consistent with that previously described for *Neoparamoeba* strains mentioned in 'Results' above. The results of phylogenetic analysis of SSU rDNA that identified the studied sea urchin strains of *N. branchiphila* are supported by the molecular proof of a close relationship between kinetoplastid endosymbionts of DE11D and DE4A strains and *Perkinsela amoebae* (Hollande, 1980) known from other strains of *N. branchiphila* lineage (Dyková et al. 2008)

The results of the present study and our previous experience based on isolation of over 40 *Neoparamoeba* strains of different origin (Dyková et al. 2005, 2007, 2008) and about 60 strains of other marine free-living amoebae suggest that *Paramoeba* spp. are not as frequent as *Neoparamoeba* spp., nor are they as easy to isolate.

The present isolation of *Neoparamoeba branchiphila* strains exclusively from coelomic fluid of moribund *Diadema* aff. *antillarum* suggests that the amoebae either caused or contributed to the observed disease condition. Similarly, Jones (1985), who sampled radial nerves (with associated water vascular radial canals) in green sea urchins *Strongylocentrotus droebachiensis*, recovered amoebae only from individuals showing signs of disease. Also, experimental infections by injection of amoebae and by exposure to water containing diseased individuals caused 'paramoebiasis' of echinoids (Jones & Scheibling 1985, Jellet et al. 1988). Jellet et al. (1988) excluded the effect of toxic products of amoebae by injection of membrane-filtered coelomic fluid from moribund, infected individuals to healthy ones; however, pathogenesis of this echinoid 'paramoebiasis' remained obscure: nothing but the activation of autolytic changes and production of degradative enzymes was mentioned as the possible mechanism of this disease.

Almost simultaneously with the above-mentioned early research of amoebic aetiology of sea urchin mortalities, some studies focused on the cause of necrotic lesions on the sea urchin tests (Maes & Jangoux 1984) or on the diversity and pathogenicity of microorganisms isolated from the affected areas of tests (Gilles & Pearse 1986). In light of the fact that necrotic lesions of sea urchins reported during mortality episodes were occasionally also observed on individuals within 'healthy' populations (Pearse et al. 1977, Maes & Jangoux 1984, Scheibling 1984), they can be considered as primary lesions that form an entry gate for amoeba infection.

The study by Gilles & Pearse (1986) clearly showed that the presence of a mixture of bacteria in water environment makes it extremely difficult to determine the proper agent of lesions. Lesions similar to those described from naturally infected sea urchins were induced experimentally by *Vibrio anguillarum* and *Aeromonas salmonicida* (Gilles & Pearse 1986).

The current effort to establish farming centers for breeding and production of edible sea urchins (Tajima et al. 2007) revives the issue of sea urchin diseases in wild populations. Damage to wild populations may result in the loss of a resource, decreased biodiversity, and a shift in the ecological balance, as exemplified by post-hurricane amoebic disease outbreaks in sea urchins in Nova Scotia (Scheibling et al. 2010).

The lack of consistent comparable data on pathogenesis of sea urchin disease in previous outbreaks, the nature of data from the present study, and the fact that *Neoparamoeba branchiphila* has previously been isolated from 2 species of healthy sea urchins (*Paracentrotus lividus* and *Heliocidaris erythrogramma*; see Dyková et al. 2007) currently make a final conclusion on what was the primary agent of *Diadema* aff. *antillarum* mortality impossible. In our opinion, the future study of sea urchin disease manifested by the loss of spines, exfoliation of epidermis, and lesions on the test should consider a possible synergy of both free-living agents, bacteria, and amoebae.

Acknowledgements. The authors are grateful to Dr. J. C. Hernández, Dr. S. Clemente, and Dr. A. Brito from the Grupo de investigación en Biodiversidad, Ecología Marina y Conservación (BIOECOMAC), Department of Animal Biology (Marine Sciences), Faculty of Biology, University of La Laguna, for assistance in the collection of specimens and for allowing us the use of their aquarium facilities. J.L.M. was funded by a postdoctoral grant from the Fundación Canaria Dr. Manuel Morales, La Palma, Canary Islands, Spain. Financial support was provided by the Grant Agency of the Czech Republic (524/09/0137), Ministry of Education, Youth and Sports of the Czech Republic (MSM 6007665801) and research projects of the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic (Z60220518 and LC522).

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Editorial responsibility: Dieter Steinhagen, Hannover, Germany

Submitted: January 6, 2011; Accepted: March 17, 2011
Proofs received from author(s): June 9, 2011