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Description of *Paramoeba atlantica* n. sp. (Amoebozoa, Dactylopodida) – a Marine Amoeba from the Eastern Atlantic, with Emendation of the Dactylopodid Families

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Summary. A strain of marine amoeba has been isolated and studied from the bottom sediments of the Great Meteor Seamount (Atlantic Ocean, 29°36.29′N; 28°59.12′W; 267.4 m deep). This amoeba has a typical dactylopodiid morphotype, a coat of delicate, boat-shaped scales, and a *Perkinsela*-like organism (PLO), an obligatory, deeply-specialized kinetoplastid symbiont near the nucleus. These characters allow us to include this species into the genus *Paramoeba*. However, it differs from its only described species, *P. eilhardi*, in the structure of scales. *P. atlantica* n. sp. is established therefore to accommodate the studied strain. SSU rRNA gene sequence analysis suggests that *P. atlantica* belongs to the Dactylopodida, and is sister to a monophyletic clade of *P. eilhardi* and all *Neoparamoeba* spp., branching separately from *P. eilhardi*. Therefore, the genera *Paramoeba* and *Neoparamoeba*, currently defined based on the cell surface ultrastructure, might be paraphyletic and probably should be synonymized, as further evidence is accumulated. Based on the data available we emend the families Vexilliferidae and Paramoebidae to make them more consistent with the current phylogenetic schemes.

Key words: Amoebozoa, Dactylopodida, deep-sea protists, *Paramoeba atlantica* n. sp., phylogeny, SSU rDNA, taxonomy, ultrastructure.

Abbreviations: BS – bootstrap support; DAPI – 4',6-diamidino-2-phenylindole; DIC – differential interference contrast; PBS – phosphate buffered saline; PLO – *Perkinsela*-like organism; PP – Bayesian posterior probability; SEM – scanning electron microscopy; SSU rRNA – small-subunit ribosomal RNA; TEM – transmission electron microscopy

INTRODUCTION

Marine and aestuarine amphizoic amoebozoans of the genera *Paramoeba* Schaudinn, 1896, *Janickina*

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Chatton, 1953 and *Neoparamoeba* Page, 1987 (Discosea, Dactylopodida) possess a deeply-specialized kinetoplastid symbiont *Perkinsela amoebae* (Hollande, 1980) Dyková *et al*., 2008 (or *Perkinsela*-like organism, PLO) located in the cytoplasm near the nucleus (Dyková *et al*. 2008, Hollande 1980). The dactylopodial locomotive morphotype (Smirnov and Brown 2004) is shared by *Paramoeba* and *Neoparamoeba* that may include both, free-living and parasitic species; entirely

parasitic *Janickina* spp. live in chaetognaths and have a limax locomotive form with a villous-bulb uroid (Janicki 1912, Chatton 1953). *Paramoeba* has been a sole genus in the group until establishment of *Janickina*, where several species, formerly members of *Paramoeba*, were transferred (Chatton 1953). In the 1970's, description of several more marine and estuarine dactylopodial amoebae, then included in *Paramoeba*, revealed differences in cell surface structure. Amoebae of the type species *Paramoeba eilhardi* Schaudinn, 1896 were covered with delicate, boat-shaped microscales (Grell and Benwitz 1966), while other species had an amorphous glycocalyx, sometimes containing hair-like structures but devoid of microscales (Page 1970, 1973; Cann and Page 1982). A thin stratified glycocalyx without scales was also demonstrated in *Janickina* (Hollande 1980). These observations allowed Page (1987) to create the genus *Neoparamoeba* to accommodate those members of *Paramoeba* without microscales. *Neoparamoeba* was included in the family Vexilliferidae, while *Paramoeba* was left in Paramoebidae, based on the differences in cell surface structure and presence of the microfilamentous core in subpseudopodia of the former genus. Molecular phylogenetic studies of the paramoebids and vexilliferids have mostly been focused on *Neoparamoeba*, as many members of this genus have been shown to cause mortal diseases in fish and invertebrates (e.g. Dyková *et al*. 2005, Fiala and Dyková 2003, Mullen *et al*. 2005, Young *et al*. 2007) and molecular diagnostics methods for these amoebae were sought (e.g. Wong *et al*. 2004). However, most of other available strains of Paramoebidae and Vexilliferidae were also sequenced (Dyková *et al*. 2011, Fahrni *et al*. 2003, Mullen *et al*. 2005, Peglar *et al*. 2003), including a strain of *Paramoeba eilhardi* on which the current description of this species and diagnosis of the genus *Paramoeba* is based (Grell 1961; Grell and Benwitz 1966, 1970; strain CCAP 1560/2). Small-subunit (SSU) rRNA gene sequence analysis (Mullen *et al*. 2005) has shown that this species branches as a sister to monophyletic *Neoparamoeba* spp., and the whole clade is classified in Dactylopodida (Smirnov *et al*. 2005). Later, Dyková *et al*. (2007, 2008) showed that *P. eilhardi* branched within a clade of *Neoparamoeba* spp., mostly as a sister to *Neoparamoeba perurans*.

The purpose of this paper is to describe a new marine dactylopodial amoeba possessing surface microscales and a PLO. This amoeba has the characteristics of the genus *Paramoeba*. Light and electron microscopy together with SSU rRNA gene sequence analysis were used to directly compare the new strain with the previously available strain of *P. eilhardi* (CCAP 1560/2) and to justify the naming of *P. atlantica* n. sp. together with the re-evaluation of the families in Dactylopodida.

MATERIAL AND METHODS

Strain isolation, culturing and microscopy

Amoebae were isolated from the sandy bottom sediments collected using a Van Veen grab from the Great Meteor Seamount (eastern Atlantic Ocean; 29°36.29′N; 28°59.12′W) at the depth of 267.4 m on August 17th, 2009 during the cruise M79/1 of the German research vessel METEOR. Sediment subsamples were removed using a sterilized cut syringe and transferred into sterile 650-ml tissue culture flasks (Saerstedt) filled with Millipore-filtered (0.2 μm) seawater (ca. 35‰). Samples were kept at 10°C after collection and during transport to the laboratory. Aliquots of sediment (1–2 ml) were inoculated into 130-mm Petri dishes with addition of filtered seawater and autoclaved wheat grains. Samples were kept at 18–20°C and regularly observed using an inverted microscope. Amoebae were isolated and cloned by transferring into the Petri dishes with fresh seawater using glass capillary pipettes. Cultures were maintained in filtered seawater (ca. 35‰) with addition of wheat grains. Living amoebae were observed and measured either in culture or on coverslips using a Zeiss Axiovert 200 inverted microscope with phase contrast and DIC optics. In total, several hundred cells were observed and the dimensions of 117 cells were measured. For DAPI-staining, cells were fixed on coverslips with 4% paraformaldehyde prepared with 1 × PBS (pH 7.4) for 10 min., washed with the same buffer (3 × 5 min.), followed by application of DAPI at a final concentration of 2.5 μg/ml in the same buffer for 15 min. After staining, cells were washed with buffer, enclosed in anti-bleaching medium and observed using a Zeiss Axiophot fluorescent microscope.

For transmission electron microscopy (TEM) the following fixation protocols were applied: (1) (All steps at room temperature.) Addition of several drops of 1% osmium tetroxide in a culture medium (5 min.); 2.5% glutaraldehyde in filtered seawater (40 min.); 1% osmium tetroxide in filtered seawater (60 min.). Cells washed with seawater (3 × 5 min.) between fixation steps and before dehydration. (2) The same as (1), but sodium cacodylate buffer (0.05 M, pH 7.4) used instead of seawater. (3) (All steps on ice.) 2% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) mixed 1 : 1 with the culture medium (60 min.). Cells washed with buffer (3 × 5 min.) before dehydration. (4) 1% osmium tetroxide in KOH-Cr buffer at pH 7.4 after Dalton (1955) (60 min. on ice). Cells washed with buffer (3 × 5 min.) before dehydration. In all cases the fixation started in culture dishes, later amoebae were scraped away from the substratum, concentrated by gentle centrifugation and embedded in 2% agar before dehydration. Small pieces of agar (ca. 1 mm³) containing amoebae were cut out and dehydrated in a graded ethanol series followed by epoxy propane and embedded in Araldite M epoxy resin (Serva). Silver to light gold sections were cut on Reichert ultramicrotome using a diamond knife and stained with

2% uranyl acetate in 70% ethanol and Reynolds' lead citrate. Negatively stained whole mounts of scales were prepared by placing the cells on formvar-coated grids, allowing them to settle and fixing with osmium tetroxide vapours for 15 min. Grids were then rinsed with distilled water, followed by negative staining with 1% aqueous phosphotungstic acid as described in Harris (1999: 20–21). Sections and negatively stained whole mounts were observed using a Philips EM208 electron microscope at 80 kV.

For scanning electron microscopy amoebae were placed on coverslips (18 × 18 mm), allowed to attach and then fixed and dehydrated. Fixation was for 30 min. in a mixture of 2.5% glutaraldehyde and 1% osmium tetroxide in seawater. After a brief wash in seawater of decreasing concentration amoebae were dehydrated in a graded ethanol series (30 to 100%) and critical-point dried with liquid CO_2, sputter-coated with gold and observed using a FEI Quanta scanning electron microscope.

For comparative purposes, a strain of *Paramoeba eilhardi* was obtained from Culture Collection of Algae and Protozoa, Oban, UK (accession number CCAP 1560/2). Culture maintenance and observations were conducted as described above.

DNA isolation, sequencing and phylogenetic analysis

For molecular phylogenetic study the genomic DNA was isolated from the cell cultures using the guanidine isothiocyanate (Maniatis *et al*. 1982) method. Small subunit ribosomal RNA gene was amplified and sequenced as a single piece essentially as described previously (Kudryavtsev *et al*. 2009, 2011) using the universal primers sAF (5'-CTGGTTGATYCTGCCAG-3') in combination with RibB (Medlin *et al*. 1988). In total, one molecular clone of full-length SSU rDNA was sequenced for *Paramoeba atlantica* in both directions (GenBank accession No JN202436). Partial sequences of the three molecular clones of the symbiont's SSU rDNA co-amplified in the same reaction were obtained (the final sequence used is a consensus of them; GenBank accession No JN202437). For *P. eilhardi* four molecular clones of full-length SSU rRNA were sequenced in both directions (GenBank accession No JN202438-JN202441). Phylogenetic analysis was done as described in Kudryavtsev *et al*. (2011). Seaview (Galtier *et al*. 1996) was used for manual alignment; RaxML Version 7.2.6 (Stamatakis 2006) and MrBayes Version 3.1.2 (Altekar *et al*. 2004, Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003) run at the Bioportal computer service (http://www.bioportal.uio.no) were used for tree reconstruction. Alternative tree topologies were produced and tested using Treefinder (Jobb 2008, http://www.treefinder.de).

RESULTS

### Culture growth, morphology and ultrastructure of *Paramoeba atlantica* n. sp.

The cells first appeared in the inoculated samples after 3 weeks of incubation, and multiplied to considerable densities in around 5–6 weeks of incubation in one of the 10 dishes inoculated. Following purification and cloning, amoebae multiplied in culture and formed dense aggregations of the cells, with very low densities of bacteria, after three weeks of incubation. Cultures could remain stable in this condition for more than three months (repeatedly observed since January 2010) if the Petri dishes were sealed with Parafilm. If dishes were not sealed, amoebae did not demonstrate fast growth and the culture degraded quickly.

Typical locomotive forms are shown in Figs 1–7. During rapid locomotion amoebae were generally oval, with length greater than breadth (all measurement data are given in the diagnosis); in slower moving cells breadth was sometimes the greatest dimension (Fig. 4). The cytoplasm was clearly separated into anterior hyaloplasm, occupying 1/4–1/3 of the cell length, and posterior granuloplasm. The anterior edge of the cell produced numerous hyaline subpseudopodia (Figs 1–4) usually up to 10 μm in length. These subpseudopodia could be withdrawn shortly after formation or moved laterally towards the uroid as the cell advanced. Most of the locomotive cells had 2–4 dorsal longitudinal ridges (Figs 1, 6, 7) bearing subpseudopodia. These extended forward, sometimes their tips reached beyond the anterior edge of the cell. Some of these subpseudopodia moved forward over the dorsal surface of the cell and ventrally towards the substratum. The posterior end of the locomotive form was usually blunt (Figs 1, 2, 6) with several small folds over the surface. Less frequently it was pointed, and the cell adopted an elongated triangular shape (Figs 3, 5). Rate of locomotion over the glass substrate at 18°C was 10–32 μm/min. (average 17.4 μm/min.) (n = 17) equaling about half of a cell length per minute.

During slower and non-directed movement amoebae were rounded and flattened, with strongly wrinkled dorsal surface and numerous hyaline subpseudopodia produced from the narrow peripheral hyaloplasm in all directions. Floating forms were adopted for a long time by some cells in dense mature cultures, and, for several minutes, when amoebae were artifitially detached from the substratum (Figs 8, 9). At the initial stages of formation they were spherical with short papillate projections (Fig. 8); mature floating forms had rounded cell body with narrow radiating hyaline pseudopodia (Fig. 9). Some of the floating forms were slightly asymmetrical, especially in cultures.

Amoebae possessed a single, spherical nucleus of vesicular type with the central nucleolus (Figs 5, 10, 12). The nucleus was located centrally in the granuloplasm. The symbiont (PLO) was clearly seen always closely associated with the nucleus (Fig. 5). It was
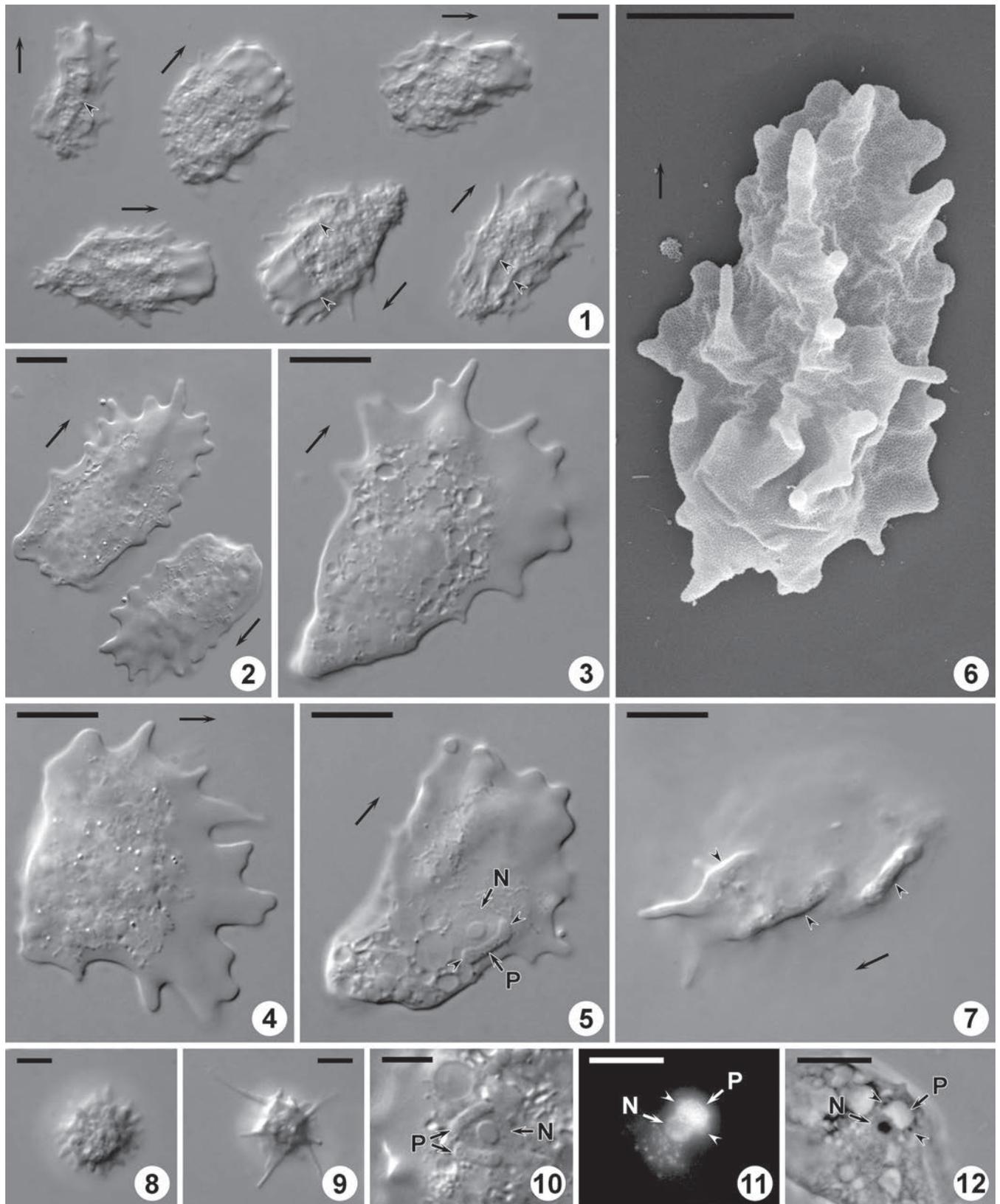
ovoid, and had a usual structure, with the large central "Mittelstück" and two "Seitenteile" (terminology according to Grell 1961). 1–2% of all cells observed had two symbionts (Fig. 10). In DAPI-stained preparations (Figs 11, 12) the "Mittelstück" was strongly positive, while "Seitenteile" demonstrated less intensive fluorescence. The nucleus showed a ring-shaped fluorescence pattern around DAPI-negative nucleolus (Fig. 11). The granuloplasm contained numerous transparent vesicles and food vacuoles with bacteria. Some of the cells contained numerous spherical, yellowish bright granules of different sizes, not exceeding 3 μm in diameter. There were neither contractile vacuole nor crystals. In our cultures amoebae never formed cysts.

Figures 13–18 show some ultrastructural features of the studied strain. In spite of various fixation protocols applied for TEM, the fixation quality of the nucleus, PLO and cytoplasm was never adequate, being slightly better when protocols (1) and (4) were used. Cell surface structure was preserved more or less identically with every protocol and corresponded well to the SEM observations. The plasma membrane surface was completely covered with a layer of delicate, boat-shaped microscales of medium electron density. The microscales could be easily detected even in the lower magnification SEM micrographs (Fig. 6). Higher magnification SEM (Fig. 13) and the ultrathin sections (Figs 14–17) have shown that there was only one type of microscales; they all were distally open boat-shaped structures consisting of the flat bases (Figs 16, 17) and slightly curved walls arising from the periphery of the bases (Figs 14–16). Bases and walls of the microscales did not contain any holes and appeared to have homogeneous structure. Diagrammatic reconstruction of the microscale is shown in Fig. 19. The nucleus in sections was rounded and showed an electron-dense central nucleolus; one or two structures resembling parasomes were often seen close to the nuclear membrane. However, the fixation quality of nucleus and parasomes was never sufficient for a detailed description. Lipid droplets and food vacuoles were regularly seen in the cytoplasm; mitochondria were probably destroyed, as a structure resembling a poorly preserved mitochondrion was seen only once in the sections. Dictyosomes were also not found, although numerous vesicles probably deriving from the dictyosomes, many of which contained scales (Fig. 18) were seen in the cytoplasm close to the nuclear envelope.

Gene sequence data analysis

Small-subunit ribosomal RNA gene of *Paramoeba atlantica* was 2100 b.p. long and had a G + C content of 37.9%. All typical eukaryotic secondary structure elements could be identified in this sequence. There were no long introns. Preliminary phylogenetic analysis of the sequence has shown that *P. atlantica* belongs to Amoebozoa and constantly groups within the Dactylopodida. For detailed analysis of the phylogenetic relationships of this species we have selected the datasets of Dactylopodida and Vannellidae (selected (79) sequences, 1426 alignment positions; Fig. 20) and Dactylopodida only (all available (66) sequences, 1556 alignment positions; Fig. 21). In both datasets branching of *Paramoeba atlantica* was the same regardless of the algorithm of tree reconstruction. This species was always sister to a monophyletic clade of *Neoparamoeba* spp. with moderate to high support (PP = 0.95–1; BS = 69–93%). The sequence of *P. eilhardi* CCAP 1560/2 never formed a clade with *P. atlantica*, branching instead in a poorly resolved position (PP = 0.6; BS = 53%, Fig. 21) at the base of the *Neoparamoeba* spp. clade, or as sister to *Neoparamoeba perurans* strains with a restricted dataset (Fig. 20). Different strains of other *Neoparamoeba* spp.: *N. aestuarina*, *N. pemaquidensis* and *N. branchiphila*, formed clades mainly corresponding to species (Fig. 21). *Korotnevella* spp. were basal to a clade of *Neoparamoeba* + *Paramoeba* spp. Relationships at the base of the dactylopodid tree were not stable and the tree topology depended on the dataset used. With a restricted dataset (Fig. 20) *Korotnevella* spp. were

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Figs 1–12. *Paramoeba atlantica* n. sp. Morphology of amoebae as demonstrated with light and scanning electron microscopy. **1–7** – amoebae during locomotion on the glass surface (6 – scanning electron micrograph, showing subpseudopodia produced also on the dorsal surface of the cell; other figures are DIC images). Arrows indicate direction of movement, arrowheads in Figs 1 and 7 (the latter represents a dorsal view of the moving amoeba), longitudinal ridges on the dorsal surface of the cell; **8–9** – floating forms shortly after detachment from the substratum (8) and after prolonged period of floating (9). Nucleus (N) and PLO (P) in living amoebae are shown in Figs 5 and 10; **10** – demonstrates a rare case of two PLOs in a single cell. Arrowheads in Fig. 5 point to "Seitenteile" of the PLO, arrow to "Mittelstück"; **11–12** – nucleus and PLO stained with DAPI: fluorescent image (11) and corresponding phase contrast micrograph (12). Signs are the same as in 5. Scale bar: 10 μm in all figures.
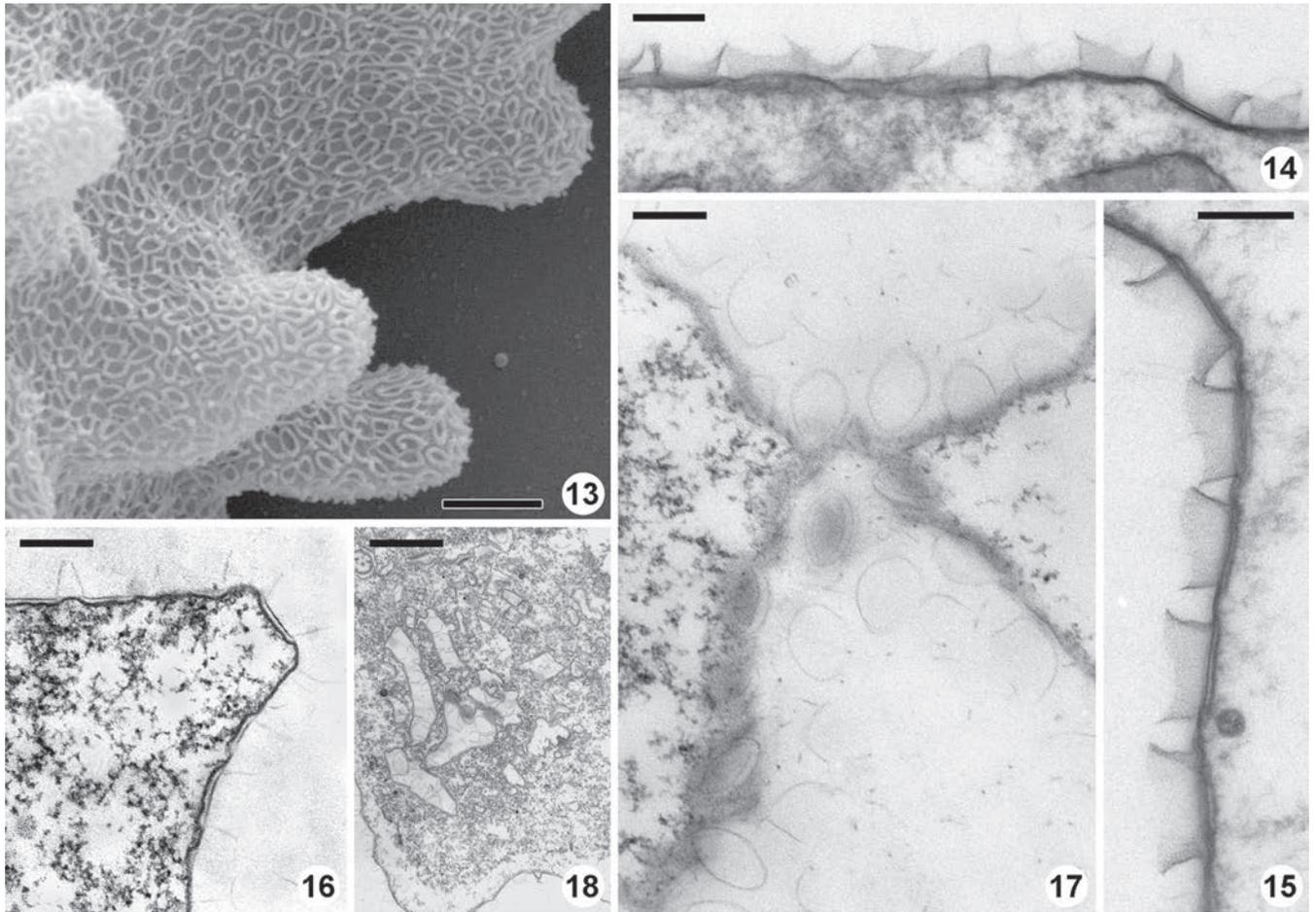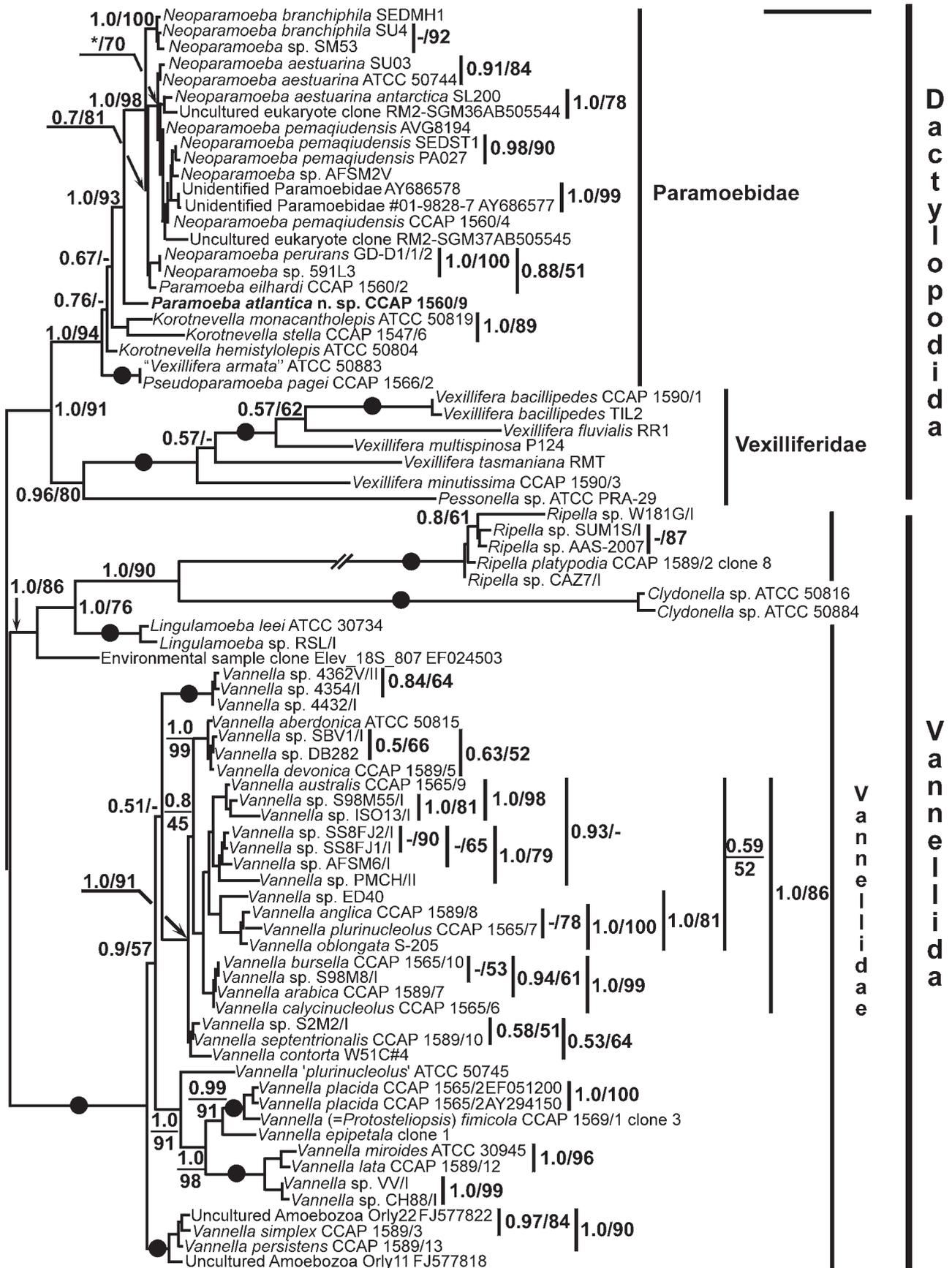
Figs 13–18. *Paramoeba atlantica* n. sp. Electron micrographs. **13** – scanning electron micrograph of the cell surface showing scales; **14–15** – scales on the thicker sections of the cell surface, transmission electron micrographs; **16** – vertical ultrathin section of the cell surface; **17** – tangential section of the cell surface; **18** – scale containing cytoplasmic vesicles, probably originating from a dictyosome. Scale bar in Figs 13, 18 : 1 μm, in Fig. 17 : 0.5 μm, in other figures : 0.25 μm.
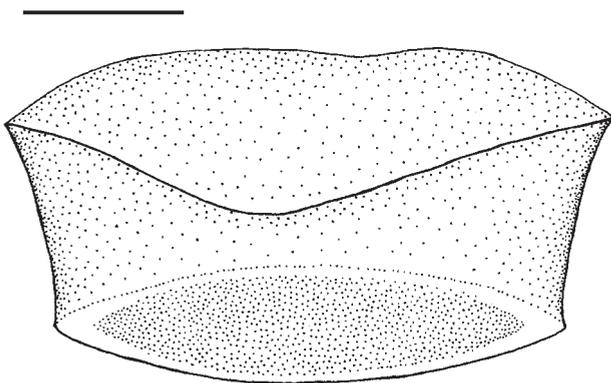
Fig. 19. Diagram illustrating the scale structure in *P. atlantica* n. sp. Scale bar: 0.1 μm.

Fig. 20. Maximum likelihood tree of of selected Flabellinia (Vannellida and Dactylopodida) based on 79 small subunit (SSU) rRNA gene sequences showing position of *Paramoeba atlantica* n. sp. (in bold). The tree shown was derived based on 1426 nucleotide positions using the program RaxML Version 7.2.6 (Stamatakis 2006) and has a LnL = –16214.53 and a gamma distribution α = 0.65. Families of Dactylopodida are indicated according to a proposed revision. Numbers at nodes indicate Bayesian posterior probabilities/ bootstrap values if above 0.5/50%. Solid circles = 1.0/100. Dash indicates value below 50%, while asterisk, that the branch does not exist in the tree derived with that algorithm. Length of the disrupted clades was reduced 2 times. Scale bar: 0.1 substitutions/site.

1.0/100 *Neoparamoeba branchiphila* SEDMH1
Neoparamoeba branchiphila SU4 **-/92**
***/70** *Neoparamoeba* sp. SM53
Neoparamoeba aestuarina SU03 **0.91/84**
Neoparamoeba aestuarina ATCC 50744
1.0/98 *Neoparamoeba aestuarina antarctica* SL200 **1.0/78**
0.7/81 Uncultured eukaryote clone RM2-SGM36AB505544
Neoparamoeba pemaqiudensis AVG8194
Neoparamoeba pemaqiudensis SEDST1 **0.98/90**
Neoparamoeba pemaqiudensis PA027
Neoparamoeba sp. AFSM2V
1.0/93 Unidentified Paramoebidae AY686578
Unidentified Paramoebidae #01-9828-7 AY686577 **1.0/99**
Neoparamoeba pemaqiudensis CCAP 1560/4
Uncultured eukaryote clone RM2-SGM37AB505545
0.67/- *Neoparamoeba perurans* GD-D1/1/2 **1.0/100** **0.88/51**
Neoparamoeba sp. 591L3
0.76/- *Paramoeba eilhardi* CCAP 1560/2
Paramoeba atlantica n. sp. CCAP 1560/9
1.0/94 *Korotnevella monacantholepis* ATCC 50819 **1.0/89**
Korotnevella stella CCAP 1547/6
Korotnevella hemistylolepis ATCC 50804

Paramoebidae

1.0/91 "*Vexillifera armata*" ATCC 50883
Pseudoparamoeba pagei CCAP 1566/2
0.57/62 *Vexillifera bacillipedes* CCAP 1590/1
Vexillifera bacillipedes TIL2
0.57/- *Vexillifera fluvialis* RR1
Vexillifera multispinosa P124
Vexillifera tasmaniana RMT
0.96/80 *Vexillifera minutissima* CCAP 1590/3
Pessonella sp. ATCC PRA-29

Vexilliferidae

0.8/61 *Ripella* sp. W181G/I
Ripella sp. SUM1S/I **-/87**
1.0/90 *Ripella* sp. AAS-2007
Ripella platypodia CCAP 1589/2 clone 8
Ripella sp. CAZ7/I
1.0/86 **1.0/76** *Clydonella* sp. ATCC 50816
Clydonella sp. ATCC 50884
Lingulamoeba leei ATCC 30734
Lingulamoeba sp. RSL/I
Environmental sample clone Elev_18S_807 EF024503

Vannella sp. 4362V/II **0.84/64**
Vannella sp. 4354/I
Vannella sp. 4432/I
1.0/99 *Vannella aberdonica* ATCC 50815
Vannella sp. SBV1/I **0.5/66** **0.63/52**
Vannella sp. DB282
Vannella devonica CCAP 1589/5
0.51/- **0.8/45** *Vannella australis* CCAP 1565/9 **1.0/98**
Vannella sp. S98M55/I **1.0/81**
Vannella sp. ISO13/I
Vannella sp. SS8FJ2/I **-/90**
Vannella sp. SS8FJ1/I **-/65** **0.93/-**
Vannella sp. AFSM6/I **1.0/79**
Vannella sp. PMCH/II
1.0/91 *Vannella* sp. ED40
Vannella anglica CCAP 1589/8 **-/78** **1.0/100** **1.0/81**
Vannella plurinucleolus CCAP 1565/7 **0.59/52** **1.0/86**
Vannella oblongata S-205
Vannella bursella CCAP 1565/10 **-/53**
Vannella sp. S98M8/I **0.94/61**
Vannella arabica CCAP 1589/7 **1.0/99**
Vannella calycinucleolus CCAP 1565/6
0.9/57 *Vannella* sp. S2M2/I
Vannella septentrionalis CCAP 1589/10 **0.58/51** **0.53/64**
Vannella contorta W51C#4
Vannella 'plurinucleolus' ATCC 50745
0.99/91 *Vannella placida* CCAP 1565/2EF051200 **1.0/100**
Vannella placida CCAP 1565/2AY294150
Vannella (=*Protosteliopsis*) *fimicola* CCAP 1569/1 clone 3
1.0/91 *Vannella epipetala* clone 1
1.0/98 *Vannella miroides* ATCC 30945 **1.0/96**
Vannella lata CCAP 1589/12
Vannella sp. VV/I **1.0/99**
Vannella sp. CH88/I
Uncultured Amoebozoa Orly22FJ577822 **0.97/84**
Vannella simplex CCAP 1589/3 **1.0/90**
Vannella persistens CCAP 1589/13
Uncultured Amoebozoa Orly11 FJ577818

Vannellidae

Dactylopodida

Vannellida

always sister to a clade of *Paramoeba* + *Neoparamoeba*, while a robust clade of "*Vexillifera armata*" and *Pseudoparamoeba pagei* was always sister to *Korotnevella* + *Paramoeba*/*Neoparamoeba*. With the more expanded dataset positions of "*Vexillifera*" + *Pseudoparamoeba* and *Korotnevella* were swapped (Fig. 21). *Korotnevella* was either paraphyletic (Figs 20, 21), or sometimes monophyletic (not shown) depending on the algorithm of analysis and the choice of nucleotide positions. The clade of *Vexillifera* spp. and "*Pessonella* sp." PRA-29 always demonstrated the same topology regardless of the dataset and the algorithm of analysis and was sister to the whole clade of *Paramoeba*/ *Neoparamoeba*/ *Korotnevella* (Fig. 20). Several molecular signatures shared by *P. atlantica* with *Korotnevella*, *Pseudoparamoeba* and *Vexillifera*, but absent in *P. eilhardi* and *Neoparamoeba* spp. were found in the sequence at positions 226, 319, 547, 1022, 1044 and 1081.

Small-subunit rRNA gene attributed to a PLO was co-amplified, cloned and sequenced together with the nuclear SSU rRNA gene of an amoeba. In phylogenetic trees this sequence was robustly a sister branch to a monophyletic clade of PLOs from different species of *Neoparamoeba* spp. (Fig. 22). The clade of PLOs from *Neoparamoeba* spp. and *P. atlantica* was sister to *Ichthyobodo* spp.

Morphology, cell coat and SSU rDNA of *P. eilhardi* CCAP 1560/2

A CCAP culture of *P. eilhardi* showed a good growth under the culturing conditions used, and no traces of the contamination with other eukaryotes were ever seen. During locomotion (Figs 23–27) amoebae were mostly longer than broad, and had a dactylopodial morphotype. Numerous blunt dactylopodia were formed from both, edge of the cell (Figs 23, 25) and its dorsal surface (Figs 24, 29). They could be as long as the entire locomotive form (Fig. 27). Many cells formed dorsal longitudinal ridges that continued anteriorly into dactylopodia (Fig. 24). An uroid was mostly plicate (Fig. 26). Length of the locomotive form was 28–63 μm (average 45 μm), breadth 11–40 μm (average 26 μm), length : breadth ratio was 0.93–3.10 (average 1.80) (n = 48). Rate of locomotion at 18°C was 14–35 μm/min (average 24 μm/min.) (n = 6) comprising approximately 0.5–1 cell length per minute. Locomotion was very unstable; amoebae often changed the direction of movement, and the locomotive rate of the same cell could change in up to ca. 2 times within minutes. Floating form formed numerous tapering, slender, hyaline pseudopods radiating

from the central mass of the cytoplasm. Amoebae had a single vesicular nucleus (Fig. 28) 5–10 μm in diameter (average 8 μm) with a central nucleolus, 3–6 μm in diameter (average 4 μm) (n = 14). About 75% of the cells contained two parasomes adjacent to the nucleus (Fig. 28), the rest of the cells had three or, rather exceptionally, one parasome. Length of the parasome was 6–8 μm (average 7 μm), breadth 3–5 μm (average 4 μm) (n = 18). Scanning electron microscopy and transmission electron microscopy of the negatively-stained whole mounts (Figs 29–31) shows that the cell surface was entirely covered with the boat-shaped scales consisting of a base plate and an upper rim connected to the periphery of the base plate with eight upright bars (Figs 30, 31). Length of the scale was 357–490 nm (average 415 nm), width 179–238 nm (average 212 nm), height 107–200 nm (average 154 nm) (n = 20).

Sequenced molecular clones of SSU rDNA were 2137–2142 base pairs long and had a G + C content of 41.46–41.97%. A slight sequence variation between clones was seen. This variation was comparable to that occurring between the newly obtained sequences and the previously published SSU rDNA sequence attributed to *P. eilhardi* (Mullen *et al.* 2005; GenBank accession No AY686575). Identity percentage between the newly obtained sequences and a previously published one was 96.9–97.5 (average 97.2) while that within the newly obtained sequences was 97–97.8 (average 97.5). Regular substitutions (i.e. those present in all molecular clones compared to a sequence AY686575) occurred in 0.5% of all nucleotide positions.

DISCUSSION

Species identification

Morphology and ultrastructure of the newly isolated amoeba fully correspond to the diagnosis of the genus *Paramoeba* as emended by Page (1987). We made a direct comparison of this strain with the only described species of this genus, *P. eilhardi*, using CCAP strain 1560/2 on which all current knowledge on *P. eilhardi* is based. A re-investigation of this strain has shown that its light microscopic and cell surface characteristics are in accordance with the published descriptions of *P. eilhardi* (Cann and Page 1982; Grell and Benwitz 1966, 1970; Page 1983), except that the size of the amoebae studied here was somewhat smaller than reported in the literature. Re-sequencing of the SSU rDNA of
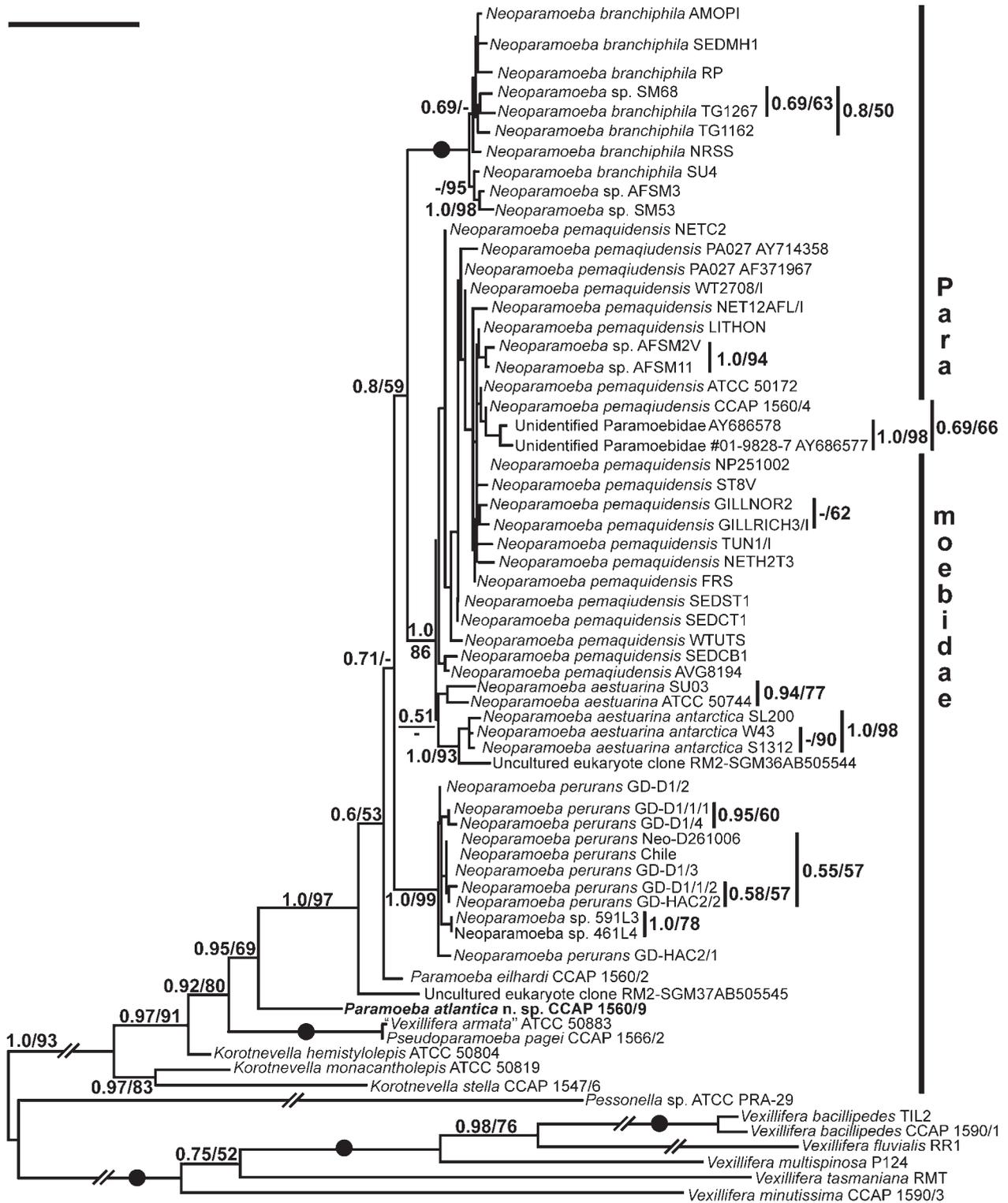
Fig. 21. Maximum likelihood tree based on 66 small subunit (SSU) rRNA gene sequences of Dactylopodida showing position of *Paramoeba atlantica* n. sp. (in bold). The tree shown was derived based on 1556 nucleotide positions using the program RaxML Version 7.2.6 (Stamatakis 2006) and has a LnL = −11130.11 and a gamma distribution α = 0.65. Numbers at nodes indicate Bayesian posterior probabilities/ bootstrap values if above 0.5/50%. Solid circles = 1.0/100. Dash indicates value below 50%, while asterisk, that the branch does not exist in the tree derived with that algorithm. Length of the disrupted clades was reduced 2 times. Scale bar: 0.05 substitutions/site.
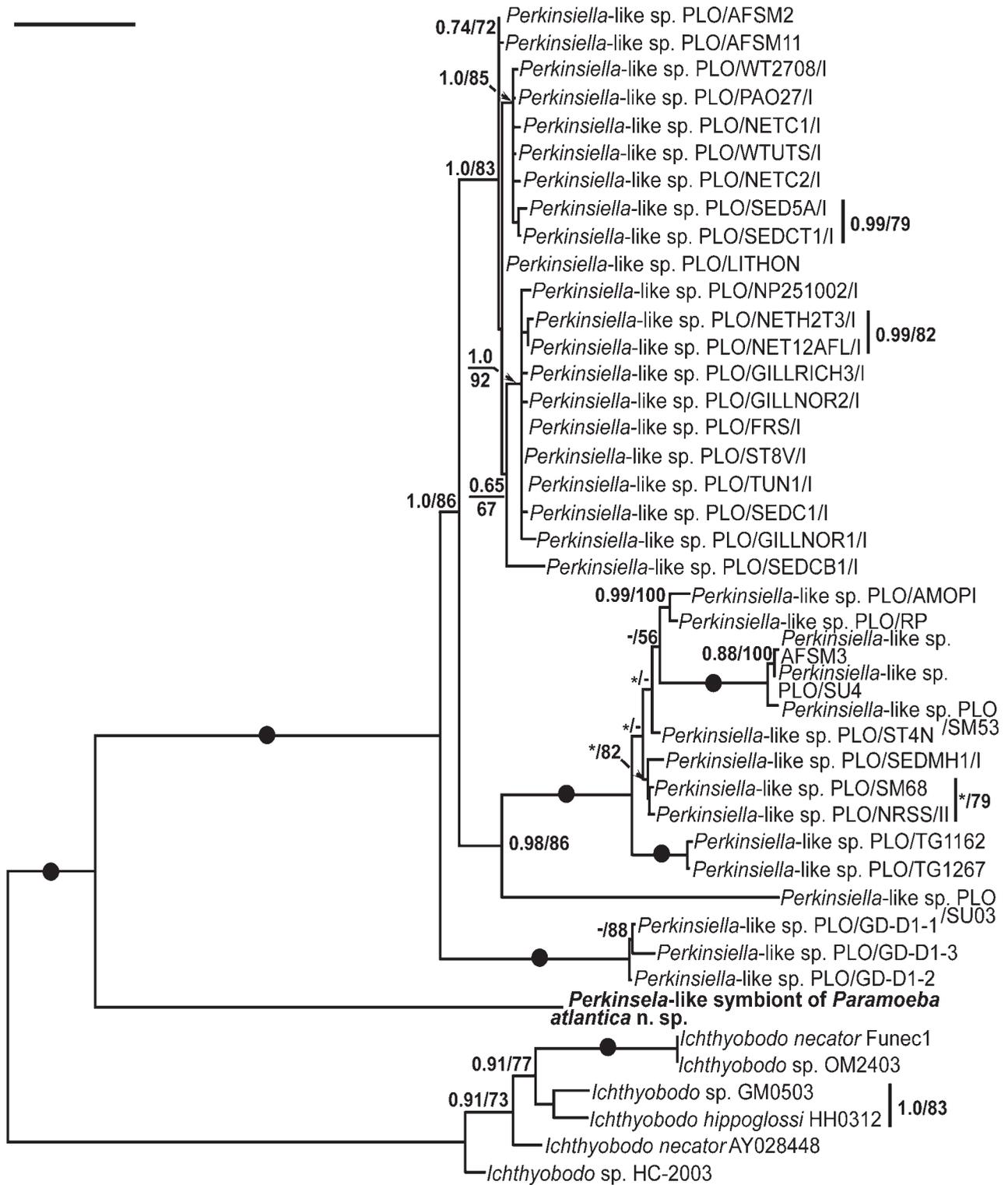
Fig. 22. Maximum likelihood phylogenetic tree of 37 small subunit (SSU) rRNA gene sequences of *Perkinsela*-like symbionts of *Neoparamoeba* spp. (here designated as *Perkinsiella* according to the current GenBank annotation), rooted with *Ichthyobodo* spp. (6 sequences). New sequence of *Perkinsela*-like symbiont of *Paramoeba atlantica* is in bold. The tree shown was derived based on 1466 nucleotide positions using the program RaxML Version 7.2.6 (Stamatakis 2006) and has a LnL = –6227.04 and a gamma distribution α = 1.18. Numbers at nodes indicate Bayesian posterior probabilities/bootstrap values if above 0.5/50%. Solid circles = 1.0/100. Dash indicates value below 50%, while asterisk, that the branch does not exist in the tree derived with that algorithm. Scale bar: 0.05 substitutions/site.
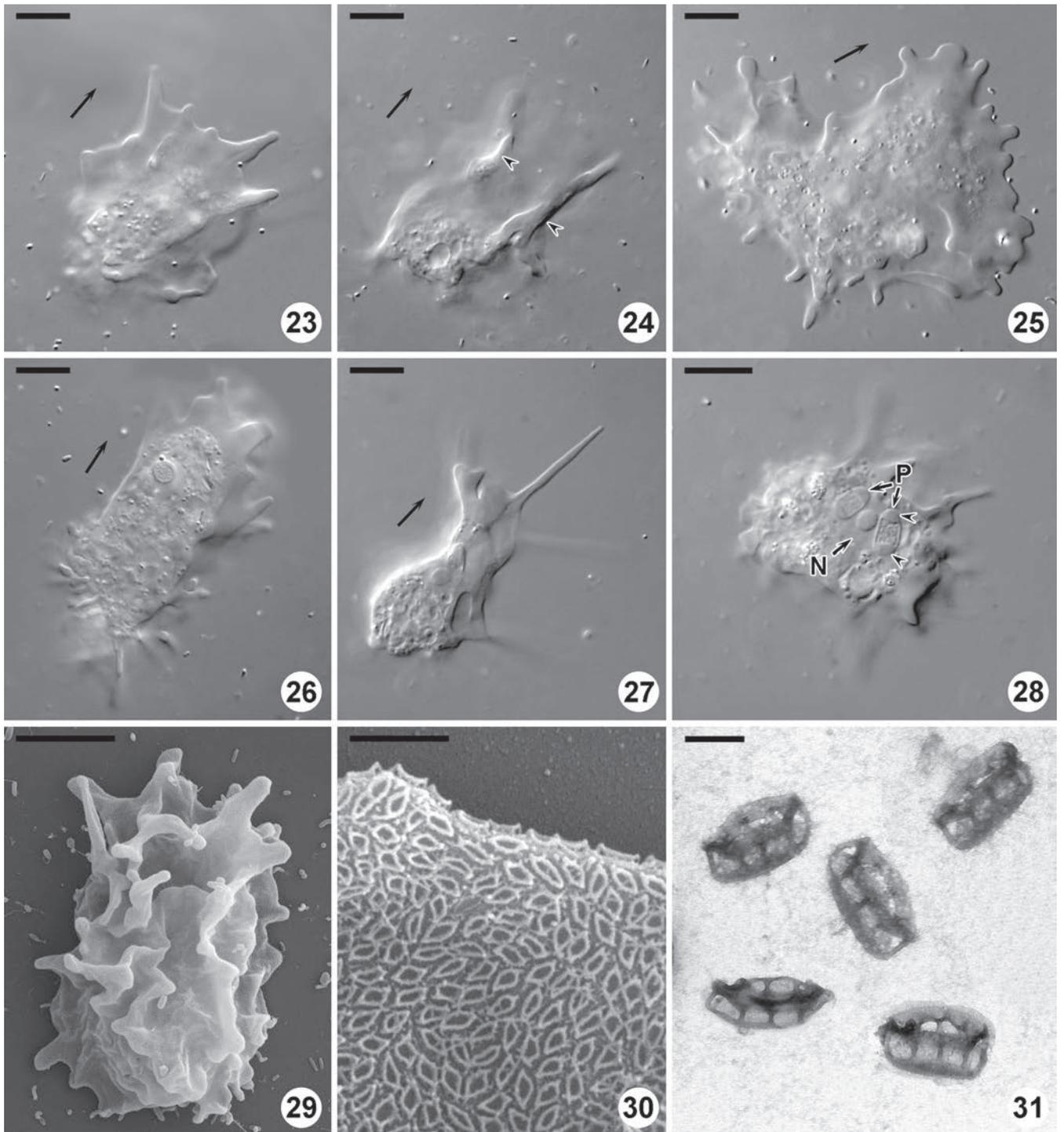
Figs 23–31. *Paramoeba eilhardi* CCAP 1560/2. **23–27** – locomotive forms on the glass surface. Fig. 24 is the dorsal view of the cell shown in 23, arrowheads indicate longitudinal ridges. Arrows indicate direction of movement; **28** – nucleus (N) and two PLOs (P) in a living cell. Arrowheads indicate "Seitenteile"; **29–30** – scanning micrographs demonstrating the whole cell (29) and microscales on the cell surface at a higher magnification (30); **31** – transmission electron micrograph of the negatively stained scales. Scale bar: 1 µm in Fig. 30, 0.25 µm in Fig. 31, 10 µm in other figures.

this strain demonstrates that it is identical with the previously obtained sequence (AY686575; Mullen *et al.* 2005), that was hence correctly attributed to *P. eilhardi*.

The new isolate differs from *P. eilhardi* in having a broader frontal hyaloplasm, not producing long subpseudopodia during locomotion (like shown in Fig. 27), smooth uroid, structure of the microscales and a very small fraction of amoebae cells hosting two symbionts as well as absence of the cells with more than two symbionts. Though the latter character may be more strain- than species-specific (e.g. Page 1983), it is still mentioned here, as the nature of host-symbiont relationships in these amoebae, and of the observed variability is not yet clear. This does not allow to exclude the species-specificity of this character completely. It is impossible to compare the details of the cytoplasmic ultrastructure, as the problems with fixation quality of a new isolate were not overcome. Interestingly, similar problems occurred during the ultrastructural study of *P. eilhardi* (Grell and Benwitz 1970), that partly could be overcome by the application of the Dalton's (1955) protocol. Yet, the application of this protocol in our study did not significantly improve the results. Small-subunit rDNA sequence analysis demonstrates remarkable differences between the strains and shows that in the phylogenetic trees (Figs 20, 21) *P. eilhardi* always branches distantly from our isolate. Therefore, we establish *Paramoeba atlantica* n. sp. to accommodate the studied amoeba. Interestingly, the strain studied here is morphologically similar to an unnamed amoeba isolated by Smirnov (1999) from the anaerobic sediments of the Nivå Bay (The Sound, Baltic Sea) and identified as *Neoparamoeba* sp. Both strains are similar in size and shape of the locomotive form, nucleus and subpseudopodia. However, the ultrastructure of the Nivå Bay strain is unknown, therefore it is not possible to conclude whether both amoebae really belong to the same morphospecies and the same genus.

Phylogenetic position of *P. atlantica* and taxonomy of Dactylopodida

In the molecular phylogenetic analysis presented here two microscale-bearing species *P. eilhardi* and *P. atlantica* branch separately. The former species is within *Neoparamoeba* spp. and forms a clade with an uncultured amoebozoan that is sister to *N. perurans* (Fig. 21), while *P. atlantica* is sister to the whole clade of *Neoparamoeba* spp./*P. eilhardi*. Only part of the tree is well-resolved: while position of *P. atlantica* as well as the clade of *Neoparamoeba* + *P. eilhardi* are supported

well, the position of *P. eilhardi* is never highly supported, but at the same time it never alters with the algorithm of tree reconstruction. An alternative branching for *P. eilhardi* shown by Dyková *et al.* (2008) in maximum parsimony trees was never reproduced in our analysis. Topology tests also reliably reject all hypotheses that imply a monophyletic clade of two *Paramoeba* spp.

Based on our re-investigation of *P. eilhardi*, we can exclude the possibility that its sequence has been misattributed to *P. eilhardi*, being instead a sequence of a *Neoparamoeba* sp. (e.g. contaminant in the culture) as suggested earlier (Dyková *et al.* 2007). Therefore, two explanations remain possible for the revealed branching of two *Paramoeba* spp. First, the poorly supported position of *P. eilhardi* in the phylogenetic tree may indicate an insufficient taxon sampling for *Paramoeba*, and the tree configuration may change substantially, once more scale-bearing species are added. In this case *P. eilhardi* may finally form a clade with *P. atlantica*. However, SSU rRNA sequence signatures shared by *P. atlantica* with *Korotnevella* and *Pseudoparamoeba* but not with *P. eilhardi* and *Neoparamoeba* spp. weaken this suggestion. Second, if the position of *P. eilhardi* in the tree is correct, the results obtained here suggest that *Paramoeba* and *Neoparamoeba* as defined by Page (1987) are paraphyletic, and the latter name should be abandoned as a junior synonym of *Paramoeba*. This explanation implies that presence of boat-shaped surface microscales is ancestral to the clade of *Paramoeba*/*Neoparamoeba* + *Korotnevella*; one or several losses of microscales must have then occurred in the clades of *Neoparamoeba* spp., probably correlated with the development of an amphizoic way of life. This is in accordance with the hypothesis of the cell coat evolution proposed by Smirnov *et al.* (2007), in the sense that the microscales of *Paramoeba*/*Neoparamoeba* might have evolved in a similar way to the glycostyles of *Vannella*/*Platyamoeba*, having been lost several times independently in different evolutionary lineages of amoebae. In this case additional differences used by Page (1987) to separate *Neoparamoeba* from *Paramoeba* (like dorsal longitudinal ridges during locomotion) also seem to be non-valid at the generic level, as both, *P. atlantica* and *P. eilhardi* have microscales and longitudinal ridges. Therefore a future re-definition of the genus *Paramoeba* could be possible based on the dactylopodial morphotype and a PLO shared by *Paramoeba* and *Neoparamoeba*, regardless of the cell surface organisation. Currently this formal change seems to be premature, as the position of *P. eilhardi* is poorly supported and its

alternative explanation is possible, but it should be kept in mind for future taxonomic work. A re-unification of *Paramoeba* and *Neoparamoeba* would not affect the validity and position of *Janickina*. It should remain *incertae sedis* until gene sequence data are available, as it has a limax locomotive form and a villous-bulb uroid (Chatton 1953, Hollande 1980) never observed in other parasome-bearing species.

Based on the present phylogenetic analysis and recent data of Dyková *et al*. (2011), the families Paramoebidae (*Paramoeba* and *Korotnevella*) and Vexilliferidae (*Vexillifera*, *Neoparamoeba* and *Pseudoparamoeba*) re-defined by Page (1987) based on the cell surface structure, the shape of subpseudopodia and presence or absence of dorsal folds during locomotion, and remaining unchanged since then (Adl *et al*. 2005, Cavalier-Smith *et al*. 2004, Smirnov *et al*. 2005, Smirnov *et al*. 2011), need a reassessment. Our data and all previously published phylogenetic trees show that if Paramoebidae includes *Korotnevella* and *Paramoeba*, it should also include *Neoparamoeba* and *Pseudoparamoeba*, otherwise both Vexilliferidae and Paramoebidae are paraphyletic. Vexilliferidae in this case should comprise *Vexillifera* spp. (but not "*V. armata*" ATCC 50883 branching with *Pseudoparamoeba pagei*, that was most probably misidentified and requires a reinvestigation; Dyková *et al*. 2011) and an amoeba PRA-29 identified as "*Pessonella* sp." (Tekle *et al*. 2008). Both families are then monophyletic, and we provide new diagnoses for them to accommodate the proposed changes in the taxonomic composition.

Being isolated from the distant and poorly accessible locality, *P. atlantica* is a good example of how the survey of amoebae from poorly studied habitats may lead to expansion of our knowledge on the diversity of this group and influence the established classification schemes. The sediment sampling method used (Van Veen grab, Van Veen 1933) did not allow a precise determination of whether amoebae were really isolated from the bottom or water column (hence whether they can be considered deep-sea), but this is further evidence highlighting the extent to which the protozoan diversity in the oceans is understudied (e.g. Atkins *et al*. 2000; Hausmann *et al*. 2002a, b; Moran *et al*. 2007), especially in the bottom sediments. For Amoebozoa, for example, only two papers are available with in total 11 morphospecies recorded (Hausmann *et al*. 2002a, Moran *et al*. 2007), and only the latter one, where 3 morphospecies were found, contains complete descriptions and illustrations of the species observed.

Diagnoses of new and emended taxa

Position in the system according to Smirnov *et al*. 2011.

Phylum Amoebozoa Lühe, 1913
Subphylum Lobosa Carpenter, 1861
Class Discosea Cavalier-Smith, 2004
Subclass Flabellinia Smirnov *et al*., 2005
Order Dactylopodida Smirnov *et al*., 2005

Family Paramoebidae Poche, 1913, emend.

Flattened dactylopodial amoebae with blunt, hyaline subpseudopodia, conical or finger-shaped in outline. Cell coat consists of microscales, dense amorphous glycocalyx that may include hair-like structures, or dome-shaped glycostyles with hexagonal bases.

Genera: *Paramoeba* Schaudinn, 1896 (type genus), *Korotnevella* Goodkov, 1988, *Neoparamoeba* Page, 1987, *Pseudoparamoeba* Page, 1979.

Paramoeba atlantica n. sp.

Diagnosis: Length of the locomotive form 23–65 μm (average 36.5 μm), breadth 12–31 μm (average 21 μm), length : breadth ratio 0.92–3.42 (average 1.78) (n = 117). During locomotion flattened, with wide anterior hyaloplasm and dorsal longitudinal ridges; conical or finger-shaped hyaline subpseudopodia produced from anterior margin and dorsal surface of the cell. Single vesicular nucleus 4–9 μm in diameter (average 6 μm), spherical central nucleolus 1.5–5 μm in diameter (average 3 μm) (n = 37). Single PLO adjacent to nucleus, ovoid, 5–8 μm long (average 12 μm) and 1–2 μm broad (average 1.6 μm) (n = 25); rarely two parasomes. Cell coat consists of boat-shaped microscales with delicate, homogeneous walls; length of the scale base 0.21–0.37 μm (average 0.32 μm), breadth 0.12–0.24 μm (average 0.18 μm) (n = 74); height of the scale 0.08–0.17 μm (average 0.13 μm) (n = 54).

Observed habitat: marine, bottom sediments of the Great Meteor Seamount, eastern Atlantic Ocean (29°36.29′N; 28°59.12′W; depth 267.4 m).

Type material: type strain is deposited with CCAP (Oban, UK), accession number 1560/9.

Etymology: *atlantica*, refers to the Atlantic Ocean where the strain was collected.

Differential diagnosis: differs from *P. eilhardi* in the structure of microscales, shape during locomotion and predominating number of parasomes per cell; from *P. perniciosa* in size of the cell, nucleus and PLO.

Family Vexilliferidae Page, 1987, emend.

Elongated flattened amoebae of acanthopodial morphotype, with one or more long, slender, hyaline subpseudopodia, rarely formed in some species. Cell coat consisting of delicate glycostyles that can be prismatic with hexagonal cross-section, or t-shaped in vertical section.

Genera: *Vexillifera* Schaeffer, 1926 (type genus); a discosean amoeba PRA-29 identified as "*Pessonella*" sp. may also belong to this family, but currently available data (Tekle *et al.* 2008) do not provide a morphological evidence for this suggestion; therefore, the presented diagnosis is based entirely on features of *Vexillifera* spp.

REFERENCES

Adl S. M., Simpson A. G. B., Farmer M. A., Andersen R. A., Anderson O. R., Barta J. R., Bowser S. S., Brugerolle G., Fensome R. A., Fredericq S., James T. Y., Karpov S., Kugrens P., Krug J., Lane C. E., Lewis L. A., Lodge J., Lynn D. H., Mann D. G., McCourt R. M., Mendoza L., Moestrup Ø., Mozley-Standridge S. E., Nerad T. A., Shearer C. A., Smirnov A. V., Spiegel F. W., Taylor M. F. J. R. (2005) The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *J. Eukaryot. Microbiol.* **52:** 399–451

Altekar G., Dwarkadas S., Huelsenbeck J. P., Ronquist F. (2004) Parallel metropolis-coupled Markov chain Monte Carlo for Bayesian phylogenetic inference. *Bioinformatics* **20:** 407–415

Atkins M. S., Teske A. P., Anderson O. R. (2000) A survey of flagellate diversity at four deep-sea hydrothermal vents in the eastern Pacific Ocean using structural and molecular approaches. *J. Eukaryot. Microbiol.* **47:** 400–411

Cann J. P., Page F. C. (1982) Fine structure of small free-living *Paramoeba* (Amoebida) and taxonomy of the genus. *J. Mar. Biol. Ass.U. K.* **62:** 25–43

Cavalier-Smith T., Chao E.-Y., Oates B. (2004) Molecular phylogeny of Amoebozoa and evolutionary significance of the unikont *Phalansterium. Eur. J. Protistol.* **40:** 21–48

Chatton E. (1953) Ordre des Amoebiens nus ou Amoebaea. In: Traité de Zoologie, (Ed. P. Grassé). Masson, Paris, **1:** 5–91

Dalton A. J. (1955) A chrome-osmium fixative for electron microscopy. *Anat. Rec.* **121:** 281

Dyková I., Nowak B. F., Crosbie P. B. B., Fiala I., Pecková H., Adams M. B., Macháčková B., Dvořáková H. (2005) *Neoparamoeba branchiphila* n. sp., and related species of the genus *Neoparamoeba* Page, 1987: morphological and molecular characterization of selected strains. *J. Fish Dis.* **28:** 49–64

Dyková I., Nowak B., Pecková H., Fiala I., Crosbie P., Dvořáková H. (2007) Phylogeny of Neoparamoeba strains isolated from marine fish and invertebrates as inferred from SSU rDNA sequences. *Dis. Aquat. Org.* **74:** 57–65

Dyková I., Fiala I., Pecková H. (2008) *Neoparamoeba* spp. and their eukaryotic endosymbionts similar to *Perkinsela amoebae* (Hollande, 1980): Coevolution demonstrated by SSU rRNA gene phylogenies. *Eur. J. Protistol.* **44:** 269–277

Dyková I., Kostka M., Pecková H. (2011) Three new species of the amoebozoan genus *Vexillifera* Schaeffer, 1926. *Acta Protozool.* **50:** 55–63

Fahrni J. F., Bolivar I., Berney C., Nassonova E., Smirnov A., Pawlowski J. (2003) Phylogeny of lobose amoebae based on actin and small-subunit ribosomal RNA genes. *Mol. Biol. Evol.* **20:** 1881–1886

Fiala I., Dyková I. (2003) Molecular characterization of *Neoparamoeba* strains isolated from gills of *Scophthalmus maximus. Dis. Aquat. Org.* **55:** 11–16

Galtier N., Gouy M., Gautier C. (1996) SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. *Comput. Appl. Biosci.* **12:** 543–548

Grell K. G. (1961) Über den Nebenkörper von *Paramoeba* Schaudinn. *Arch. Protistenkd.* **105:** 303–312

Grell K. G., Benwitz G. (1966) Die Zellhülle von *Paramoeba eilhardi* Schaudinn. *Z. Naturforsch.* **21b:** 600–601

Grell K. G., Benwitz G. (1970) Ultrastruktur mariner Amöben. I. *Paramoeba eilhardi* Schaudinn. *Arch. Protistenkd.* **112:** 119–137

Harris J. R. (1999) Negative staining of thinly spread biological particulates. In: Electron microscopy methods and protocols, (Ed. M. A. Nasser Hajibagheri). Methods in molecular biology, Humana Press, Totowa, NJ, **117:** 13–30

Hausmann K., Hülsmann N., Polianski I., Schade S., Weitere M. (2002a) Composition of benthic protozoan communities along a depth transect in the eastern Mediterranean Sea. *Deep-Sea Res. I* **49:** 1959–1970

Hausmann K., Weitere M., Wolf M., Arndt H. (2002b) *Meteora sporadica* gen. nov. et sp. nov. (Protista incertae sedis) – an extraordinary free-living protist from the Mediterranean deep sea. *Eur. J. Protistol.* **38:** 171–177

Hollande A. (1980) Identification du parasome (Nebenkern) de *Janickina pigmentifera* à un symbionte *(Perkinsiella amoebae* nov. gen.-nov. sp.) apparenté aux flagellés kinetoplastidies. *Protistologica* **16:** 613–625

Huelsenbeck J. P., Ronquist F. (2001) MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* **17:** 754–755

Janicki C. (1912) Paramoebenstudien *(P. pigmentifera* Grassi und *P. chaetognatha* Grassi). *Z. Wiss. Zool.* **103:** 449–518

Jobb G. (2008) *TREEFINDER version of June 2008.* Munich, Germany. Distributed by the author at www.treefinder.de

Kudryavtsev A., Pawlowski J., Hausmann K. (2009) Description and phylogenetic relationships of *Spumochlamys perforata* n. sp. and *Spumochlamys bryora* n. sp. (Amoebozoa, Arcellinida). *J. Eukaryot. Microbiol.* **56:** 495–503

Kudryavtsev A., Wylezich C., Pawlowski J. (2011) *Ovalopodium desertum* n. sp. and the phylogenetic relationships of Cochliopodiidae (Amoebozoa). *Protist* **162:** 571–589

Maniatis T., Fritsch E. F., Sambrook J. (1982) Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Medlin L., Elwood H. J., Stickel S., Sogin M. L. (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA coding regions. *Gene* **71:** 491–499

Moran D. M., Anderson O. R., Dennett M. R., Caron D. A., Gast R. J. (2007) A description of seven Antarctic marine gymnamoebae including a new subspecies, two new species and a new genus: *Neoparamoeba aestuarina antarctica* n. subsp., *Platyamoeba oblongata* n. sp., *Platyamoeba contorta* n. sp. and *Vermistella antarctica* n. gen. n. sp. *J. Eukaryot. Microbiol.* **54:** 169–183

Mullen T. E., Nevis K. R., O'Kelly C. J., Gast R. J., Frasca S. (2005) Nuclear small-subunit ribosomal RNA gene-based characterization, molecular phylogeny and PCR detection of the *Neoparamoeba* from western Long Island Sound lobster. *J. Shellfish Res.* **24:** 719–731

Page F. C. (1970) Two new species of *Paramoeba* from Maine. *J. Protozool.* **17:** 421–427

Page F. C. (1973) *Paramoeba*: a common marine genus. *Hydrobiologia* **41:** 183–188

Page F. C. (1983) Marine Gymnamoebae. Inst. Terr. Ecol., Cambridge

Page F. C. (1987) The classification of 'naked' amoebae (phylum Rhizopoda). *Arch. Protistenkd.* **133:** 199–217

Peglar M. T., Amaral Zettler L. A., Anderson O. R., Nerad T. A., Gillevet P. M., Mullen T. E., Frasca S., Jr., Silberman J. D., O'Kelly C. J., Sogin M. L. (2003) Two new small-subunit ribosomal RNA gene lineages within the subclass Gymnamoebia. *J. Eukaryot. Microbiol.* **50:** 224–232

Ronquist F., Huelsenbeck J. P. (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19:** 1572–1574

Smirnov A. V. (1999) An illustrated survey of gymnamoebae – Euamoebida and Leptomyxida (Rhizopoda, Lobosea), isolated from anaerobic sediments of the Nivå Bay (Baltic Sea, The Sound). *Ophelia* **50:** 113–148

Smirnov A. V., Brown S. (2004) Guide to the methods of study and identification of soil gymnamoebae. *Protistology* **3:** 148–190

Smirnov A., Nassonova E., Berney C., Fahrni J., Bolivar I., Pawlowski J. (2005) Molecular phylogeny and classification of the lobose amoebae. *Protist* **156:** 129–142

Smirnov A. V., Nassonova E. S., Chao E., Cavalier-Smith T. (2007) Phylogeny, evolution and taxonomy of vannellid amoebae. *Protist* **158:** 295–324

Smirnov A. V., Chao E., Nassonova E. S., Cavalier-Smith T. (2011) A revised classification of naked lobose amoebae (Amoebozoa: Lobosa). *Protist* **162:** 545–570

Sprague V., Beckett R. L., Sawyer T. K. (1969) A new species of *Paramoeba* (Amoebida, Paramoebidae) parasitic in the crab *Callinectes sapidus. J. Invertebr. Pathol.* **14:** 167–174

Stamatakis A. (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22:** 2688–2690

Tekle Y., Grant J., Anderson O. R., Nerad T. A., Cole J. C., Patterson D. J., Katz L. A. (2008) Phylogenetic placement of diverse amoebae inferred from multigene analyses and assessment of clade stability within 'Amoebozoa' upon removal of varying rate classes of SSU-rDNA. *Mol. Phylogenet. Evol.* **47:** 339–352

Van Veen J. (1933) Onderzoek naar het zandtransport van rivieren. *De Ingenieur. B. Bouw- en Waterbouwkunde* **27:** 151–159

Wong F. Y. K., Carson J., Elliott N. G. (2004) 18S ribosomal DNA-based PCR identification of *Neoparamoeba pemaquidensis*, the agent of amoebic gill disease in sea-farmed salmonids. *Dis. Aquat. Org.* **60:** 65–76

Young N. D., Crosbie P. B. B., Adams M. B., Nowak B. F., Morrison R. N. (2007) *Neoparamoeba perurans* n. sp., an agent of amoebic gill disease of Atlantic salmon (*Salmo salar*). *Int. J. Parasitol.* **37:** 1469–1481