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Phylogeny of *Neoparamoeba* strains isolated from marine fish and invertebrates as inferred from SSU rDNA sequences

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ABSTRACT: We characterised 9 strains selected from primary isolates referable to *Paramoeba*/*Neoparamoeba* spp. Based on ultrastructural study, 5 strains isolated from fish (amoebic gill disease [AGD]-affected Atlantic salmon and dead southern bluefin tuna), 1 strain from netting of a floating sea cage and 3 strains isolated from invertebrates (sea urchins and crab) were assigned to the genus *Neoparamoeba* Page, 1987. Phylogenetic analyses based on SSU rDNA sequences revealed affiliations of newly introduced and previously analysed *Neoparamoeba* strains. Three strains from the invertebrates and 2 out of 3 strains from gills of southern bluefin tunas were members of the *N. branchiphila* clade, while the remaining, fish-isolated strains, as well as the fish cage strain, clustered within the clade of *N. pemaquidensis*. These findings and previous reports point to the possibility that *N. pemaquidensis* and *N. branchiphila* can affect both fish and invertebrates. A new potential fish host, southern bluefin tuna, was included in the list of farmed fish endangered by *N. branchiphila*. The sequence of *P. eilhardi* (Culture Collection of Algae and Protozoa [CCAP] strain 1560/2) appeared in all analyses among sequences of strain representatives of *Neoparamoeba* species, in a position well supported by bootstrap value, Bremer index and Bayesian posterior probability. Our research shows that isolation of additional strains from invertebrates and further analyses of relations between molecular data and morphological characters of the genera *Paramoeba* and *Neoparamoeba* are required. This complexity needs to be considered when attempting to define molecular markers for identification of *Paramoeba*/*Neoparamoeba* species in tissues of fish and invertebrates.

KEY WORDS: *Neoparamoeba* strains · *Paramoeba eilhardi* · Phylogeny · Invertebrate infections

INTRODUCTION

Over the past 20 yr, new data on amoebic gill disease (AGD) in farmed marine fish have become available at an increasing rate. Three important steps have been made to date in investigations on the aetiology of this disease: (1) The causative agent of gill infections of coho salmon *Oncorhynchus kisutch* (Walbaum, 1792) reared in seawater was diagnosed as *Paramoeba pemaquidensis* Page, 1970 by Kent et al. (1988) and the same agent of AGD was reported in turbot *Scophthalmus maximus* (L.) and Atlantic salmon *Salmo salar* (L.) (Roubal et al.1989, Munday et al. 1990, Dyková et al. 1998). (2) Amoeba strains isolated from gills of different fish hosts and referred to originally as *Paramoeba* species have been assigned to *Neoparamoeba* Page, 1987 (Dyková et al. 2000); attention has focused on ultrastructural features used by Page (1987) to discriminate *Neoparamoeba* from *Paramoeba*; the value of morphometric data for species diagnosis within the

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genus *Neoparamoeba* is considered questionable (Dyková et al. 2000, 2005b). (3) Due to size differences and a great diversity of shape within clonal cell populations of *Neoparamoeba* strains, comparison of strains has concentrated on their molecular characteristics. Taxonomic relatedness of *Neoparamoeba* strains has been inferred from phylogenetic analyses of SSU rDNA sequences, and branching pattern has been explored for the establishment of a new *Neoparamoeba* species (Fiala & Dyková 2003, Dyková et al. 2005b)

When first conclusions on the aetiology of AGD were reported by Kent et al. (1988), species of the genus *Paramoeba* Schaudinn, 1896 were already known as causative agents of invertebrate mortalities. More than 70 yr after the description of the type species of the genus *Paramoeba* Schaudinn, 1896 (*P. eilhardi* Schaudinn 1896), which was isolated from the water of a marine aquarium (information taken from Chatton 1953), another species, *P. perniciosa* Sprague, Beckett & Sawyer, 1969 was identified. It repeatedly caused mortalities of blue crabs *Callinectes sapidus* along the coast of North Carolina (USA), and was also identified as a parasite of the crustaceans *Cancer irroratus* and *Homarus americanus* (Sawyer 1976, Johnson 1977). *P. invadens* Jones, 1985, recovered from the green sea urchin *Strongylocentrotus droebachiensis*, inflicted massive kills along the Atlantic coast of Nova Scotia, Canada through the years 1980 to 1983 (Jones 1985).

Paramoeba and *Neoparamoeba* species, together with *Janickina pigmentifera* (Grassi, 1881) and *J. chaetognathi* (Grassi, 1881) (Chatton 1953), which were described as parasites of *Spadella* spp. (Chaetognatha), form a group of amoebae of extraordinary importance, because of their potential pathogenicity for marine fish and invertebrates. Page included them in the category of parasitic marine gymnamoebae (Page 1983).

The pathogenicity of *Paramoeba* and *Neoparamoeba* spp., the epizootic nature of infections and impacts of mass mortalities on the fishing economy and environment are strong reasons for research commitment. Moreover, 6 species of *Paramoeba*, *Neoparamoeba* and *Janickina* have in common another important phenomenon: they live in permanent association with eukaryotic symbionts located in their cytoplasms (Grell & Benwitz 1970, Page 1970, Perkins & Castagna 1971, Hollande 1980, Dyková et al. 2000, 2003). This in itself provides a strong motivation for a meticulous comparison of strain representatives of this group of naked amoebae and also of their eukaryotic endosymbionts.

Because a large number of strains have been isolated from AGD, considerably more information is available on agents of this disease than on agents of amoebic infections of invertebrates. Unfortunately, the epizootic agents found in crustaceans and echinoids (Sprague et al. 1969, Johnson 1977, Jones 1985) were not preserved in culture collections, and agents of more recent mortalities of American lobsters were not isolated (Mullen et al. 2004, 2005). The first and only SSU rDNA sequence of a strain representative of the genus *Paramoeba* (*P. eilhardi*, Culture Collection of Algae and Protozoa [CCAP] strain 1560/2) was deposited in GenBank in 2004. A sudden surge of interest in researching the amoebic diseases of invertebrates which was triggered by outbreaks of disease in American lobsters (Mullen et al. 2004, 2005) brought new insights into the etiology of amoebic diseases in invertebrates, and at the same time, opened a broad field for complementary investigations.

The present study started as an attempt to isolate *Paramoeba* strains from invertebrates and obtain information about their relationships with *Neoparamoeba* strains characterised previously. Data we have acquired on new strains from invertebrates and fish, supplemented by those retrieved from the GenBank database, are used to present phylogenetic relationships inferred from SSU rDNA sequences.

MATERIALS AND METHODS

Nine newly isolated strains were included in the study; 5 of them were isolated from fish, 1 from netting of floating sea cages in Atlantic salmon farms, and 3 from invertebrates. The designations and origins of strains are specified in Table 1. All successful isolations from southern bluefin tuna were from dead fish recovered by divers. The isolation success rate was 33.3% (isolates from 3 of 9 dead specimens sampled between summer 2004 and winter 2005). No amoebae were isolated from gills of harvested tuna (4 attempts).

The handling of primary isolates, culturing and harvesting for ultrastructural and molecular studies followed procedures described for fish-isolated strains by Dyková et al. (2000) and routinely used in our previous study (Dyková et al. 2005b). All strains were subcultured weekly using non-seeded malt and yeast extract in 75% seawater agar (MY75S, UK National Culture Collection [UKNCC] 2001 catalogue).

For morphological and molecular studies, purified cell populations were used, either derived from one cell, or, when classical clonal procedures failed, from several cells of presumably common origin (growing as a separate group of closely adjacent cells on the agar surface). Light and electron microscopical methods used on newly isolated strains were those of Dyková et al. (2005b).

DNA extraction, amplification and sequencing were performed according to the protocol of Fiala & Dyková (2003), also used in our latest report on *Neoparamoeba* species (Dyková et al. 2005b). The set of SSU rDNA se-

Table 1. Amoeba strains included in the present study

Host species Amoeba strain/clone	Origin
Thunnus maccoyii, dead (gills)	
TUN1/I	Port Lincoln, Australia
TG1162	Port Lincoln, Australia
TG1267	Port Lincoln, Australia
Salmo salar (gills)	
GILLRICH3/I	Tasmania, Australia
WT2708/I	Tasmania, Australia
Salmo salar (net material of sea cages)	
NET12AFL/I	Tasmania, Australia
Paracentrotus lividus	
AMOPI	Cretan Sea, Kárpathos Island, Greece
Heliocidaris erythrogramma	
SU4	Tamar River, Georgetown, Tasmania, Australia
Callinectes sapidus	
RP	Gulf of Mexico, MS, USA

quences aligned the present study included the whole data set in Dyková et al. (2005b), supplemented with 9 sequences obtained from our study, and another 4 retrieved from the GenBank database. The latter included the first sequence for the reference strain of *Paramoeba eilhardi* (CCAP 1560/2), the duplicated sequence of *N. aestuarina* strain CCAP 1560/7, and 2 sequences from amoebae isolated from lobsters from Long Island Sound, USA, along with those of *Korotnevella* and *Vexillifera* as representatives of paramoebid and vexilliferid (PV) lineages of Gymnamoebae (Peglar et al. 2003), respectively. *Vannella anglica* and *V. aberdonica* represented an outgroup. In total, 52 sequences were aligned in the Clustal_X program (Thompson et al. 1997) with a gap opening/gap extension penalty of 8/2. Corrections were done by eye using the BioEdit sequence alignment editor (Hall 1999). The alignment is available from the authors upon request. Phylogenetic analyses were performed using the maximum parsimony (MP) and maximum likelihood (ML) methods. In addition, Bayesian inference of phylogeny (BI) was applied. MP and ML procedures were carried out with the PAUP* package, version 4.0b10 (Swofford 2001). For BI procedures, the MrBayes program v. 3.0 (Ronquist & Huelsenbeck 2003) was used. Models of nucleotide substitution were evaluated using MrModeltest v. 2.2 (Nylander et al. 2004). The model GTR+I+Γ was chosen. Posterior probabilities were computed using the Markov Chain Monte Carlo method with 500 000 generations. The length of burn-in period was 100 000 generations. MP analysis was done using a heuristic search with random addition of taxa (10 replications) and the ACCTRAN-option. Gaps were treated as missing data. Transition/transversion (Ts/Tv) ratios were 1:1, 1:2 and 1:3. In addition to clade support assessed with bootstrapping of 1000 replicates, Bremer decay indices were established. For the ML analysis, the likelihood ratio test (LRT) implemented in Modeltest v. 3.06 (Posada & Crandall 1998) was used to determine the best model of evolution. Based on the LRT, the ML was performed with the GTR+I+Γ model of evolution. The estimated α-parameter was 0.4523, the number of substitution types was 6 and the proportion of invariable sites was 0.2934. The best tree was determined using Tree Bisection-Reconnection (TBR) rearrangements. The bootstrap analysis (500 replicates) was done using the Seqboot in PHYLIP, v. 3.6a3 (Felsenstein 2002) and the PHYML program (Guindon & Gascuel 2003).

Newly introduced strains, as well as those from our previous study, are cryopreserved and stored in liquid nitrogen in the culture collection of the Institute of Parasitology (Biology Centre of the Academy of Sciences of the Czech Republic).

RESULTS

Morphology and fine structure of trophozoites

Light microscopical observation of 16 primary isolates growing on the surface of agar qualified suitable groups of cells for subculturing and detailed study. As a result of subculturing, with attention focused on step-by-step purification of cell populations, 9 strains (listed in Table 1) having characteristics of the order Dactylopodida (Smirnov et al. 2005) and family Paramoebidae (Poche, 1913) Page, 1987 were selected (Fig. 1). Each strain in Fig. 1 is represented by the most typical trophozoites seen in hanging drop preparations using Nomarski DIC (differential interference contrast) light microscopy. They were selected from a series of images taken during the long-term culturing. Trophozoites of individual strains differed in size only; their morphotypes were identical. All of them contained endosymbionts, occasionally with more than 2 per host cell. Using descriptions given by Page (1987) and previous ultrastructural studies (Dyková et al. 2000, 2005b), our transmission electron microscopic analyses assigned all strains listed in Table 1 to the genus *Neoparamoeba* Page, 1987. Basic ultrastructural characteristics common to these strains are shown in Figs. 2 to 8 (all specimens isolated from invertebrates).

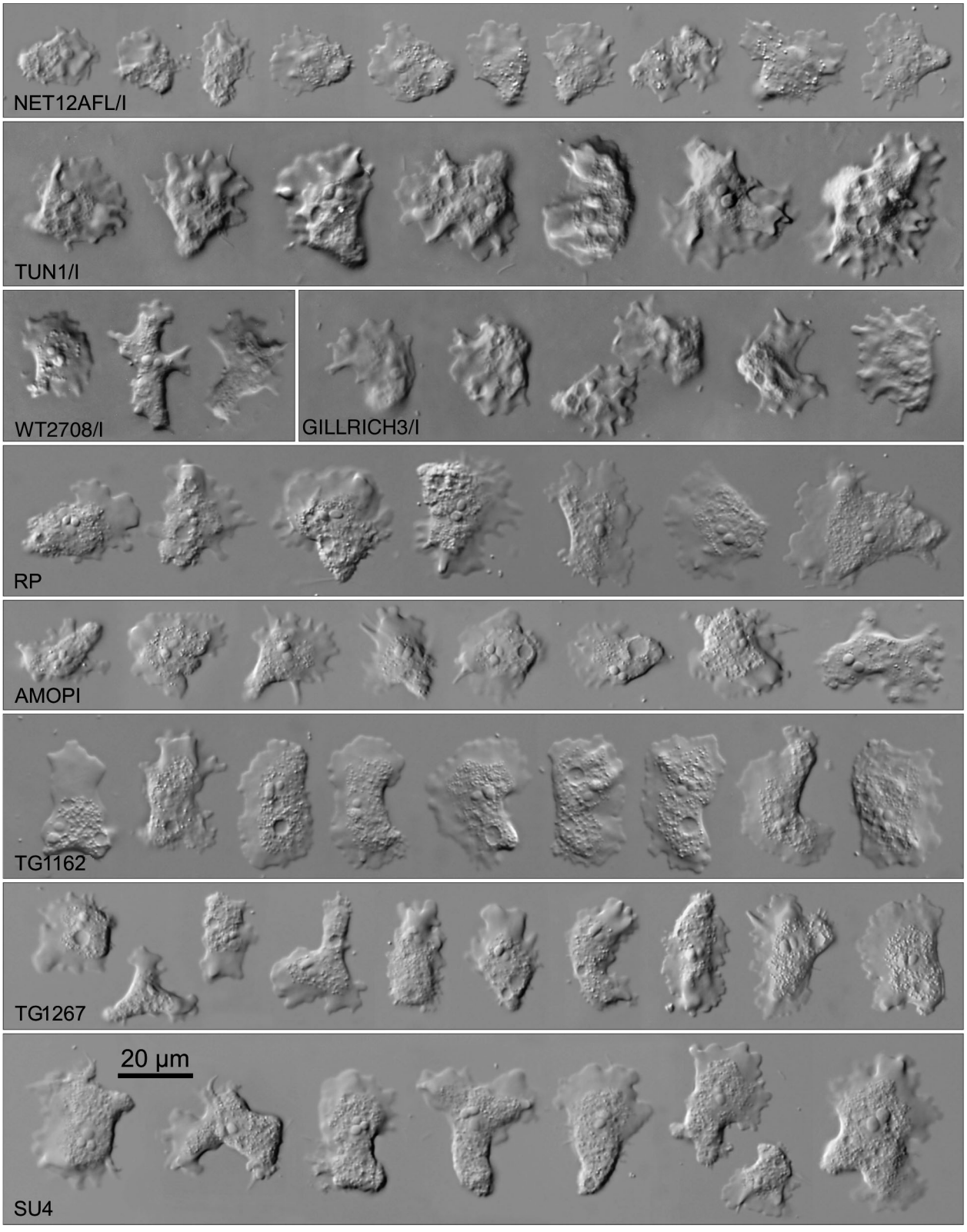
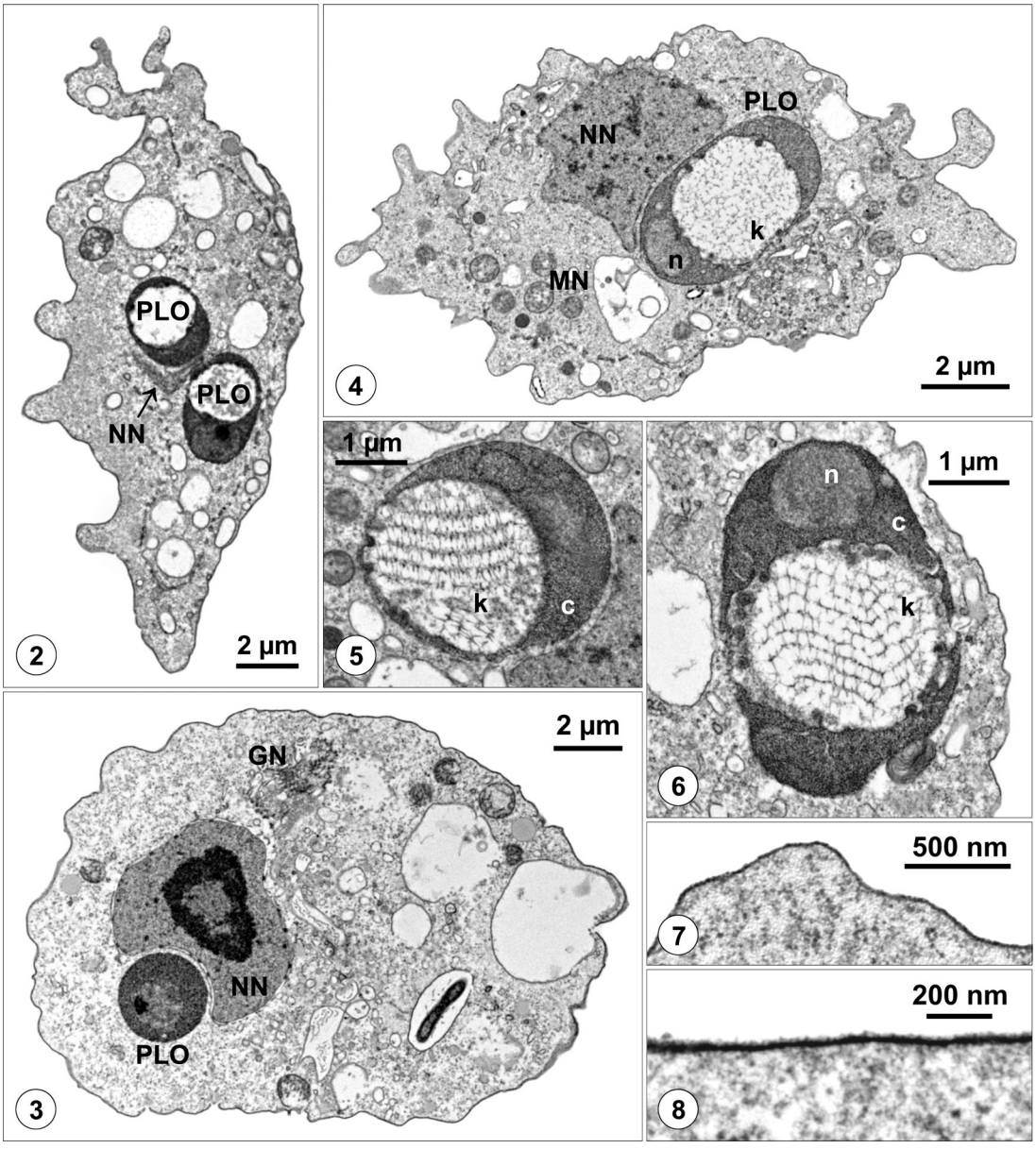


Fig. 1. Trophozoites representative of newly characterised *Neoparamoeba* strains. Images are marked with codes of strains (see Table 1); codes are used in phylogenetic trees. Scale bar applies to all strains shown. Nomarksi differential interference contrast microscopy



Figs. 2 to 8. Trophozoites representative of *Neoparamoeba* strains isolated from invertebrate hosts and details of their ultrastructure; transmission electron microscopy; GN = Golgi apparatus, MN = mitochondria, NN = *Neoparamoeba* nucleus, PLO = *Perkinsiella amoebae*-like endosymbiont, c = cytoplasm of PLO, k = kinetoplast of PLO, n = nucleus of PLO. Fig. 2 & Fig. 3. Trophozoites of RP strain isolated from *Callinectes sapidus*; PLO sections in Figs. 2 & 3 were cut at different levels. Fig. 4. Trophozoite of AMOPI strain isolated from *Paracentrotus lividus*; PLO with bipolar symmetry is located alongside host nucleus. Fig. 5 & Fig. 6. PLOs with typical arrangement of kDNA network. Fig. 7. Trophozoite cell surface of RP strain. Fig. 8. Amorphous glycocalyx of SU4 strain trophozoite isolated from *Centrostephanus rodgersii*

Molecular phylogeny and taxonomy based on SSU rDNA sequences

Phylogenetic relationships of strains inferred from sequence-based analyses are presented in the MP tree supplemented with nodal support values and Bremer decay indices (Fig. 9). The branching pattern of the tree clearly defines 3 clades, those that are known from previous analyses of sequences from fish-isolated and environmental *Neoparamoeba* strains (Dyková et al. 2005b). Three strains that we isolated from invertebrates (2 from sea urchins and 1 from crab) collected in localities far from one another (Greece, Tasmania, Australia and the Gulf Coast, USA, respectively) clus-

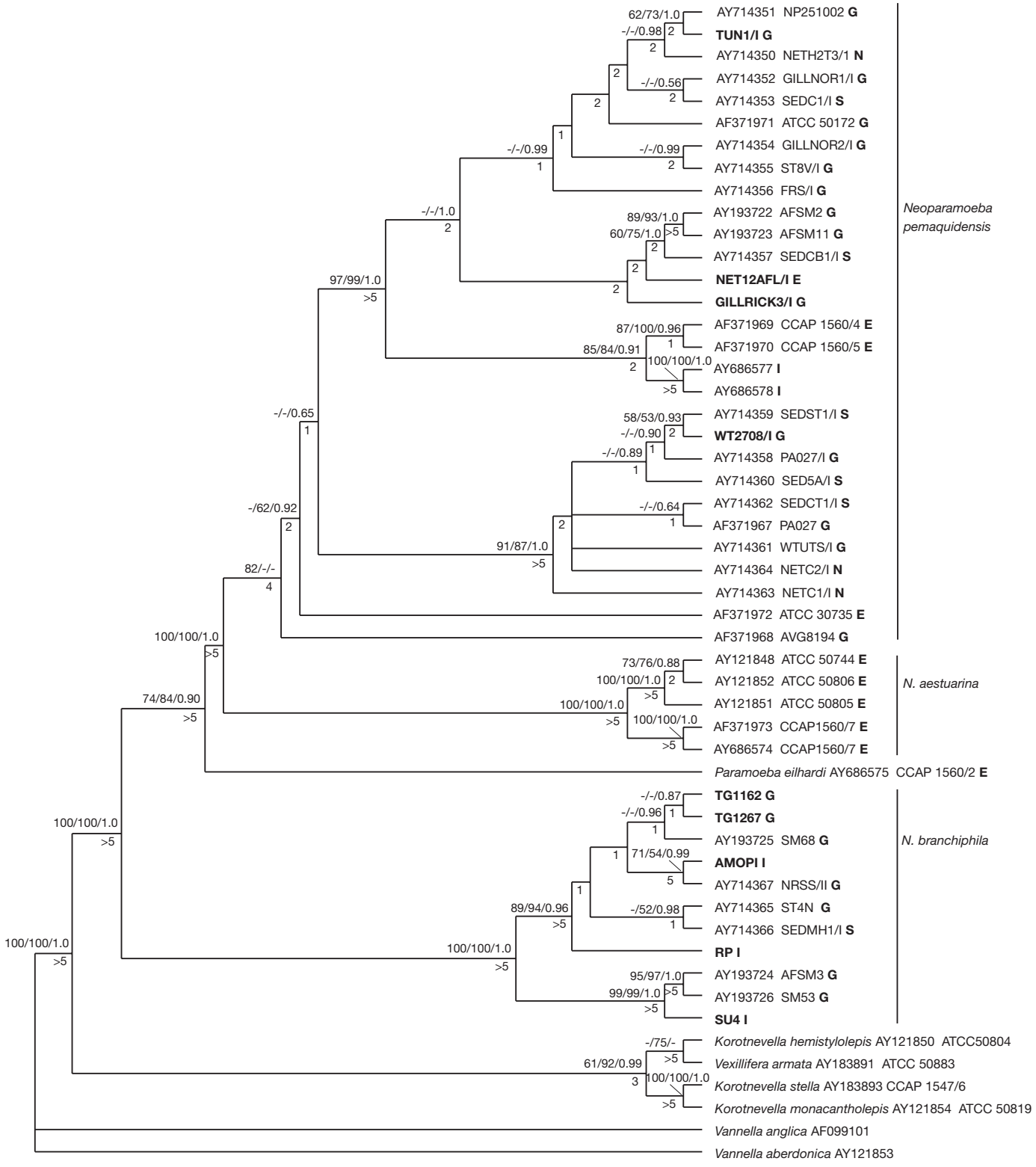


Fig. 9. Maximum parsimony (MP) tree of the SSU rDNA sequences; strict consensus of 2 most parsimonious trees (transition/transversion [Ts:Tv] = 1:2, 3288 steps, consistency index [CI] = 0.79, retention index [RI] = 0.64). Values above the lines indicate nodal support (MP with Ts:Tv = 1:2/maximum likelihood [ML]/Bayesian inference [BI]); Bremer decay indices are given below the lines. Data retrieved from the GenBank database are presented as accession numbers of sequences, followed by codes of strains, while the origin of each newly acquired sequence is given as bold-faced code of strain. Abbreviations used for origin of strains are as follows: G = gills, S = sediments, N = net material, E = environmental origin and I = invertebrates

ter together with 2 of 3 strains isolated from gills of southern bluefin tunas (Australia) within the clade of *N. branchiphila*. The clade of *N. pemaquidensis* is enlarged by the inclusion of 4 strains we have introduced, i.e. 1 strain isolated from gills of southern bluefin tuna, 2 from gills of Atlantic salmon, and 1 from netting of a floating Atlantic salmon farm cage. The clade of *N. aestuarina* is enlarged by the inclusion of the new sequence for the CCAP 1560/7 strain.

Our work does not change the existing concept of potential candidates for agents of AGD. We identified specimens belonging to *Neoparamoeba pemaquidensis* and *N. branchiphila* among 5 new strains isolated from fish. Mullen et al. (2005) identified sequences generated from lobster amoebae which corresponded to *N. pemaquidensis* (closely related to sequences of reference strains CCAP 1560/4 and CCAP 1560/5), and in our study *N. branchiphila* was identified as a possible agent of infections in invertebrates.

The position of the strain representative of *Paramoeba eilhardi* (CCAP 1560/2) among the clades of *Neoparamoeba* was surprising, but it was stable in all analyses performed and well supported by the bootstrap value, Bremer index and Bayesian posterior probability. The only tree position that differed depending on the method of phylogenetic analysis was that of strain AVG8194 (AF371968). Contrary to results of all MP analyses (Ts/Tv 1:1, 1:2 and 1:3) and ML and BI computed with models in which gamma distribution is not used to account for rate variation among sites (JC69, F81 or HKY), in ML and BI (both GTR+I+Γ), AVG8194 joined *Neoparamoeba aestuarina* strains.

DISCUSSION

Our study, stimulated by a general interest in the pathogenicity of free-living amoebae in aquatic organisms, by progress made toward an understanding of fish infections, and by the lack of information on agents of amoebic infections of invertebrates, brought new and unexpected information. Our expectation that the set of SSU rDNA sequences of *Neoparamoeba* strains could be extended to include those belonging to morphologically different strains of the genus *Paramoeba* failed. The aim of including the sequence of the *Paramoeba* strain into an extended sequence set for *Neoparamoeba* was restricted to the environmental strain of *P. eilhardi* CCAP 1560/2, also used as unique *Paramoeba* strain in the study of Mullen et al. (2005). However, our analysis of the extended set of *Neoparamoeba* sequences resulting in assignment of our strains from invertebrates into *N. branchiphila*, introduces a second species of the genus that is pathogenic in invertebrates, along with *N. pemaquidensis* (Mullen et al. 2005). Unfortunately, *N. pemaquidensis*, the agent of amoebic disease of American lobster (Mullen et al. 2004, 2005) was not isolated and stored for future studies. Its identification was not simple, as it is based on phylogenetic analyses of sequences of genomic DNA extracted from host tissue samples.

To the best of our knowledge, the number of amoeba strains of interest isolated from invertebrates during the present study is unique. The lack of strains isolated from invertebrates that are stored in culture collections is surprising considering the fact that *Paramobea invadens*, for example, was isolated from radial nerves and water vessels of the green sea urchin *Strongylocentrotus droebachiensis*, then readily cultured on agar plates and used for experimental infections (Jellet et al. 1988). It is possible that some tissue-invading amoebae are more difficult to maintain in agar plate cultures or liquid media than those isolated directly from water. The individual fishes and invertebrates we have attempted to isolate were randomly selected from asymptomatic specimens. Based on our experience with AGD, we consider this sampling procedure reasonable. Jellet et al. (1988) also proved experimentally that trophozoites of *P. invadens* are present in the radial nerves of echinoids well before the onset of visible symptoms of the disease. This shows that even sampling that is not related to outbreaks of amoebic diseases can help us accumulate new data in the field. However, it is possible that free-living *Neoparamoeba* strains colonised southern bluefin tuna after death, as we were not able to culture amoebae from harvested (healthy) fish, and no AGD-like lesions have been seen so far in this fish species (B. Nowak unpubl.). *Neoparamoeba* strains can colonise gills of previously uninfected dead Atlantic salmon (Douglas-Helders 2000), so it is possible that the gills of southern bluefin tuna were colonised after death. Further evidence is needed to confirm whether *Neoparamoeba* spp. are potential pathogens of southern bluefin tuna.

The data set enlarged with sequences of *Neoparamoeba branchiphila* pointed out a strange position of the SSU rDNA sequence of *Paramoeba eilhardi*, i.e. its incorporation into sequences of *Neoparamoeba* strains. The controversial phylogenetic position of *P. eilhardi* can be interpreted in various ways. If more sequences of strains assigned morphologically to *P. eilhardi* were available, and if they clustered together in the same tree position, this would likely be convincing evidence that phylogenetic relationships inferred from SSU rRNA gene sequences do not reflect ultrastructural features discriminating *Paramoeba* from *Neoparamoeba*. This is the case for *Vannella* and *Platyamoeba* species (Dyková et al. 2005a). With only one sequence of a strain representative of *Paramoeba* available, analysis of the organism sequenced should

be done. The CCAP (now known as the UKNCC) acquired this strain in 1960 from a reputable investigator, K. G. Grell, who published several papers on *P. eilhardi* (Grell 1961, Grell & Benwitz 1966, 1970) and documented the ultrastructural details (boat-shaped scales observed on the cell surface) rather well. However, we know of no reports on the deposition of clonal cultures in protist collections, or of recent ultrastructural verification of species determination made before the sequence was prepared in 2004. Our previous attempt to characterise this strain more than 40 yr after K. G. Grell deposited it in the CCAP failed due to an extreme overload of the culture with bacteria. Working previously with several 'strains' obtained from the UKNCC, we found that some of them were actually mixed cultures of 2 to 3 different species. This is an expected consequence of cryopreservation and deposition of non-clonal cultures, and sometimes may result from long-term storage of isolates subcultured for only a short period of time. Since the sequence for *P. eilhardi* is the only one available for the genus *Paramoeba*, there is an urgent need for more information about the reference strain, as well as comparisons with sequences from strains corresponding to *P. invadens* and *P. perniciosa*. It is possible that the strains described as representatives of the latter species were assigned to the genus *Paramoeba* primarily on the basis of the presence of *Perkinsiella amoebae*-like organisms (PLOs) in their cytoplasm.

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