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Molecular characterisation of *Neoparamoeba* strains isolated from gills of *Scophthalmus maximus*

Ivan Fiala[1,2], Iva Dyková[1,2,*]

[1]Institute of Parasitology, Academy of Sciences of the Czech Republic and [2]Faculty of Biological Sciences, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic

ABSTRACT: Small subunit ribosomal RNA gene sequences were determined for 5 amoeba strains of the genus *Neoparamoeba* Page, 1987 that were isolated from gills of *Scophthalmus maximus* (Linnaeus, 1758). Phylogenetic analyses revealed that 2 of 5 morphologically indistinguishable strains clustered with 6 strains identified previously as *N. pemaquidensis* (Page, 1970). Three strains branched as a clade separated from *N. pemaquidenis* and *N. aestuarina* (Page, 1970) clades. Our analyses suggest that these 3 strains could be representatives of an independent species. In a more comprehensive eukaryotic tree, strains belonging to *Neoparamoeba* spp. formed a monophyletic group with a sister-group relationship to *Vannella anglica* Page, 1980. They did not cluster with Gymnamoebae of the families Hartmannellidae, Flabellulidae, Leptomyxidae or Amoebidae presently available in GenBank.

KEY WORDS: *Paramoeba* · *Neoparamoeba* · SSU rDNA · Phylogenetic position

INTRODUCTION

Amoebic gill disease (AGD), repeatedly declared one of the most serious diseases affecting farmed salmonids *Salmo salar* Linnaeus, 1758 and *Oncorhynchus mykiss* (Walbaum, 1792) in the last 2 decades (Kent et al. 1988, Munday et al. 1988, 2001, Roubal et al. 1989, Rodger & McArdle 1996, Findlay & Munday 1998, Clark & Nowak 1999, Douglas-Helders et al. 2001), has also been diagnosed in turbot *Scophthalmus maximus* (Linnaeus, 1758) (Dyková et al. 1998, 2000). Proper identification and systematic classification of the agent and determining its relationship to similar marine amoebae was regarded an important step in the strategy employed to reduce significant stock losses both in salmonids and flatfishes. The small subunit ribosomal ribonucleic acid (SSU rRNA) gene of 6 strains of *Neoparamoeba pemaquidensis* (Page, 1970) and 1 strain of *N. aestuarina* (Page, 1970) has recently been sequenced in order to develop PCR for detection of the causative agent of AGD (Elliott et al. 2001). Sequences of the SSU rRNA gene were made accessible in GenBank in May 2002.

As a first step, aimed at unravelling the biology and taxonomy of the agent of AGD in turbot *Scophthalmus maximus*, comparative light and transmission electron microscopical studies of 6 *Neoparamoeba* strains indicated that 2 morphologically similar species, *N. pemaquidensis* and *N. aestuarina* have to be considered as agents of AGD in turbot (Dyková et al. 2000).

This communication is intended to describe the molecular characteristics of morphologically identified *Neoparamoeba* Page, 1987 strains isolated from gills of *Scophthalmus maximus* to make possible comparison with isolates from other fish and invertebrate hosts.

MATERIALS AND METHODS

Five *Neoparamoeba* strains (AFSM2V, AFSM3, AFSM11, SM53 and SM68) isolated from the gills of the turbots *Scophthalmus maximus* farmed in NW Spain

*Corresponding author. Email: iva@paru.cas.cz

were used in this study: 2 strains (SM53, SM68), isolated in 1998, displayed the morphological characteristics described by Dyková et al. (2000); the other 3 strains (AFSM2V, AFSM3, AFSM11) were isolated in 2001. Handling of primary isolates, culture purification, clonal procedures and harvesting of trophozoites followed the methods described by Dyková et al. (2000). The assignment of the strains to the genus *Neoparamoeba* as defined by Page (1987) was undertaken on fresh mounts (hanging drop preparations) and thin sections prepared for transmission electron microscopy.

Total DNA from samples containing a small amount of cells was extracted using the DNeasy™ Tissue Kit (Quiagen) according to the manufacturer's protocol. Phenol/chloroform extractions of cell lysates following the protocol of Maslov et al. (1996) were used when a large number of cells ($>10^6$) were harvested.

Universal eukaryotic primers (5′-ACCTGGTTGATCCTGCCAG-3′ and 5′-CTTCCGCTGGTTCACCTACGG-3′) (Medlin et al. 1988) were used for amplification of the SSU rRNA gene. PCR was carried out in a 25 µl reaction volume using 10 pmol of each primer, 250 µM of each deoxyribonucleotide triphosphate (dNTP), and 2.5 µl 10 × PCR buffer (Takara) and 1 Unit of TaqDNA polymerase (Takara). The reactions were run on a T3 thermocycler (Biometra). The thermal cycling pattern was 95°C, 5 min (×1); 94°C, 1 min; 43°C, 2 min; 72°C, 2 min (×5); 94°C, 1 min; 48°C, 1 min; 72°C, 2 min (×25); 72°C, 10 min (×1). Amplification products were gel-isolated and cloned into pCR® 2,1 TOPO cloning vector using the TOPO-TA Cloning Kit (Invitrogen), and sequenced from both strands on an automatic sequencer, CEQ™ 2000 (Beckman Coulter), using the CEQ DTCS Dye Kit (Beckman Coulter) according to the manufacturer's protocol.

For phylogenetic analyses, 2 alignments were performed. For the first alignment the SSU rRNA gene sequences of 5 *Neoparamoeba* strains from turbot were compared with sequences of 7 *Neoparamoeba* strains available in GenBank and *Vannella anglica* Page, 1980 as an outgroup. The second alignment comprised other Gymnamoebia sequences and a set of eukaryotic sequences selected to cover the major groups of eukaryotic taxa. The sequence of a diplomonad species, *Hexamita inflata* Dujardin, which is considered among the earliest diverging eukaryotes (Leipe et al. 1993), was set as an outgroup. Sequences were aligned in the Clustal X program (Thompson et al. 1997) with various alignment parameters and corrected by eye using the BioEdit sequence alignment editor (Hall 1999). From the second alignment, 7 ambiguously aligned regions (711 sites) were excluded. The alignments are accessible on request. Phylogenetic analyses were performed using the maximum parsimony (MP), maximum likelihood (ML) and distance (minimum evolution, ME) methods. All methods were carried out with the program package PAUP*, version 4.0b10 (Swofford 2001). The MP analysis was done using heuristic search with random addition of taxa (10 replications) and the ACCTRAN-option. Gaps were treated as missing data. Transversion:transition (Tv:Ts) ratios were 1:1, 1:2, 1:3, 1:4 and 1:5. For the ML analysis the likelihood ratio test (LRT), implemented in the Modeltest version 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 + G + I evolution model for both alignments (GTR = General Time Reversible model; G = gamma distributed site-to-site variation; and I = proportion of invariable sites; Lanave et al. 1984). The distance method was executed using heuristic search with the minimum evolution as the objective setting. The K2P (Kimura two parameter; Kimura 1980) and HKY (Hasegawa-Kishino-Yuno; Hasegawa et al. 1985) 85 substitution models were used. Genetic distances were calculated with the K2P algorithm. Clade support was assessed by bootstrapping (MP and ML = 100 replicates; ME = 1000 replicates). The Kishino-Hasegawa (KH) and Shimodaira-Hasegawa (SH) tests (Kishino & Hasegawa 1989) were performed by phylogenetic analysis using parsimony (PAUP*) using resampling estimated log-likelihood (RELL) bootstrap (1000 replicates) to assess the significance of differences in likelihood scores of the tree topologies.

RESULTS

The morphology of the 3 more recently isolated *Neoparamoeba* strains included in the study (AFSM2V, AFSM3 and AFSM11) corresponded to strains, characterised previously (SM53, SM68) (Dyková et al. 2000). No differences were observed among these 4 strains either at the cellular or ultrastructural level. Also the range in the average length of attached trophozoites did not differ from other *Neoparamoeba* strains thus far isolated from turbot.

The total length of the SSU rRNA gene in the 5 *Neoparamoeba* strains was 2065 bp (AFSM2V), 2048 bp (AFSM3), 2061 bp (AFSM11), 2051 bp (SM53) and 2049 bp (SM68). The GenBank accession nos. are: AFSM2V = AY193722, AFSM3 = AY193724, AFSM11 = AY193723, SM53 = AY193726, SM68 = AY193725. The guanine plus cytosine content was 40.97% (AFSM2V), 39.40% (AFSM3), 41.19% (AFSM11), 39.15% (SM53) and 40.41% (SM68). This low content correlates with other sequences of most Gymnamoebia (Bolivar et al. 2001).

The first alignment which comprised 12 *Neoparamoeba* sequences and 1 sequence of *Vannella*

anglica, consisted of 2107 nucleotide sites; of these, 208 sites were parsimony-informative. The distances computed from the first alignment are summarised in Table 1. MP, ML and ME analyses of the data set resulted in trees with the same topology (Fig. 1). AFSM2V and AFSM11 were the most derivative strains clustered within the Clade A (Fig. 1) as a sister branch to 2 strains isolated from seawater in Wales (UK). Clade A (Fig. 1) is well bootstrap-supported by MP and ME (100%). Using ML, the same clade has low bootstrap support (57%). Strains AFSM3, SM53 and SM68 branched out of Clades A and B (*N. aestuarina*) and composed a monophyletic group—Clade C (Fig. 1) with high bootstrap support.

The second, more comprehensive, analysis was based on alignment of 1730 nucleotide sites and included 52 taxa; 7 ambiguously aligned regions were removed (711 sites). The number of parsimony characters was 1135. The *Neoparamoeba* strains clustered together in all analyses performed (MP, ML and ME) and this monophyly was well supported with a 100% bootstrap value. MP analysis, with Tv:Ts ratios of 1:3, 1:4 and 1:5, and ML analysis (GTR + G + I model) revealed the topology of the tree shown in Fig. 2. *Vannella anglica* formed the sister branch to the *Neoparamoeba* spp. clade, but this relation was not bootstrap-supported. The *Neoparamoeba* spp. clade diverged after division of the Gymnamoebia species and before rapid diversification of the eukaryotic taxa. The branching pattern was different in MP, with a Tv:Ts of 1:1; *V. anglica* appeared as an independent lineage diverging just before the *Neoparamoeba* spp. clade and before the Gym-

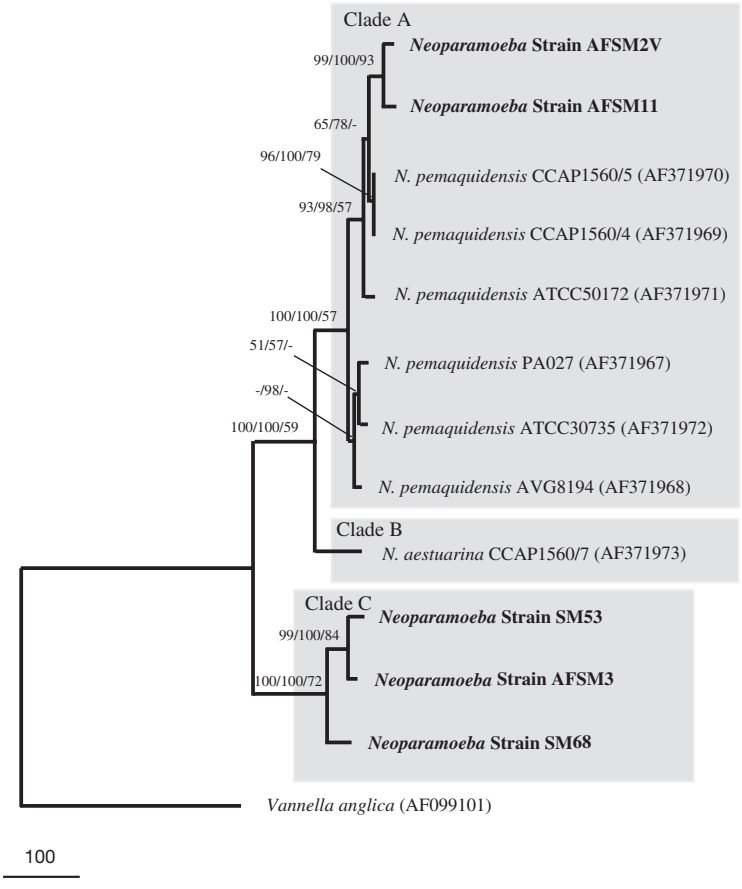

Fig. 1. Phylogenetic analysis comparing gene sequences of 12 *Neoparamoeba* species with sequence for *Vannella anglica*. Maximum parsimony (MP) tree (transversion:transition, Tv:Ts ratio = 1:2; 1100 steps; CI = 0.87; Retention Index [RI] = 0.80) rooted at *V. anglica*. Bootstrap values (MP Tv:Ts 1:2; Kimura two parameter distance method [K2P]; maximum likelihood method [ML]) are indicated for nodes gaining more than 50% support. GenBank accession nos. in parentheses; distance scale is given under tree

Table 1. Genetic distances (K2P) among 12 *Neoparamoeba* strains and *Vannella anglica* strain based on SSU rDNA alignment (2107 nucleotide sites). *NP*: *N. pemaquidensis*; *NA*: *N. aestuarina*

	1	2	3	4	5	6	7	8	9	10	11	12
(1) Strain SM53												
(2) Strain AFSM3	0.0133											
(3) Strain SM68	0.0358	0.0317										
(4) Strain AFSM2	0.0944	0.0922	0.0944									
(5) Strain AFSM11	0.0923	0.0911	0.0894	0.0122								
(6) *NA* - CCAP1560/7	0.0939	0.0878	0.0914	0.0495	0.0496							
(7) *NP* - CCAP 1560/5	0.0888	0.0888	0.0894	0.0158	0.0168	0.0456						
(8) *NP* - CCAP 1560/4	0.0888	0.0888	0.0894	0.0158	0.0168	0.0456	0.0000					
(9) *NP* - ATCC 50172	0.0921	0.0921	0.0921	0.0192	0.0183	0.0456	0.0093	0.0093				
(10) *NP* - PA 027	0.0905	0.0905	0.0922	0.0278	0.0258	0.0439	0.0172	0.0172	0.0187			
(11) *NP* - ATCC 30735	0.0866	0.0855	0.0854	0.0258	0.0228	0.0428	0.0177	0.0177	0.0192	0.0108		
(12 *NP* - AVG 8194	0.0888	0.0888	0.0888	0.0304	0.0263	0.0428	0.0197	0.0197	0.0207	0.0127	0.0118	
(13) *Vannella anglica*	0.2676	0.2677	0.2650	0.2748	0.2705	0.2713	0.2661	0.2661	0.2673	0.2678	0.2662	0.2654

namoebia species. ME analysis positioned the *Neoparamoeba* spp. clade within rapid diversification of eukaryotic taxa. None of the relationships mentioned above was supported by a bootstrap value higher than 50%. In order to clarify the branching pattern of *Neoparamoeba* spp. + *V. anglica* + Gymnamoebia sequences tenuously supported by bootstrapping, KH and SH tests were performed (Table 2). The topologies

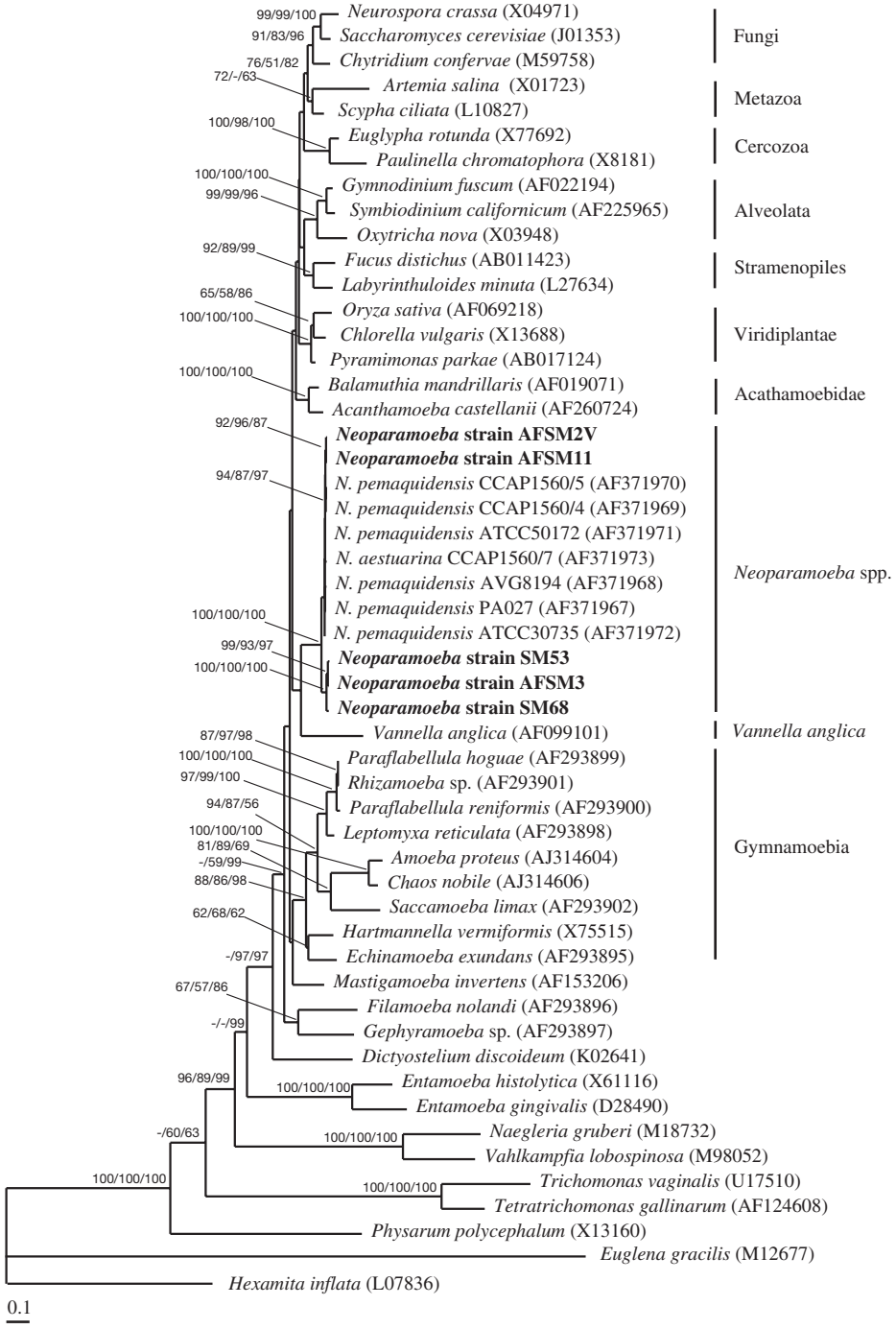

Fig. 2. Phylogenetic analysis comparing gene sequences of major eukaryotic taxa. Maximum likelihood (ML) tree (GTR + G + I; $\alpha = 0.510338$, proportion of invariable sites = 0.005179) (–ln = 30 643.6814) based on 18S rRNA gene sequences. The tree is rooted on *Hexamita inflata* (Diplomonadida). Bootstrap values (ML; MP Tv:Ts = 1:3; distance method K2P) are indicated for nodes gaining more than 50% support. GenBank accession nos. in parentheses; distance scale is given under tree

Table 2. Kishino-Hasegawa (KH) and Shimodaira-Hasegawa (SH) tests for constrained trees using RELL bootstrap (1000 replicates). Text form of a tree topology used in the PAUP* program (see also Votýpka et al. 2002). c: eukaryotic taxa with rapid diversification; e: early diverging taxa (*Euglena gracilis*, *Physarum polycephalum*, *Trichomonas gallinarum*, *Tetratrichomonas vaginalis*, *Vahlkampfia lobospinosa*, *Naegleria gruberi*, *Entamoeba gingivalis*, *E. histolytica*, *Dictyostelium discoideum*, *Gephyramoeba* sp., *Filamoeba nolandi*); g: Gymnamoebia; h: *Hexamita inflata*; m: *Mastigamoeba invertens*; n: *Neoparamoeba* strains; v: *Vannella anglica*

Tree	−ln L	Diff −ln L	KH-test p	SH-test p
((h),((e),(v,((n),((m,(g)),(c))))))	16976.11381	(best)		
((h),((e),((m,(g)),((v,(n)),(c)))))	16978.39424	2.28043	0.705	0.841
((h),((e),((g),((m),((v,(n)),(c))))))	16983.29615	7.18234	0.333	0.506
((h),((e),(v,((g),((n),(m,(c))))))	16996.62980	20.51599	0.054	0.080
((h),((e),(v,((m,(g)),((n),(c)))))	16977.44562	1.33181	0.684	0.855
((h),((e),(v,((g),(m,((n),(c))))))	16982.36173	6.24792	0.271	0.571
((h),((e),((g),(m,(v,(n),(c)))))	16988.34037	12.22656	0.075	0.204
((h),((e),(m,(v,(n),(g),(c)))))	16989.46696	13.35316	0.058	0.171
((h),((e),(v,(n),m,(g),(c))))	16990.06993	13.95612	0.046*	0.154
((h),((e),(v,((m,((g),(n))),(c)))))	16982.77805	6.66424	0.279	0.563
((h),((e),(m,(v,(g),(n))),(c)))	16989.13446	13.02065	0.132	0.222
((h),((e),((m,v,(g),(n)),(c))))	16990.06993	13.95612	0.046*	0.154
(h,((e),(v,((g),((n),(m,((c))))))))	16980.23762	4.12381	0.540	0.791
(h,((e),(v,((n),((g),(m,((c))))))))	16978.87615	2.76235	0.662	0.865
(h,((e),((v,(n)),((g),(m,((c)))))))	16980.61881	4.50500	0.561	0.724
((h),((e),((v,(n)),((m,(g)),(c)))))	16977.44706	1.33325	0.788	0.892
((h),((e),((n),((m,(g)),(v,(c)))))	16983.51360	7.39979	0.116	0.486
((h),((e),((n),(v,((m,(g)),(c)))))	16980.73244	4.61863	0.243	0.668

*$p < 0.05$

described above plus the alternative tree topologies determined for ML under the (GTR + G + I) evolution model were tested. Only the topologies with *Mastigamoeba invertens* branching together with the clade of *Neoparamoeba*, Gymnamoebia and *V. anglica* and also with the 'crown taxa' clade were rejected by the KH test ($p < 0.05$).

DISCUSSION

Our phylogenetic analyses confirmed a close relationship of 5 mutually morphologically indistinguishable *Neoparamoeba* strains from *Scophthalmus maximus* with 6 strains of *N. pemaquidensis*, and 1 strain of *N. aestuarina* characterised by Elliot et al. (2001). The topology of the tree (Fig. 1) and genetic distances (Table 1) revealed a very close relationship between 2 turbot strains (AFSM2V and AFSM11) and *N. pemaquidensis* strains. The closest was the affinity with CCAP 1560/4 and CCAP 1560/5 strains of *N. pemaquidensis* isolated from seawater in Wales, and also with ATCC 50172 strain of the same species isolated from *Oncorhynchus kisutch* (Walbaum, 1792) in the USA. The remaining 3 strains under study (Clade C) clearly branched out of *N. pemaquidensis* and *N. aestuarina* clades. Although morphologically indistinguishable, they represented a lineage distinctly separated from the other *Neoparamoeba* strains and thus could be independent species.

The phylogenetic position of Gymnamoebae and their relation to other organisms has been thoroughly discussed by Bolivar et al. (2001) and earlier investigations (Silberman et al. 1999, Sims et al. 1999, Amaral Zettler et al. 2000). Our SSU rDNA analyses indicate a common evolutionary history of *Neoparamoeba* spp. and *Vannella anglica*. Previous phylogenetic studies (Sims et al. 1999, Bolivar et al. 2001) classed *V. anglica* as an independent lineage diverging before the Gymnamoebia species sequenced thus far. Our results show a sister-group relation of the recently sequenced *Neoparamoeba* strains with *V. anglica*. Both genera have morphological features typical of Gymnamoebae (Page 1987). The results of our phylogenetic analyses of SSU rRNA gene sequences indicate that *Neoparamoeba* sequences do not cluster with species of the Gymnamoebia (sensu stricto according to Bolivar et al. 2001). Depending on the model of evolution, the branching pattern of the tree changed and the overall bootstrap support was low, so that we could not accurately determine the real position of the *Neoparamoeba* clade. KH and SH tests also failed to clarify this. Further studies focused on sequencing early diverging taxa will hopefully solve some of these problems.

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