Acta Protozool. (2011) 50: 57–65
http://www.eko.uj.edu.pl/ap

ACTA
PROTOZOOLOGICA

Three New Species of the Amoebozoan Genus *Vexillifera* Schaeffer, 1926

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Summary. Five amoeba strains isolated from organs of fish were selected by morphology as possible members of the genus *Vexillifera* Schaeffer, 1926. Phylogenetic analyses of SSU rDNA sequences revealed that four of these strains form a best supported clade together with *Vexillifera minutissima* (strain CCAP 1590/3) and *V. bacillipedes* (sequence of the type culture was newly generated in this study). Comparison of trophozoite morphology and SSU rDNA sequences identified one of the five fish-isolated strains as closely related to *V. bacillipedes*, and characteristics of another three strains allowed to establish three new species: *Vexillifera multispinosa*, *V. fluvialis* and *V. tasmaniana*. The enlargement of dataset of *Vexillifera* SSU rDNA sequences enables us to doubt the identification of ATCC strain 50883 designated as *V. armata*. Since sequence of this strain branched in our analysis in a distant phylogenetic position within *Korotnevela* and *Neoparamoeba* clade, the *Vexillifera* origin of this sequence is called in question. The same applies to the newly generated sequence of the type strain of *V. expectata*, previously characterised by its morphology only.

Key words: *Vexillifera multispinosa*, *Vexillifera fluvialis*, *Vexillifera tasmaniana*, new species, taxonomy, phylogeny.

INTRODUCTION

The early systematic history of the genus *Vexillifera* Schaeffer, 1926 was summarized by authors who subsequently described more than 20 *Vexillifera* species from freshwater and marine environments (Bovee 1951, 1956, 1985; Page 1969, 1972, 1979a, b, 1991). As quoted by Page (1969), the genus *Vexillifera* Schaeffer, 1926 was erected for amoebae with long slender subpseudopodia extending from the anterior end during locomotion, which are capable of moving about in the water "after the manner of tentacles." Majority of the species listed within this genus was described on the basis of light microscopic observations, results of which were documented with line drawings. The ultrastructure was studied in five species, *V. armata* (see Page 1979b), *V. bacillipedes* (see Page 1979a), *V. minutissima* (see Page 1983), *V. granatensis* (see Mascaro *et al.* 1985), and *V. telmathalassa* (see Anderson 1994). Similarly, limited data are available on molecular markers. To date, only two sequences declared to represent *Vexillifera* species (*V. armata* and *V. minutissima*) have been used for phylogenetic reconstructions, either individually (Fahrni *et al.* 2003, Peglar *et al.* 2003, Nikolaev *et al.* 2005) or jointly (Nikolaev *et al.* 2006, Smirnov

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et al. 2005, Tekle *et al.* 2008), but none of those was generated from the type strains. Two *Vexillifera* strains (*V. bacillipedes* and *V. minutissima*) are available in the UK National Culture Collection, of which only one (*V. bacillipedes*) is the type strain (CCAP 1590/1).

In the course of morphological sorting of amoeba strains that we isolated from organs of fish, several strains attracted our attention as they exhibited essential morphological features for their allocation to *Vexillifera*. Detailed examination of their characters with light and electron microscope and research of correlation of their morphology and phylogenetic relationships with vexilliferid amoebae previously characterized at molecular level are presented in this contribution.

MATERIALS AND METHODS

Five fish-isolated amoeba strains (P124, RR1, RMT, TIL2 and 4730) were included in the study together with the type strain of *Vexillifera bacillipedes* Page, 1969 obtained from UK National Culture Collection (UKNCC). The strains are listed in Table 1 that provides data on their geographic, host and organ origin.

The isolation of amoeba strains from freshwater fish was based on sterile sampling of organs followed by inoculation on 1.5% non-nutrient amoeba saline agar (see Rp. in Catalogue of the UK National Culture Collection, 2001), whereas 2.5% seawater agar (average water salinity 27 ppt) was used in marine fish tissue sampling. Primary isolates were subcultured using selected groups of several (4–8) morphologically identical cells, in week intervals.

Light-microscopical observations were made on live amoebae attached to the under side of cover slip in hanging drops, using Nomarski optics (Olympus BX51). Material for electron microscopy was collected and processed in the same way as described by Dyková *et al.* (2010b). Ultrathin sections were examined with a JEOL JEM 1010 electron microscope operating at 80 kV. Images were collected with Megaview II soft imaging system using analySIS software.

Samples of DNA of six amoeba strains (P124, RR1, RMT, TIL2, 4730 and CCAP 1590/1) were extracted from cell pellets using the JETQUICK Tissue DNA Spin Kit (Genomed, Germany) according to the manufacturer's protocol. PCR procedure described in Dyková *et al.* (2010a) was used for the amplification of SSU rRNA genes of two strains whereas procedure described in Dyková *et al.* (2010b) was used for amplification of another three strains. The amplicons were gel-purified and cloned into pCR® 2.1 TOPO Cloning Vector (Invitrogen, USA) or pDrive Cloning Vector (Qiagen GmbH, Germany) using the protocols suggested by the manufacturers. Sequencing reactions were performed using an automatic sequencer ABI 3130 × 1 with the ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA).

To infer the phylogenetic positions of six sequences obtained in this study and to ascertain the generic affiliation of the respective strains, a dataset was assembled that contained 44 SSU rDNA sequences of diverse amoebozoans representing 25 genera, with an accent on flabellineans. The final alignment of the length of 1,353 bp was prepared and analyzed by maximum likelihood, maximum parsimony, Fitch-Margoliash methods with LogDet distances and Bayesian method as described in Dyková *et al.* (2010b). Percent identities among the six sequences were calculated from an alignment trimmed to 2,199 bp. The same region was compared among the four *Paramoeba* / *Neoparamoeba* sequences included in our phylogenetic analysis and between *Korotnevella hemistylolepis* and *K. stella* for comparison.

RESULTS

Light microscopy and ultrastructure

When observed in hanging drop preparations, trophozoites of four fish-isolated strains (Fig. 1) displayed the same morphotype as trophozoites of the type culture of *Vexillifera bacillipedes* (CCAP 150/1) (Fig. 2) in that they had an irregular shape (triangular, spatulate, ovoid or oblong shape) with non-furcated, linear, actively waving subpseudopodia projecting from a flattened hyaloplasmic rim. Trophozoites of individual strains differed in size, proportion of granuloplasm and hyaloplasm, number of subpseudopodia and number of refractile inclusions located in the cytoplasm. The length of trophozoites (not including subpseudopodia) exceeded 20 µm in two strains, whereas the other two strains had smaller trophozoites (see below). The proportion of granuloplasm in individual strains increased due to its load with bacteria that correlated with the

Table 1. Amoeba strains included in the study.

Strain	Host and organ origin	Local origin
P124	*Leporinus fasciatus* (Bloch), gills	Amazon River, Peru, Iquitos
RR1	*Rutilus rutilus* (L.), spleen	Lužnice river, Czech Republic
RMT	*Salmo salar* L., gills	Salmon farm, Tasmania, Australia
TIL2	*Oreochromis niloticus* (L.), gills	Farm, Czech Republic
4730	*Perca fluviatilis* (L.), liver	Černovický brook, Czech Republic
CCAP1590/1	N.A. (environmental strain)	Rock River, Janesville, WI, USA

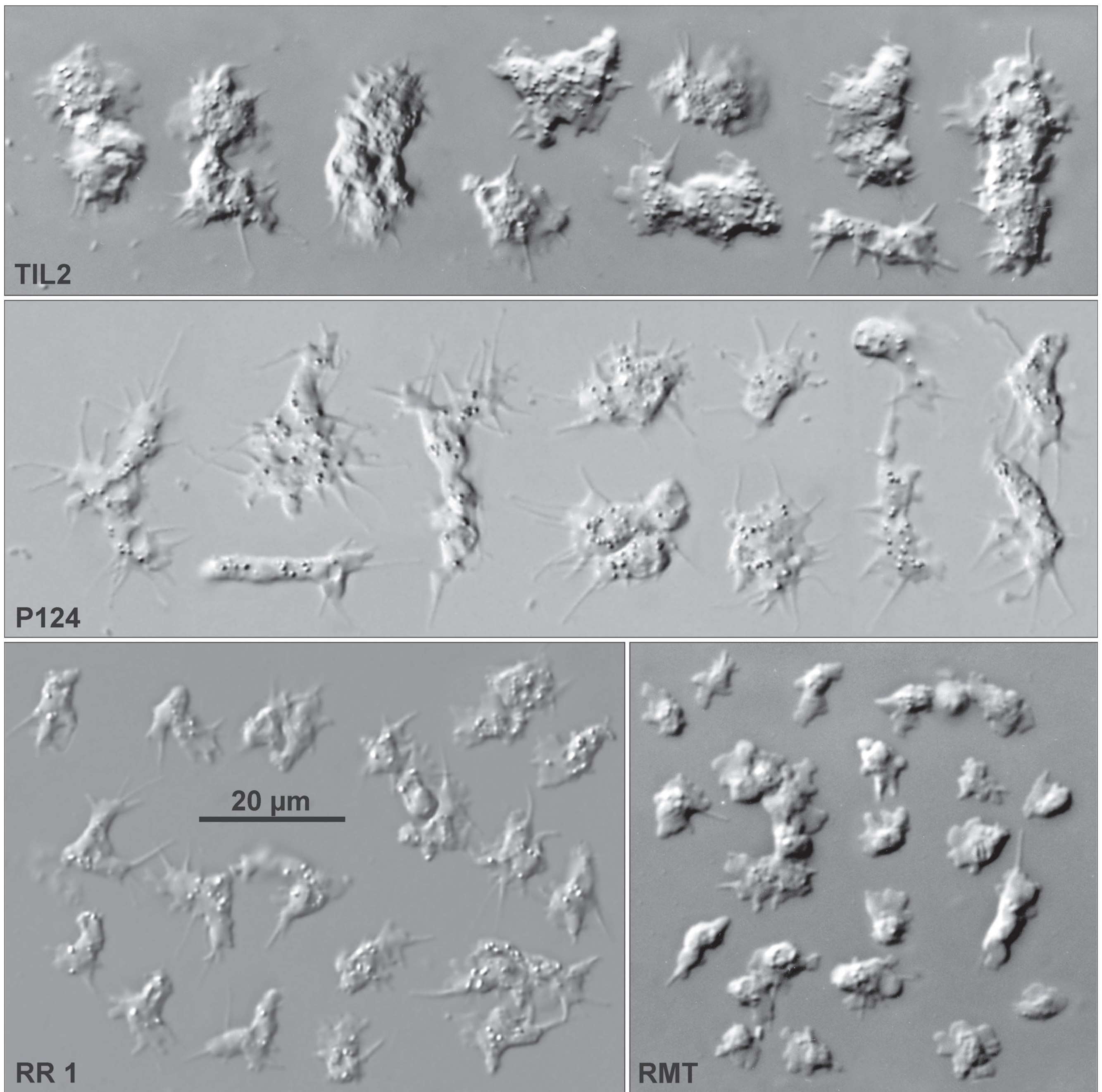


Fig. 1. Trophozoites of *Vexillifera* strains as seen in Nomarski differential interference contrast. TIL2 – closely related to *Vexillifera bacillipedes*, P124 – type strain of *V. multispinosa* sp. n., RR1 – type strain of *V. fluvialis* sp. n., RMT – type strain of *V. tasmaniana* sp. n. Scale bar applies to all strains.

length of subculturing period. The latter also influenced the visibility of nuclei in the cytoplasm when observed in the light microscope. Cysts were not observed in any of the strains studied.

Comparison of ultrastructure of individual strains pointed out to differences in the cell surface (Figs 3–7). They consisted in that a thin outer electron-dense layer and a glycocalyx either was amorphous or differentiated into glycostyles arranged in regular manner. Glycostyles looked either like cylinders in longitudinal sections and hexagons in cross sections, or they had T-shaped form in longitudinal sections. Unlike peculiarities observed

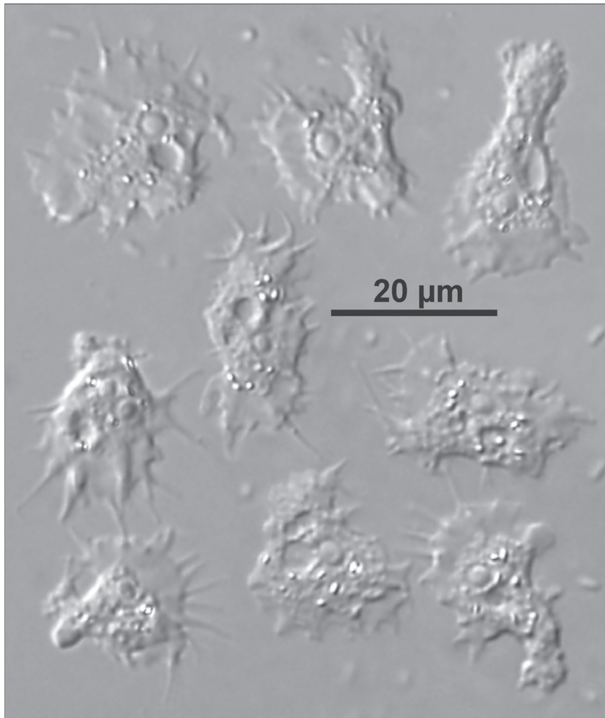


Fig. 2. Trophozoites of the type strain of *Vexillifera bacillipedes* (CCAP 1590/1).

on the cell surface, the other ultrastructural details were rather uniform in all strains under study. The cytoplasm contained ovoid, bean- or sausage-shaped mitochondria that possessed tubular, occasionally branching cristae (Fig. 8). Golgi complex consisted of parallel arrays of stacks of cisternae. If present in thin sections, contractile vacuole was surrounded by small vesicles. Regardless of the level of sectioning, nucleus had mostly an irregular outline with clearly visible nuclear membrane and electron-dense nucleolus in the interfase (Fig. 10).

Disappearance of nuclear membrane and corresponding changes of chromatin arrangement were also observed in several trophozoites (Fig. 11). Refractile bodies, conspicuous in the cytoplasm of trophozoites in the light microscope, could not be d in detail since they were extremely scarce in thin sections.

Re-examination of live trophozoites of *Vexillifera expectata* (strain 4730), species originally described using ultrastructural features and light microscopy of fixed material only (Dyková *et al.* 1998), corrected our previous opinion on the morphotype of this strain. Contrary to previous observations of fixed trophozoites, live individuals observed in hanging drop preparations were uniform, limax-like with anterior hyaline cap and differed substantially from the other four fish-isolated *Vexillifera* strains. In order to correct this error, a newly obtained SSU rDNA sequence was included in phylogenetic analysis.

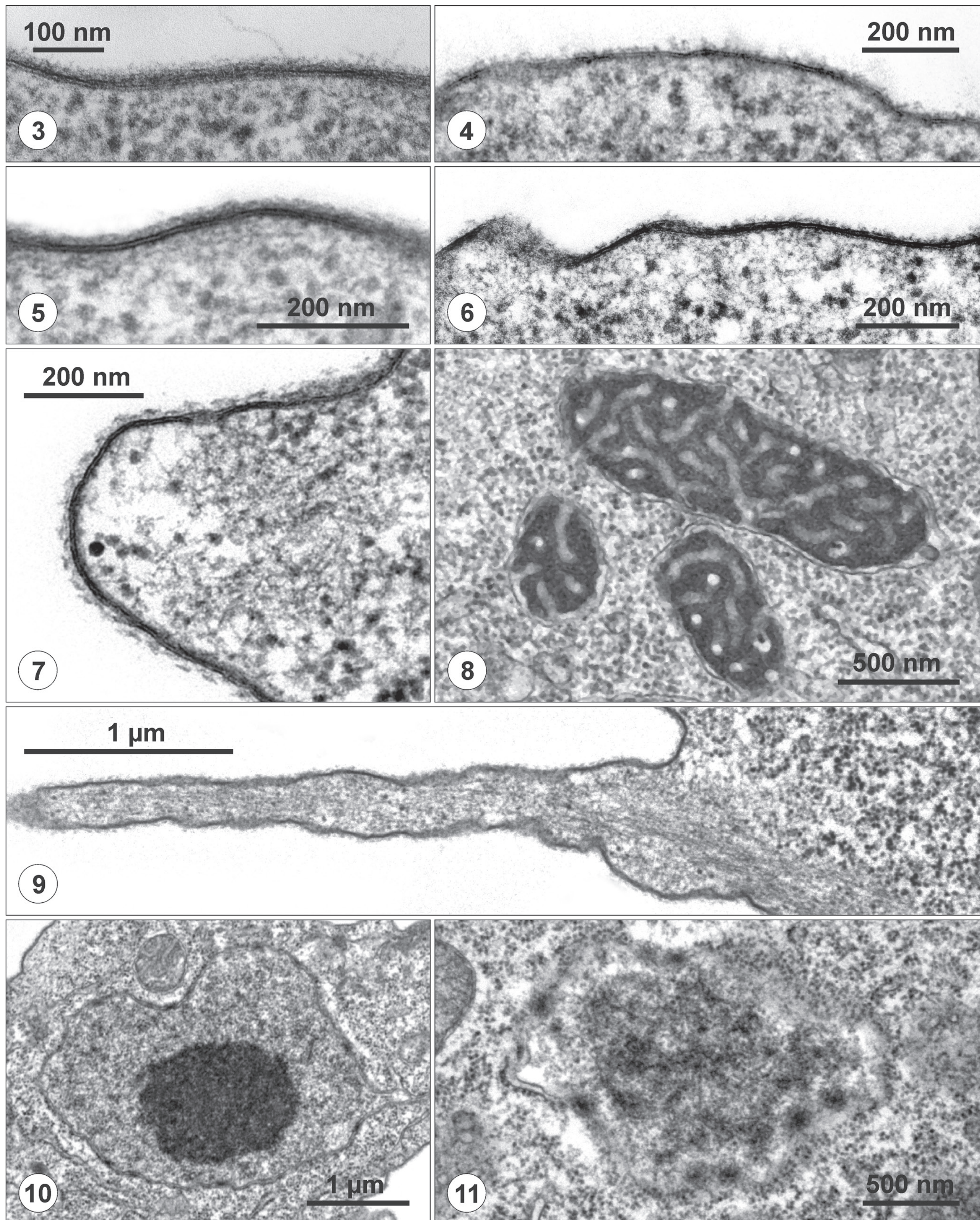
SSU rDNA sequences and phylogenetic analysis

The sequences obtained in this study and belonging to *Vexillifera* strains are deposited in GenBank database under Acc. Nos. HQ687481– HQ687485. Acc. No. of amoeba strain 4730 is HQ687486. The length of the five *Vexillifera* sequences ranges from 1,898 to 2,133 bp (see Table 2). Percent identities (see Table 2) of these sequences are quite low, from 63 to 74 % with one exception – the pair of *V. bacillipedes* and strain TIL2 (90% identical). The same region of SSU rDNA sequence is much less divergent in related genera of amoebae – the sequences of the four species of *Paramoeba* / *Neoparamoeba* included in our study are identical in at least 87% of their sequences, and the sequences of *Korotnevella hemistylolepis* and *K. stella* are 83% identical. SSU rDNA sequence of the third *Korotnevella* species is shorter and was not compared to the other two.

The results of phylogenetic analyses of a broad dataset of amoeba sequences are presented in Fig. 12.

Table 2. Sequence identity among *Vexillifera* strains under study and the lengths of their SSU rDNA sequences. *V. min* – *Vexillifera minutissima*, *V. bac*. – *V. bacillipedes*.

Strain	Length (bp)	Percent identities of the SSU rDNA sequences				
		RR1	P124	TIL2	*V. bac*.	*V. min*.
RMT	1952	66.3	67.6	63.1	62.9	74.2
RR1	1898	ID	73.4	68.0	68.0	66.3
P124	1925	73.4	ID	71.7	71.8	67.5
TIL2	2131	68.0	71.7	ID	90.2	63.8
V. bac.	2133	68.0	71.8	90.2	ID	63.4
V. min.	1960	66.3	67.5	63.8	63.4	ID



Figs 3–11. Details of ultrastructure of *Vexillifera* strains under study. **3** – cell surface of trophozoites of TIL2 strain; **4** – cell surface of *V. bacillipedes* (CCAP 1590/1); **5** – cell surface of *V. fluvialis* sp. n. (RR1 strain); **6** – cell surface of *V. tasmaniana* sp. n. (RMT strain); **7** – cell surface of *V. multispinosa* sp. n. (P124 strain); **8** – tubular branching mitochondria in the cytoplasm of *V. fluvialis*; **9** – an oriented core of microfilaments in a subpseudopodium of *V. multispinosa* trophozoite; **10** – typically irregular nucleus with electron-dense nucleolus as seen in *V. multispinosa* trophozoite; **11** – disappearance of nuclear membrane and irregular arrangement of chromatin material during nuclear division, strain P124.

The branching pattern of the phylogenetic tree clearly shows that SSU rDNA sequences of the four fish-isolated strains (RMT, RR1, P124 and TIL2), together with sequences of *Vexillifera minutissima* and type strain of *V. bacillipedes*, form a very well supported clade within Flabellinea. Phylogenetic relationships within this clade together with morphological diversification of individual strains advocate the recognition of three new species of the genus *Vexillifera* Schaeffer, 1926.

The sequence of ATCC strain 50883 denominated as *V. armata* appears in our analysis outside the aforementioned *Vexillifera* clade. It forms a sister clade to *Pseudoparamoeba* within another well supported group including genera *Pseudoparamoeba* + *Paramoeba* / *Neoparamoeba* + *Korotnevella*. The sequence of the fifth fish-isolated strain 4730 (formerly considered the type strain of *V. expectata*) branches in a sister position to *Copromyxa cantabrigiensis*.

Taxonomic conclusions

On the basis of light microscopical and ultrastructural features and the SSU rDNA sequence evidence, one of the fish-isolated strains included in the study (TIL2) is identified with *Vexillifera bacillipedes* described by Page (1969) and three fish-isolated strains are described below as three *Vexillifera* species new to science.

***Vexillifera bacillipedes* Page, 1969**

Strain TIL2 (Fig. 1) was isolated from gills of the Nile tilapia *Oreochromis niloticus* (L.) collected in a farm supplied with warmed-up water from the cooling system of an electric power station, Czech Republic. Although trophozoites were subcultured for a relatively short period of time compared to the type culture of *V. bacillipedes* (CCAP 1590/1) (Fig. 2) and due to this were overloaded with bacteria, morphological similarity of both strains was evident. The SSU rDNA sequences obtained in this study are deposited in GenBank database under Acc Nos. HQ687484 (strain TIL2) and HQ687485 (strain CCAP 1590/1). The calculated sequence similarity was the highest one (90.2%) within the set of compared *Vexillifera* sequences (Table 2).

***Vexillifera multispinosa* sp. n. (Fig. 1)**

Trophozoites of acanthopodial morphotype, variable in shape, with numerous (up to 15), sharp pointed hyaline subpseudopodia of different length (up to 15 µm). Length of trophozoites (not including subpseudopodia) between 10 and 20 µm. Actively moving trophozoites always longer than broad. Granuloplasm with clearly visible refractile crystalline inclusions, nucleus of variable shape and contractile vacuole surrounded by small vesicles resembling narrow spongiom. Rim of hyaloplasm that gives rise to subpseudopodia relatively narrow. Outer layer of cell surface with regularly arranged T-shaped 20 nm long glycostyles (Fig. 7); subpseudopodia with microfilamentous core (Fig. 9). Nuclear membrane disappearing during nuclear division (Fig. 11).

Type strain: P124 isolated from gills of the banded leporinus *Leporinus fasciatus* (Bloch, 1784) caught in the Amazon River (Peru, Iquitos), November 2004.

Material deposition: Cryo-collection of the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic. GenBank Acc. No.: HQ687481.

Etymology: The specific name refers to the multiple subpseudopodia that give a spiny appearance to trophozoites (*spinosa* L., adj. = spiny).

***Vexillifera fluvialis* sp. n. (Fig. 1)**

Trophozoites of acanthopodial morphotype, variable in shape, with sharp, on average five, pointed hyaline subpseudopodia not longer than 7 µm. Length of trophozoites (not including subpseudopodia) between 8 and 13 µm. Although irregular in shape, moving trophozoites mostly longer than broad; cytoplasm with refractile bodies. Cell surface coated with amorphous 15 nm thick glycocalyx (Fig. 5).

Type strain: RR1 isolated from spleen of the roach *Rutilus rutilus* (L.) caught in the Lužnice River, Czech Republic, August 1989.

Material deposition: Cryo-collection of the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic. GenBank Acc. No.: HQ687482.

Etymology: The specific name (*fluvialis* L., adj. = of or pertaining to a river) refers to the origin of the fish host.

***Vexillifera tasmaniana* sp. n. (Fig. 1)**

Trophozoites mostly of irregular triangular shape with conspicuous flattened hyaloplasmic rim tapering to several points; tentacle-like subpseudopodia rarely present. Length of trophozoites (not including subpseudopodia) between 6 and 10 µm. Thin, dense glycocalyx with regularly arranged, in longitudinal section cylindrical, 12 nm long glycostyles (Fig. 6).

Type strain: RMT isolated from gills of the Atlantic salmon *Salmo salar* L. used for Amoebic Gill Disease

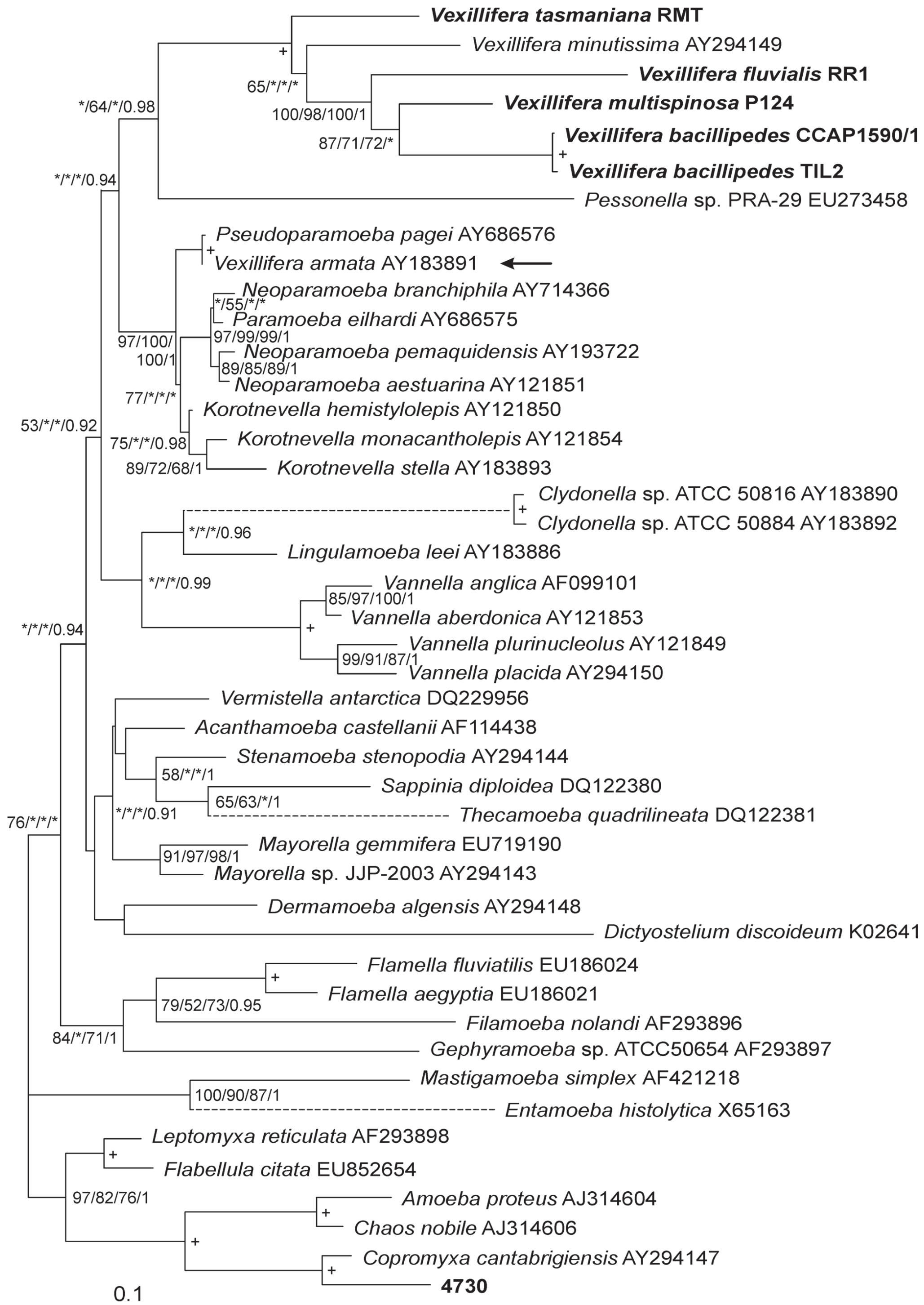


Fig. 12. Maximum likelihood (ML) tree based on SSU rDNA sequences of 44 amoebozoans. The tree is rooted with amoebae from the group Tubulinea. Five new sequences are in bold, GenBank accession numbers are shown for other sequences. The numbers at the nodes are bootstrap values for ML, maximum parsimony and Fitch-Margoliash method with LogDet distances, and posterior probabilities of the Bayesian analysis. Asterisks represent bootstrap values lower than 50% or posterior probabilities lower than 0.9 for the respective method. If all three methods scored bootstrap support 99% or higher and the posterior probability was 1 for a given node, it is indicated by "+" symbol in the tree. Conversely, if all three bootstrap values are lower than 50% and posterior probability lower than 0.9, they are not shown at all. Note the position of *Vexillifera armata* (arrowhead) and of strain 4730 (at the bottom of the tree). Three branches (dashed lines) were shortened to half, others are drawn to the scale.

(AGD) experiments, Launceston, Tasmania, Australia, November 2000.

Material deposition: Cryo-collection of the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic. GenBank Acc. No.: HQ687483.

Etymology: The specific name refers to the geographic origin of the fish host.

DISCUSSION

Although this paper deals with a small group of fish-isolated strains of amoebae, the collected data have brought new insights into phylogeny of *Vexillifera* species and provided opportunity for future correction of generic assignment of two strains. Our study benefitted from the fact that one type strain (CCAP 1590/1 strain of *V. bacillipedes*) was available for comparative morphological and molecular studies. Surprisingly, this strain that was described in detail at light and ultrastructural levels decades ago (Page 1969, 1979a) has not been sequenced prior to this study.

The study of fish-isolated *Vexillifera* strains demonstrated difficulties faced in evaluation of light microscopical features and ultrastructural details. The set of studied strains comprised those easily distinguished by conspicuous generic diagnostic features (e.g. *V. multispinosa*) as well as those that displayed generic characters in a less obvious or a subtle form (e.g. *V. fluvialis* and *V. tasmaniana*, respectively). This fact stressed necessity of repeated observation of live trophozoites in different phases of subculturing. The lack of such experience in the beginning of our amoeba studies and the application of methods of previous authors (Mascaro *et al.* 1985) caused the previous misinterpretation of the light microscopy of strain 4730, features of which evidently changed under the influence of fixative. In addition, importance its ultrastructural features were previously overestimated (Dyková *et al.* 1998).

Three types of the fine structure of glycocalyx which we observed in strains whose assignment to *Vexillifera* was proved by molecular markers supported a conclusion drawn by Page (1979a) in the pre-molecular era. He underlined presence/absence of glycocalyx as a generic or familiar character and demonstrated that the detailed structure of the surface coating can vary within a genus. Thus the type strain of *V. fluvialis* has the same amorphous glycocalyx as the type strain of *V. bacillipedes*, the fine structure of glycocalyx of *V. multispinosa* corresponds to that of *V. granatensis* Mascaro, Osuna *et* Mascaro, 1985, and the type of glycocalyx of *V. tasmaniana* can be identified with that characteristic of *V. lemani* Page, 1976. The latter type was observed also in trophozoites of strain 4730.

Similarly as in the fine structure study of *V. bacillipedes* (see Page 1979), in the presently studied species, subpseudopodia with oriented core of microfilaments were observed rather exceptionally. A nice example of microfilamentous core was seen in trophozoites of *V. multispinosa*, which has numerous subpseudopodia of which several could be observed at one level of ultrathin sectioning.

Descriptions of numerous *Vexillifera* species contain information about the presence/ absence of refractile crystals or crystalloids in the cytoplasm of trophozoites (Bovee 1985; Mascaro *et al.* 1985; Anderson 1994). For some species, number of crystals was reported and the shape characterised as geometrical bodies (e.g. truncated bipyramids in *V. anapes* and quadrangular or rectangular truncated bipyramids in *V. granatensis*), whereas for others, the shape of refractile bodies was not clearly determined or they were characterised as amorphous granules (Bovee 1985). Mascaro *et al.* (1985) searched for these structures in thin sections and concluded that they disappear due to processing for electron microscopy, leaving behind empty lacunar spaces. For each species studied we examined numerous thin sections prepared from several different blocks, but failed to obtain conclusive observation as far as refractile bodies are concerned. Our observations, however, suggest that some refractile bodies may be imitated by bacteria.

We believe that results of this study, including information on the wrong identification of two strains formerly assigned to *Vexillifera*, can motivate re-examination of the generic placement of their sequences that branch in phylogenetic trees in distant positions. *Vexillifera armata* and *V. minutissima* occurred in a number of phylogenetic trees of other authors, where they did not form a single branch, but rather indicated that this genus would be at least paraphyletic to *Neoparamoeba* + *Korotnevella* (Smirnov *et al.* 2005, Nikolaev *et al.* 2006) or more likely polyphyletic as in Tekle *et al.* (2008), where broader taxon sampling was used. Our inclusion of the type strain of *V. bacillipedes* and of three new species in analyses suggests that the identification and generic assignment of *V. armata* should be revised. Moreover, its SSU rDNA sequence is very similar to that of *Pseudoparamoeba pagei* (99.6% identity

when 1,934 bp of the same region as in other *Vexillifera* species included in this study were compared). The newly obtained sequence of "*Vexillifera*" *expectata* and its branching with *Hartmannella cantabrigiensis* raises again the question of generic placement of "*Hartmannella*" sequences that are branching in positions distant from *H. vermiformis* (Tekle *et al.* 2008). We are convinced that until paraphyletic or polyphyletic origin of species of a same genus is declared, meticulous comprehensive examination of the sequenced strains should be done.

Acknowledgements. Financial support was provided by the Grant Agency of the Czech Republic (524/09/0137), the Ministry of Education, Youth and Sports of the Czech Republic (MSM 6007665801) and by Research projects of the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic (Z60220518 and LC522).

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