

Morphological and molecular investigation of *Vexillifera* cf. *armata* Page, 1979 (Amoebozoa: Dactylopodida) isolated from the Pacific Ocean

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ABSTRACT: A strain of *Vexillifera* Schaeffer, 1926 was isolated from the bottom sediments of the Vostok Bay of the Sea of Japan and showed close similarity to *V. armata* Page, 1979. The new strain shares several morphological characters of this morphospecies, in particular, cell coat structure and the presence of unique “trichocyst-like bodies” in the cytoplasm. The studied strain branches in one of the clades of marine *Vexillifera* species, with the unnamed Mediterranean *Vexillifera* strain K9 as its closest relative. Unfortunately, the type strain of *V. armata* was lost before any molecular data were obtained. Therefore, no information is available on this species for molecular comparison. The studied strain was isolated from the habitat geographically very distant from the type one. The type strain of *V. armata* was estuarine, while the new strain was isolated from the lower sublittoral benthos and appears to be stenohaline based on the results of an experimental study. It also showed some elusive morphological differences that may be regarded as intraspecific variation. Although currently known extent of cryptic speciation in the naked lobose amoebae is relatively high, the differences between the studied strain and *V. armata* may be too subtle to warrant a description of a separate species. Therefore, we conclude that the new strain should be identified as *Vexillifera* cf. *armata*. Re-isolation of *V. armata* from its type locality is highly desirable to evaluate a degree of molecular variability within this morphospecies.

How to cite this article: Kudryavtsev A.A., Volkova E.N., Voytinsky F.P.. 2020. Morphological and molecular investigation of *Vexillifera* cf. *armata* Page, 1979 (Amoebozoa: Dactylopodida) isolated from the Pacific Ocean // Invert. Zool. Vol.17. No.4. P.385–402, video. doi: 10.15298/invertzool.17.4.04

KEY WORDS: Amoebozoa, cryptic species, Dactylopodida, molecular phylogeny, morphology, *Vexillifera*.

Морфологическое и молекулярное исследование амёбы *Vexillifera* cf. *armata* Page, 1979 (Amoebozoa: Dactylopodida), изолированной из Тихого океана

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РЕЗЮМЕ: Штамм амёб, идентифицированных как представители рода *Vexillifera* Schaeffer, 1926, был изолирован из донного грунта залива Восток Японского моря. Исследованные амёбы были сходны с видом *V. armata* Page, 1979. Изученный штамм обладает несколькими морфологическими признаками этого вида, в частности, похожим строением покрова клетки и уникальными «трихоцистоподобными структурами» в цитоплазме. Изученный штамм на филогенетическом древе входит в состав одной из ветвей, объединяющих морских представителей рода *Vexillifera*; его ближайшим родственником является неидентифицированный штамм K9 из Средиземного моря. К сожалению, типовой штамм *V. armata* был утрачен до того, как появилась возможность провести какие-либо молекулярные исследования этих амёб. Поэтому сравнить обнаруженный нами штамм и *V. armata* на основании молекулярных последовательностей невозможно. Изученный в данной работе штамм изолирован из биотопа, географически очень удаленного от того, где был найден штамм Пэйджа. Типовой штамм *V. armata* выделен из эстуария, в то время как штамм, изученный в данной работе, обнаружен в бентосе нижней сублиторали и по результатам проведенных экспериментальных исследований, возможно, является стеногаллиным. Этот штамм также обладает очень незначительными морфологическими отличиями от типового штамма *V. armata*, которые можно трактовать как внутривидовые. Хотя в настоящее время среди голых лобозных амёб известны случаи видов-двойников, различия между изученным штаммом и типовым штаммом *V. armata*, вероятно, слишком незначительны для описания отдельного вида. Поэтому мы делаем вывод о том, что изученный штамм следует обозначить как *Vexillifera* cf. *armata*. Для оценки уровня варьирования молекулярных маркеров в пределах морфологического вида *Vexillifera armata* крайне желательна реизоляция амёб, относящихся к этому виду, из типового местообитания.

Как цитировать эту статью: Kudryavtsev A.A., Volkova E.N., Voytinsky F.P.. 2020. Morphological and molecular investigation of *Vexillifera* cf. *armata* Page, 1979 (Amoebozoa, Dactylopodida) isolated from the Pacific Ocean // *Invert. Zool.* Vol.17. No.4. P.385–402, video. doi: 10.15298/invertzool.17.4.04

КЛЮЧЕВЫЕ СЛОВА: Amoebozoa, виды-двойники, Dactylopodida, молекулярная филогения, морфология, *Vexillifera*.

Introduction

The genus *Vexillifera* Schaeffer, 1926 (Amoebozoa: Dactylopodida) comprises 25 named species with morphological and molecular details described to a very different degree (Penard, 1890, 1902; Schaeffer, 1926; Wailes, 1932; Page, 1969, 1972, 1979a, b; Sawyer, 1975; Bovee, 1985; Dyková *et al.*, 2011; Van Wichelen *et al.*, 2016; Kudryavtsev *et al.*, 2018). The genus is very heterogeneous morphologically, yet all its members form a single clade on the molecular phylogenetic tree of Amoebozoa based on the small subunit (SSU) rRNA gene (Dyková *et al.*, 2011; Van Wichelen *et al.*, 2016; Kudryavtsev *et al.*, 2018). In addition, they are characterized by specific sequence motifs in this marker, in particular, a very divergent, unusually short and AT-rich part of the hypervariable region V4 corresponding to the helices 23/e1 and 23/e2 (Kudryavtsev *et al.*, 2018). The clade of *Vexillifera* spp. branches at the base of the Dactylopodida (Smirnov *et al.*, 2005; Dyková *et al.*, 2011; Kudryavtsev, Pawlowski, 2015; Van Wichelen *et al.*, 2016; Kudryavtsev *et al.*, 2018) and is one of the most stable and longest branches among the Discosea due to a significant divergence of its sequences from the rest of this clade. Regardless of the robustness of this clade, its morphological and ecological heterogeneity may prompt for description of several genera, once more species are investigated using ultrastructural and molecular methods. Only a small fraction of named species of *Vexillifera* has been simultaneously studied using microscopic and molecular methods (Kudryavtsev *et al.*, 2018). Several species of the genus *Vexillifera* have previously been studied using microscopic methods only (e.g. Bovee, 1985; Bovee, Sawyer, 1979; Page, 1979b), but it is based on these data that the current concept of the genus *Vexillifera* has partly been elaborated (Kudryavtsev *et al.*, 2018). Therefore, molecular phylogenetic data have to be collected for these species to incorporate them in the current scheme of the phylogeny of the genus. As it is often the case among the naked lobose amoebae, this task may be

challenging when no reference cultures of these previously described species are preserved from these studies (Smirnov, Brown, 2004).

Vexillifera armata Page, 1979 is the species with neither phylogenetic data nor type culture available. This amoeba was isolated by Page (1979a) from the Frogmore Creek off the Kingsbridge estuary (South Devon, England, UK) and investigated using light and electron microscopy. This is the only species of *Vexillifera* where the enigmatic trichocyst-like bodies in the cytoplasm were described, and one of the two with the glycocalyx composed of hexagonal glycostyles. The culture was deposited with the Culture Collection of Algae and Protozoa (CCAP) in UK, but subsequently lost before any molecular data could be obtained. Later, a strain identified as *V. armata* was isolated by T. Nerad in 2000 from the salt marsh on the USA east coast and deposited in American Type Culture Collection as strain ATCC® 50883™. Only SSU-rRNA gene sequence was obtained for this strain (Peglar *et al.*, 2003), but as it quickly became clear (Mullen *et al.*, 2005), this sequence is almost identical to the one of *Pseudoparamoeba pagei* (Sawyer, 1975). The genuine members of *Vexillifera* always form a clade distinct from *Pseudoparamoeba* (Dyková *et al.*, 2011; Kudryavtsev *et al.*, 2018). Later on, a strain of *Vexillifera* was isolated and studied from the marine habitat in the Mediterranean Sea, that resembled *V. armata* possessing similar trichocyst-like bodies, but had a different cell coat (Pizzetti *et al.*, 2016). The purpose of this paper is to report an isolation and a molecular investigation of another strain of *Vexillifera* that is morphologically almost identical to *V. armata* Page, 1979.

Materials and Methods

Sampling, isolation and cultivation

A strain of amoeba designated VO16.21.I.5.1 was isolated from the sample of the upper layer (1 cm) of bottom sediments collected in Vostok Bay of the Sea of Japan (42.69579°N, 132.58841°E) at the depth of 70 m on September, 13th 2016. The seawater salinity at the time of

sampling was 34‰. Samples were collected using a modified Van Veen grab, and subsampling of the sediment performed immediately using a sterile plastic spoon into sterile 100-ml plastic container. Ca. 0.5 ml portions of sediment were aseptically inoculated into 90-mm plastic Petri dishes filled with filter-sterilized (0.22 µm) seawater with addition of three autoclaved wheat grains per dish. Amoebae were detected in inoculated samples using a Leica DM IL inverted microscope. The culture was purified and cloned by transferring single cells with a glass capillary pipette into the plastic Petri dishes filled with artificial medium obtained by dissolving sea salt (Red Sea Coral Pro) in double-distilled water to a concentration of 30‰ with addition of two sterile wheat grains per 60-mm Petri dish. Bacteria co-isolated with amoebae from the sample served as the food source. The clonal cultures were maintained in 60-mm plastic Petri dishes for investigations and in 50-ml tissue culture flasks with aerated caps (TPP) for long-term storage. Light microscopic investigations were performed as described in Kudryavtsev *et al.* (2019).

For transmission electron microscopic studies amoebae were fixed using 2.5% (v/v) solution of glutaraldehyde in 0.05M Na cacodylate buffer (pH 7.4) prepared with 30‰ salinity medium for 40 min followed by a 1% (w/v) solution of osmium tetroxide in the same buffer mixture for one hour. All other details of fixation and further sample processing protocol were done as in Kudryavtsev *et al.* (2019). Ultrathin sections were observed using a JEOL Jem-1400 transmission electron microscope operated at 80 kV.

Salinity tolerance experiments

To test for survival and growth, small amounts of amoebae taken from the cultures in their growth phase were inoculated into the media with different salinities in three repetitions. Cells in a culture flask containing 15 ml of the medium were suspended by vigorous shaking, and a small portion of suspension (approximately two ml) was put into 50-ml plastic centrifuge tubes filled with seawater of required

salinity. After mixing thoroughly by inversion, the resulting suspension was distributed equally between three 60 mm Petri dishes. This method was used with all the salinities in this experiment. The experimental salinity values tested were: 0.5, 5, 10, 15, 30, 40, 50, 70, and 90‰. The inoculated dishes were checked in approximately 30 minutes after inoculation to make sure enough amoebae were inoculated into the dish. Control cultures inoculated in a similar way into the original salinity (30‰) were included in every experiment. Petri dishes were sealed with Parafilm, and condition of amoebae was checked with the inverted microscope in two hours after inoculation, followed by regular observations during subsequent 2–3 weeks.

Molecular phylogenetic analyses

The total DNA was isolated from densely growing cultures using the guanidine isothiocyanate method of Maniatis *et al.* (1982). PCR amplification of the SSU rRNA gene was performed using a primer pair A10s1 and RibB (Medlin *et al.*, 1988; Kudryavtsev *et al.*, 2009) with annealing temperature +50 °C. The amplicons obtained from two independently isolated DNA samples were purified using spin columns of the CleanUp Standard PCR purification kit (Evrogen) and sequenced directly using terminal and universal internal primers for the SSU rRNA gene. A 715-bp barcoding fragment of the cytochrome C oxidase subunit I (COI) gene was amplified as described in Nassonova *et al.* (2010). The amplicon was purified as indicated above, and sequenced directly using PCR primers. The initial steps of the phylogenetic analyses were performed as described in Kudryavtsev *et al.* (2019). The final alignments consisted of 81 SSU rRNA gene sequences of ‘Flabellinia’ (i.e. Vannellida and Dactylopodida) including all currently available sequences of *Vexillifera* spp. and several sequences of uncultured lobose amoebae known from the previous studies (Berney *et al.*, 2004; Poitelon *et al.*, 2009) that showed a tendency to group with *Vexillifera* in preliminary analyses. A total of 1516 bps unambiguously aligned sites were analysed. RAxML v. 8.2.12 (Stamatakis, 2014)

was used for the maximum likelihood (ML) tree inference with GTRGAMMAI substitution model and bootstrap with 1000 pseudoreplicates. Bayesian analysis was performed using MrBayes 3.2.6 (Ronquist *et al.*, 2012) with GTR substitution model, Covarion model and a Gamma distribution model for the site rate variation, with eight rate categories and a proportion of invariable sites. Markov chain Monte Carlo analysis was run for 10000000 generations with a burn in fraction of 0.25 samples. Two runs of four simultaneous chains finally converged with the average standard deviation of the split frequencies of 0.002548. Chain results were analysed using Tracer v. 1.6 (Rambaut *et al.*, 2014) to ensure sufficient sampling of the parameters. The second SSU rRNA gene alignment (1450 unambiguously aligned positions) analysed using the same parameters consisted of all available sequences of the genus *Vexillifera* with three sequences of *Cuneia* spp. as outgroup. COI gene alignment included 48 nucleotide sequences (all currently available species of Dactylopodida and Vannellida as an outgroup, with 606 nucleotide positions. Tree reconstruction was performed similarly to the SSU rRNA gene analysis, but the Bayesian inference used 20000000 generations and the chains converged to the average standard deviation of the split frequencies of 0.001252.

Results

Morphology of the strain VO16.211.5.1

During locomotion amoebae of this strain were 14.8–32.0 μm long by 7.4–19.3 μm broad (average 20.5 by 12.1 μm), with length: breadth ratio 0.9:1–2.9:1 (average 1.8:1). The locomotive forms were flattened, usually the anterior part of the cells was the broadest, and amoebae were tapering towards the pointed uroid (Fig. 1A, D). Sometimes cells were ovoid and equally broad in the anterior and posterior parts, with smooth, rounded uroid (Fig. 1C). The cytoplasm consisted of a flattened anterior hyaline area that occupied up to a half of the cell, and the granuloplasm located posteriorly. The anterior margin of the hyaloplasm was uneven, with

short, blunt subpseudopodia. Amoebae also produced longer subpseudopodia that were formed on the dorsal surface of the cell (Fig. 1D, E). Their maximal length equaled approximately half of the length of the cell. Some of these subpseudopodia were thickened at their tips and had a clavate appearance. These subpseudopodia were usually directed anteriorly and actively waved in the medium while the cell was moving forward (Supplementary Video S1). After relocation towards the posterior end of the cell, they bent at their bases and started to point towards a posterior direction (Fig. 1D, F) that was usually followed by their fast withdrawal. The uroid sometimes carried the remnants of these subpseudopodia (Fig. 1A), but no other differentiated uroidal structures were produced. Some of the cells temporarily expanded during locomotion in the direction of movement by adhesion of their posterior end to the substratum (Fig. 1F). Locomotion on the glass surface was usually unstable, with frequent stops and changes in the direction of movement. When changing the direction, the cells expanded their anterior hyaline area in the new direction of movement (Fig. 1G). The average locomotive rate at +22...+25 °C was 25.7 $\mu\text{m min}^{-1}$ (18.0–35.4 $\mu\text{m min}^{-1}$; $n=7$) that comprised about one cell length per minute. Floating forms were frequently produced in cultures. They had an irregular shape with the cytoplasm contracted in the form of irregularly spherical or ovoid mass producing two to six slender, hyaline pseudopodia that could be up to three times as long as the diameter of the central cytoplasmic mass (Fig. 2). The nucleus was spherical, of vesicular type, with the large, central nucleolus (Fig. 1E, F). The diameter of nucleus and nucleolus was 3.3–5.7 μm (average 4.3 μm) and 1.8–3.1 μm (average 2.6 μm), $n=29$, respectively. During locomotion the nucleus was usually located either in the central part of the granuloplasm or in its anterior part, close to the border between hyaloplasm and granuloplasm. The granuloplasm contained numerous food vacuoles enclosing bacteria (Fig. 1F) and several prominent large vacuoles that contained the remnants of digested material and appeared to be the derivatives of the food vac-

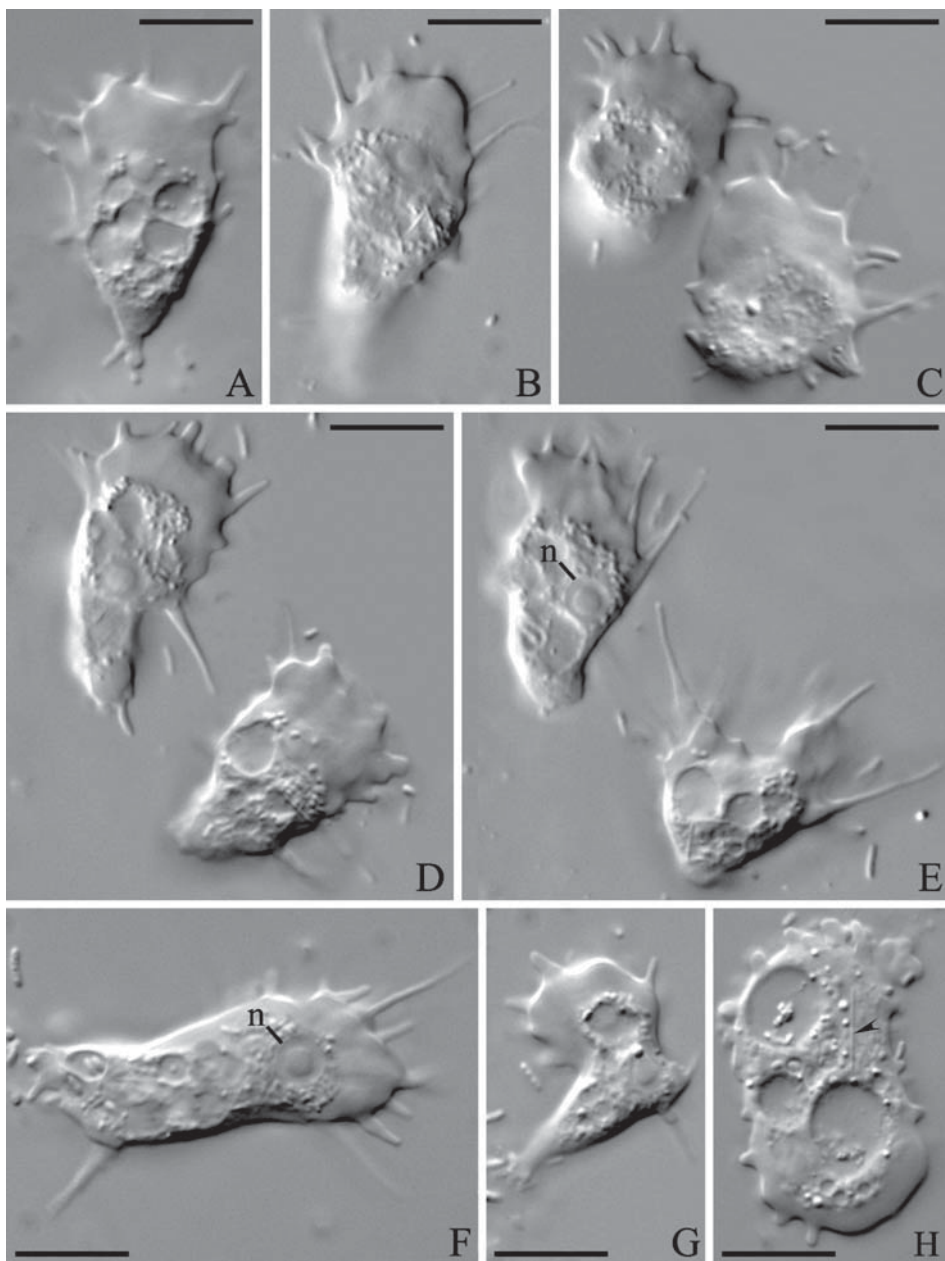


Fig. 1. Light microscopic micrographs of *Vexillifera* cf. *armata* strain VO16.211.5.1 in vivo (DIC). A–F — locomotive forms; G — non-directly moving amoeba; H — amoeba squeezed by the coverslip showing a trichocyst-like body (arrowhead). D–E — amoebae photographed in two different focal planes to show dorsal subpseudopodia (in E). Abbreviations: n — nucleus. Scale bar: 10 μ m.

Рис. 1. Прижизненные светооптические микрофотографии *Vexillifera* cf. *armata* штамм VO16.211.5.1 (дифференциальный интерференционный контраст). А–F — локомоторные формы; G — ненаправленно перемещающаяся амеба; H — амеба, придавленная покровным стеклом, видна трихоцистоподобная структура (стрелка). D–E — амебы, сфотографированные в двух разных оптических плоскостях для демонстрации дорзальных субпсевдоподий (на E). Обозначения: n — ядро. Масштабная черта: 10 мкм.

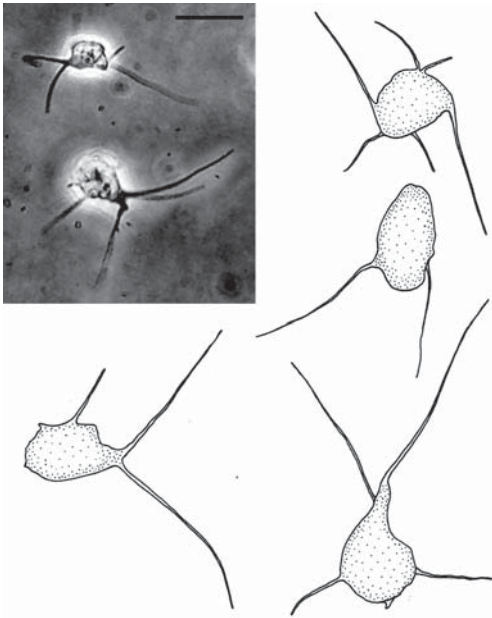


Fig. 2. Light microscopic micrograph (upper left) and schematic drawings of the floating forms of *Vexillifera* cf. *armata* strain VO16.211.5.1. Scale bar: 20 μm .

Рис. 2. Светооптическая микрофотография (левый верхний угол) и схематические изображения флотирующих форм *Vexillifera* cf. *armata* штамм VO16.211.5.1. Масштабная черта: 20 мкм.

uoles (Fig. 1H). The granuloplasm in all amoebae observed with light microscopy also contained about five rod-shaped structures without a permanent position in the cell, best seen when an amoeba was slightly squeezed with a coverslip (Fig. 1H). The length of these structures measured on slides was 3.3–7.2 μm (average 4.9 μm), $n=11$.

Transmission electron microscopy demonstrated a plasma membrane uniformly covered with the glycocalyx consisting of the prismatic glycostyles (Fig. 3A, B). The glycostyles appeared to be attached to the plasma membrane with plate-like bases, and consisted of six stalks raising over the base plate and forming a characteristic hexagonal structure in tangential sections. The stalks appeared to be interconnected with each other at the top of a glycostyle. The cytoplasm contained electron-dense structures

adjacent to the plasma membrane that were rounded or ovoid, 56–132 nm across (average 81.9 nm), $n=45$ (Fig. 3A, B, D). These structures appeared to be unevenly distributed across the surface, and were most numerous in the parts of the sections that corresponded to the peripheral areas of the cell. The nucleus in sections was irregularly rounded, with the central, electron-dense nucleolus (Fig. 3C). Subpseudopodia, when present in the sections, contained longitudinal bundles of microfilamentous material (Fig. 3D). Mitochondria in sections were spherical or ovoid, with tubular cristae (Fig. 3E). The Golgi dictyosome was about 2 μm across and consisted of five or six stacked cisternae (Fig. 3F). Numerous cytoplasmic microtubules were seen adjacent to the dictyosome in favorable sections (Fig. 3G). The rod-shaped structures were regularly seen in the granuloplasm and had a quadrangular (approximately square) shape in cross-section (Fig. 3H). Average length of the sides of this quadrangular structure was 98.75–152.00 nm, $n=8$. The core of these structures was finely granular, and surrounded by several layers of membranous material. These structures appeared to be surrounded by the outermost membrane that looked similar to the membrane of rough endoplasmic reticulum, especially in the longitudinal sections (Fig. 3I).

Molecular phylogenetic relationships

Amplified and sequenced portion of the SSU rRNA of the studied strain was 1809 bp long for both fragments independently obtained from two DNA samples. There were no internal indels in these sequences, and they were almost identical, except for three nucleotide positions close to the 3' end. The G + C content of the sequences was 31.6%. Three molecular clones of partial cytochrome C oxidase subunit 1 (COI) gene amplicon were 666 base pairs long excluding primers that corresponded to 221 amino acid residues (the first nucleotide after forward primer corresponded to the third codon position, therefore the full coding sequence obtained effectively consisted of 663 nucleotides). Sequences of the mo-

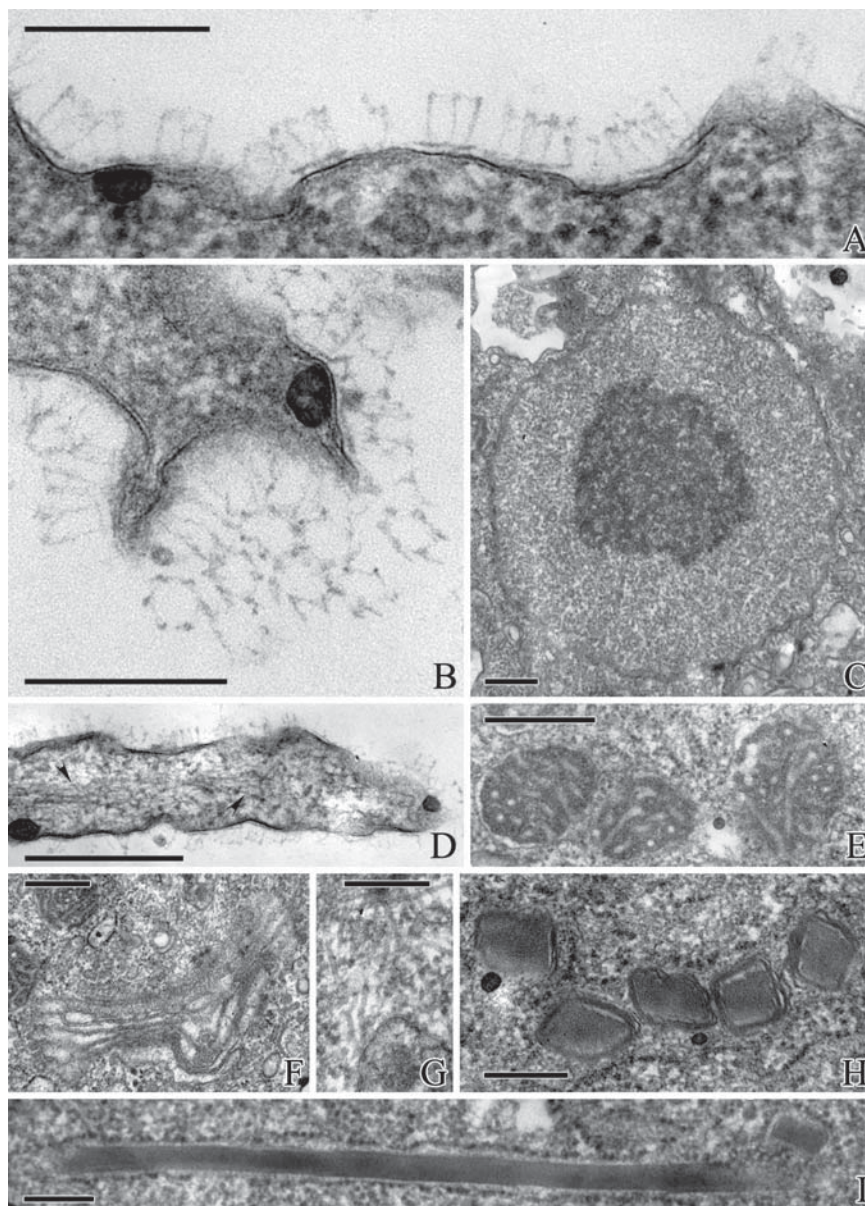


Fig. 3. Transmission electron micrographs *Vexillifera* cf. *armata* strain VO16.211.5.1. A — vertical section of the plasma membrane and glycocalyx; B — tangential section of the glycocalyx; C — nucleus; D — subpseudopodium containing axial bundle of microfilaments (arrowheads); E — mitochondria; F — Golgi dictyosome; G — cytoplasmic microtubules near the dictyosome; H — “trichocyst-like bodies” in a cross-section; I — “trichocyst-like body” in a longitudinal section. Scale bars: A, B, G–I — 0.2 μ m; C–F — 0.5 μ m.

Рис. 3. Трансмиссионная электронная микроскопия: *Vexillifera* cf. *armata*, штамм VO16.211.5.1. А — вертикальный срез плазматической мембраны и гликокаликса; В — гликокаликс на тангентальном срезе; С — ядро; D — продольный срез субпсевдоподия с аксиальным пучком микрофиламентов (показан стрелками); E — митохондрии; F — диктиосома аппарата Гольджи; G — цитоплазматические микротрубочки рядом с диктиосомой; H — “трихоцистоподобные структуры” на поперечном срезе; I — “трихоцистоподобная структура” на продольном срезе. Масштабная черта: А, В, G–I — 0,2 мкм; C–F — 0,5 мкм.

lecular clones of the COI gene amplicon differed from each other in 0.5–1.1% of the nucleotide positions (up to eight variable nucleotides) that corresponded to 1–2.3% differences between the amino acid positions (five variable positions) showing that most of the detected nucleotide substitutions were non-synonymous within the genome. The G + C content in the COI gene was 27.37% and 27.52% depending on the molecular clone. Differences between the studied strain and other species of *Vexillifera* in the COI gene sequences were in 9.6–17.8% of the 417 nucleotide position (average 14.17%; n=30); *V. bacillipedes* was the most distant (17.1–17.8%), while *Vexillifera* sp. strain MX6 isolated from AGD-infected Atlantic salmon (English *et al.*, 2019) was the closest one (9.6–10.8%).

Phylogenetic analysis based on the SSU rRNA gene robustly placed the strain VO16.21I5.1 in the clade corresponding to the order Dactylopodida, in particular, within the genus *Vexillifera*. This clade contained several stable branches that are unified by their origin. In particular, a derived “freshwater” branch contained all freshwater species of *Vexillifera* including those isolated from freshwater hosts and, surprisingly, four sequences of uncultured amoebozoans from freshwater biotopes (Fig. 4). Two clades branching at the base of *Vexillifera* spp. comprised exclusively marine and brackish water species. The studied strain was in a clade comprising *V. tasmaniana*, *V. abyssalis* and two unnamed strains S2M1 and K9; the former isolated from the turbot (*Scophthalmus maximus*), while the latter, from the marine lagoon at the eastern Italian coast (Dyková, Kostka, 2013; Pizzetti *et al.*, 2016). The latter strain was the closest relative to VO16.21I5.1 (support 1.0/100). It is noteworthy that the clade topology for *Vexillifera* spp. is not stable against the algorithm of analysis. In particular, two clades comprising marine species swapped their positions in the maximum likelihood and Bayesian trees (Fig. 4), however, both positions had a maximal support. When the phylogenetic tree was reconstructed on the basis of the 18S rRNA gene dataset comprising only all sequences of

Vexillifera with *Cuneo* spp. as outgroup, the branching order of the basal clades was stable against the algorithm of analysis (Fig. 5). The clade comprising *V. tasmaniana*, *V. abyssalis*, unnamed strains K9 and S2M1, and the strain VO16.21I5.1 was the most basal in both, ML and Bayesian analysis.

The phylogenetic trees based on the COI gene sequences had topologies that differed from those yielded in maximum likelihood analysis, however, the clades corresponding to Vannellida and Dactylopodida were always monophyletic. In the Dactylopodida clade *Cuneo* spp. was the most basal branch, and *Vexillifera* spp. grouped between *Cuneo* spp. and the rest of the clade (Fig. 6). Three molecular clones sequenced for the studied strain grouped with the other marine species, in particular, *V. abyssalis* and *Vexillifera* sp. MX6. *Vexillifera minutissima* and *V. kereti* formed separate branches, and *V. bacillipedes* intermingled between them, but this position was not supported. An application of the codon model in the tree reconstruction based on the COI gene yielded even more different topology compared to the SSU rRNA gene, in particular, the genus *Vexillifera* was not monophyletic, as *V. bacillipedes* branched separately from other members of the genus. However, the studied strain grouped in the same clade and with the same topology as in the COI gene tree based on the GTR model (not shown).

Salinity tolerance of the strain VO16.21I5.1

Experiments on the strain VO16.21I5.1, show that this species so far demonstrates very narrow salinity tolerance range, as amoebae die or significantly slow down their growth in every tested salinity level above 40 and below 20‰. Amoebae showed no survival and no growth in the salinities of 0.5 and 5‰. In 10‰ amoeba showed some growth initially, but after a short time the growth stopped, and cells did not survive. The cultures started showing faster growth in 15‰, and reached their peak in 30 and 40‰ slowing down again in 50‰, and showing almost no growth in 70 and 90‰.

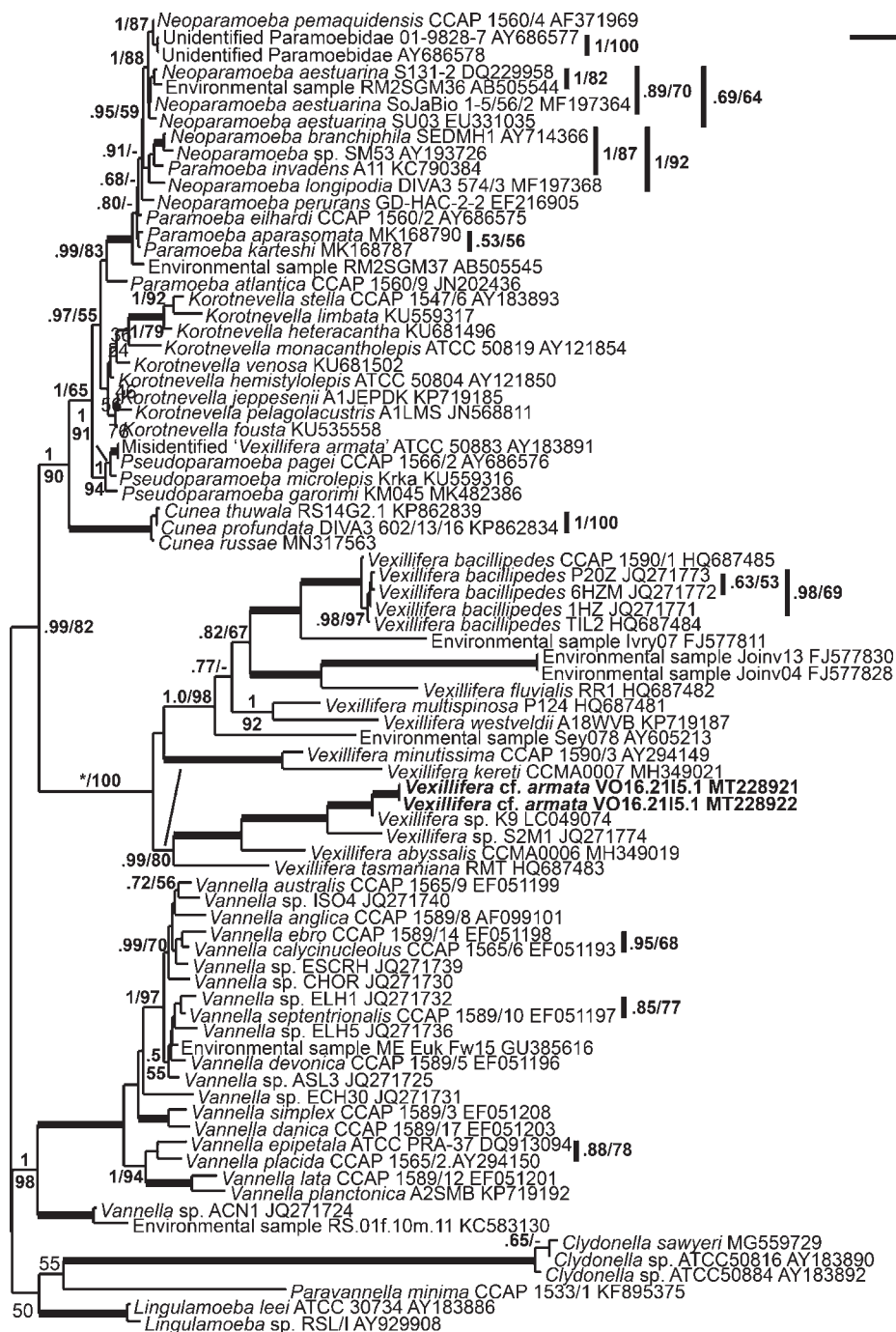


Fig. 4. Maximum likelihood phylogenetic tree of Dactylopodida with selected species of Vannellida as outgroup. The tree is based on 81 SSU rRNA gene sequences (1516 unambiguously aligned nucleotide positions). New sequences of *Vexillifera cf. armata* are in bold. Numbers at nodes indicate Bayesian posterior probabilities/bootstrap values if above 0.5/50. Thick lines = 1.0/100. The line between two clades of *Vexillifera* indicates that they are swapped in the Bayesian tree. Scale bar — 0.1 substitutions/site.

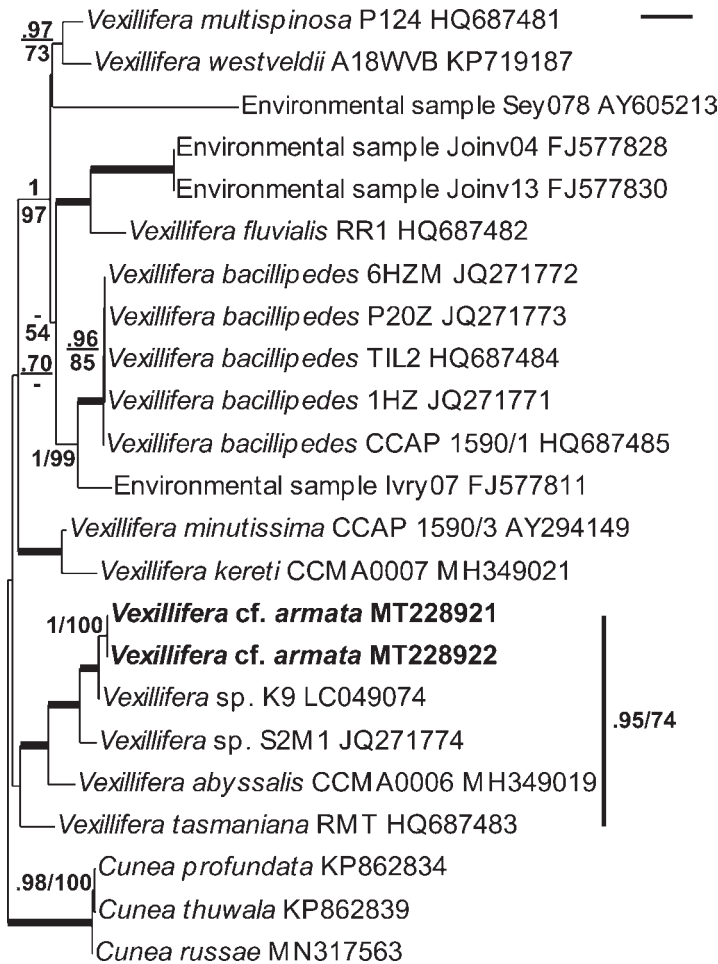


Fig. 5. Maximum likelihood phylogenetic tree of *Vexillifera* with *Cunea* spp. as outgroup. The tree is based on 23 SSU rRNA gene sequences (1450 unambiguously aligned nucleotide positions). New sequences of *Vexillifera* cf. *armata* are in bold. Numbers at nodes indicate Bayesian posterior probabilities/bootstrap values if above 0.5/50. Thick lines = 1.0/100. Scale bar — 0.1 substitutions/site.

Рис. 5. Филогенетическое древо представителей рода *Vexillifera* и представителей рода *Cunea* в качестве внешней группы. Древо построено по 23 последовательностям гена малой субъединицы рибосомной РНК (1450 надежно выровненных нуклеотидных позиций). Новые последовательности *Vexillifera* cf. *armata* обозначены жирным шрифтом. Числа показывают байесовские апостериорные вероятности/значения бутстрепа для соответствующих ветвей, если они превышают 0.5/50. Жирные линии соответствуют значениям 1.0/100. Масштабная черта соответствует 0,1 замене на нуклеотидную позицию.

Рис. 4. Филогенетическое древо Dactylopodida, построенное по алгоритму максимального правдоподобия, некоторые виды Vannellida использованы в качестве внешней группы. Древо построено по 81 последовательности гена малой субъединицы рибосомной РНК (1516 надежно выровненных нуклеотидных позиций). Новые последовательности *Vexillifera* cf. *armata* обозначены жирным шрифтом. Числа показывают байесовские апостериорные вероятности/значения бутстрепа для соответствующих ветвей, если они превышают 0.5/50. Жирные линии соответствуют значениям 1.0/100. Косая линия между двумя кладами представителей рода *Vexillifera* означает, что в дереве, построенном в соответствии с байесовским алгоритмом, они поменялись местами. Масштабная черта соответствует 0,1 замене на нуклеотидную позицию.

Discussion

Taxonomic affinity of the studied *Vexillifera* isolate

Based on the characters demonstrated by the light microscopic, ultrastructural and molecular investigation, the studied strain is assignable to the genus *Vexillifera* (Bovee, 1985; Kudryavtsev *et al.*, 2018), and in particular looks very similar to *Vexillifera armata* Page, 1979. Amoebae of our strain and members of this species have an identical shape during locomotion, and similar floating forms. The glycocalyx of *V. armata* consists of prismatic hexagonal glycostyles that also have a similar size range to the glycostyles of the strain VO16.2115.1 (Page, 1979a). In addition, amoebae share identical rod-like structures in the cytoplasm designated by Page (1979a) as “trichocyst-like bodies”. However, there are several differences between the two amoebae that prevent us from a straightforward assignment of the strain VO16.2115.1 to *V. armata*. Amoebae studied by Page were slightly smaller and “shorter” than those of VO16.2115.1 (length 10–23 μm with the average 15.5 μm , length:breadth ratio 1–2.2, average 1.6). Amoebae studied here had a broader range of length:breadth ratio, although it overlapped with that, measured by Page. The ranges of nuclear diameter also overlap, but the nuclei measured by Page were generally smaller. These morphological differences may be too small for assignment of our strain to a different morphospecies, but in addition, there are several indirect pieces of evidence that identification of the strain studied here as *Vexillifera armata* is uncertain. Unfortunately, no molecular data were obtained for the type strain of *V. armata*, and this strain was lost, therefore, we cannot perform a sequence comparison. However, there are unnamed strains of *Vexillifera* that have been recently studied using molecular data and belong to the same clade as the strain studied here. In this case morphological and molecular comparison can be performed. The strain which is most similar to VO16.2115.1 was designated K9 without an assignment of a species name (Pizzetti *et al.*, 2016). The sequenced part of its

SSU rRNA gene shows differences from the strain studied here in 6.1 and 6.3% of the nucleotide positions. Most of the differences are restricted to several local areas of the gene, in particular, there are clearly different nucleotide patterns in the helices 23e12 of the hypervariable region V4, 43e of the hypervariable region V7, 45e and 46e of the hypervariable region V8, and 49 of the hypervariable region V9. Unfortunately, no sequence data is available for the barcoding part of the V4 region of the SSU rRNA gene in the K9 strain. Morphologically this strain is similar to the one studied here, and *V. armata*. Size of its trophic cells varies within the similar range as that of *V. armata* (Table 1), but it had much higher length to breadth ratio. This strain also differed in the glycocalyx structure: its glycocalyx is described as amorphous, but its image shows an uneven fuzzy layer (Pizzetti *et al.*, 2016). At least, it does not show any glycostyles. *Vexillifera* sp. strain K9 also demonstrated inclusions that were described as “wedge-shaped filamentous structures” without any fixed number or position in the cytoplasm (Pizzetti *et al.*, 2016). These structures strongly resemble “trichocyst-like bodies” present in our strain and in *V. armata* Page, 1979, and may have the same nature. *Vexillifera* sp. K9 was also isolated from a marine habitat, a coastal lake on the Tyrrhenian coast of Italy with salinity 28.4–33.7‰ (Pizzetti *et al.*, 2016), but there are no data on its salinity tolerance. The strain studied here was isolated from the Far Eastern benthic marine habitat with the salinity of 34‰, while *V. armata*, from Kingsbridge estuary (UK) where salinity was not measured during collection (Page, 1979), but comprises about 30‰ according to other records (http://apps.environment-agency.gov.uk/static/documents/nvz/NVZ2017_ET8_Kingsbridge_Estuary_Datasheet.pdf). The estuarine nature of this habitat suggests that its salinity may be variable.

In conclusion, there are three marine strains of *Vexillifera* with the significantly overlapping ranges of variation of their morphological characters. They all were isolated from marine habitats separated by long distances. Two most similar strains are *V. armata* Page, 1979 and

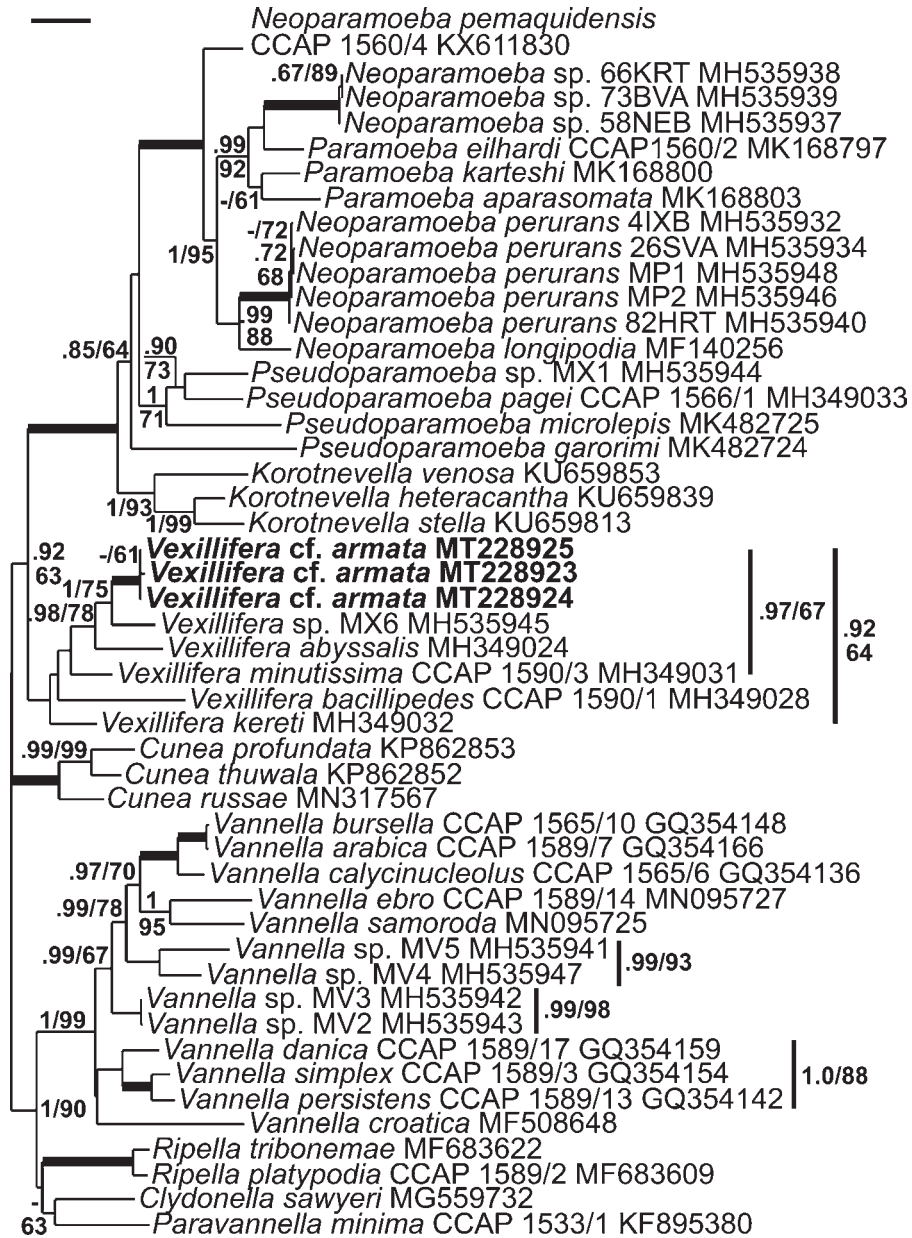


Fig. 6. Maximum likelihood phylogenetic tree of Dactylopodida with Vannellida as outgroup. The tree is based on 48 COI gene sequences (606 unambiguously aligned nucleotide positions). New sequences of *Vexillifera* cf. *armata* are in bold. Numbers at nodes indicate Bayesian posterior probabilities/bootstrap values if above 0.5/50. Thick lines = 1.0/100. Scale bar — 0.1 substitutions/site.

Рис. 6. Филогенетическое древо Dactylopodida, построенное по алгоритму максимального правдоподобия, Vannellida использованы в качестве внешней группы. Дерево построено по 48 последовательностям гена COI (606 надежно выровненных нуклеотидных позиций). Новые последовательности *Vexillifera* cf. *armata* обозначены жирным шрифтом. Числа показывают байесовские апостериорные вероятности/значения бутстрепа для соответствующих ветвей, если они превышают 0.5/50. Жирные линии соответствуют значениям 1.0/100. Масштабная черта соответствует 0,1 замене на нуклеотидную позицию.

Table 1. Morphometric comparison of the strain VO16.21I.5.1, *Vexillifera armata* Page, 1979, and *Vexillifera* sp., strain K9 (Pizzetti *et al.*, 2016).
Таблица 1. Сравнение морфометрических характеристик VO16.21I.5.1, *Vexillifera armata* Page, 1979 и *Vexillifera* sp., штамм K9 (Pizzetti *et al.*, 2016)

	VO16.21I.5.1	<i>V. armata</i> Page, 1979	<i>Vexillifera</i> sp. K9
Locomotive form size (length X breadth, μm)	14.8–32.0 X 7.4–19.3 (average 20.5 X 12.1)	Length 10–23 (average 15.5)	10.1–21 X 4.4–13 (average 14.8 X 8.4)
Length:breadth ratio	0.9:1–2.9:1 (average 1.8:1)	1.0:1–2.2:1 (average 1.6:1)	3.9:1–9.5:1 (average 5.9:1)
Nucleus X nucleolus (μm)	3.3–5.7 X 1.8–3.1 (average 4.3 X 2.6)	2.8–4.2 X 1.9–2.8	Not provided
Glycocalyx, nm	Glycostyles: height 46–75 (58.4); diameter 48.7–61.7 (54.4)	Glycostyles: height 60–70; diameter 50	“Amorphous glycocalyx”, 10–20 nm thick

Vexillifera sp. VO16.21I5.1: their size ranges overlap, cell coat structure and ultrastructure of “trichocyst-like bodies” are almost identical. On the other hand, their habitats are geographically remote, and no molecular data are available for Page’s strain. The extent of cryptic speciation has been getting more and more evident among the naked lobose amoebae recently (Kudryavtsev, Pawlowski, 2015; Tekle, Wood, 2018; Mesentsev, Smirnov, 2019; Kudryavtsev, Volkova, 2020; Lara *et al.*, 2020; Mesentsev *et al.*, 2020). For example, among the small dactylopodid amoebae that belong to the genus *Cunea* no sound morphological differences were found between three strains showing significant variation in their molecular sequences and ecological preferences (Kudryavtsev, Pawlowski, 2015; Kudryavtsev, Volkova, 2020). We have no reason to be confident that the same situation would not occur in the other genera of Amoebozoa, in particular, in *Vexillifera*. Therefore, a re-isolation of *V. armata* from its type location is highly desirable in order to perform molecular comparison with the strain studied here that until then has to be designated as *Vexillifera* cf. *armata*.

Novel ultrastructural characteristics of *Vexillifera*

A detailed comparison of the revealed glycocalyx structure of *V. cf. armata* VO16.21I5.1

with the original description of *V. armata* (Page, 1979) shows some structural differences that, if confirmed further, may clarify several aspects of this type of cell coat in *Vexillifera*. Page (1979a: 116) describes a basal layer of glycocalyx in *V. armata* that in cross-section looks like a line in ca. 12 nm above the plasma membrane. The hexagonal glycostyles are described as emerging from this basal layer. The results of our observation of *V. cf. armata* strain VO16.21I5.1 show clearly that the basal parts of the hexagonal glycostyles are separated from each other, and contrasting to the description of Page (1979a) there is no basal layer of glycocalyx that looks like a continuous line, but is rather made up of separate structures that serve as basal parts of each glycostyle (Fig. 3A). Under this interpretation, these glycostyles are rather attached to the plasma membrane surface in a way similar to the microscales. Interestingly, these structural features seem to look the same in some of the electron micrographs by Page (1979a), e.g., in his Fig. 12, however, his interpretation of these structures was different. Given the present data, we can question whether the glycocalyx structures of *Vexillifera* have the same structural characteristics as the glycostyles of Vannellida, i.e. whether they are truly embedded in the plasma membrane (Page, Blakey, 1979; Smirnov *et al.*, 2007). It is prob-

able, that the nature of their contact with the plasma membrane is similar to that of the microscapes in scale-bearing dactylopodid amoebae, like species of the genera *Paramoeba* and *Korotnevella*. However, the question whether these structures are separable from the plasma membrane without destruction, which is the main criterion of the glycostyles, or not (Page, Blakey, 1979), remains open.

Electron-dense submembrane bodies (Fig. 3A, B, D) are similar in size and structure to the collosome-like structures previously described among the dactylopodids in *V. abyssalis* and *V. kereti* (Kudryavtsev *et al.*, 2018), and *Cunea* spp. (Kudryavtsev, Pawlowski, 2015; Kudryavtsev, Volkova, 2020). Initially, these structures were described by Page (1980) and Page and Willumsen (1983) in the members of the genera *Rhizamoeba* and *Flabellula*. The function of these structures has not yet been demonstrated.

Molecular phylogeny of the genus *Vexillifera*

The presented phylogenetic tree based on the SSU rRNA gene (Fig. 4) is to date the most complete for the genus *Vexillifera*, and shows several peculiar features. In particular, there are three stable clades of *Vexillifera* as shown previously (Kudryavtsev *et al.*, 2018) that differ in their origin. Two basal clades are from brackish water/marine habitats, and one derived clade comprises freshwater species. Interestingly, a discrepancy was observed between ML and Bayesian phylogenetic trees with respect to the position of the two marine branches of *Vexillifera* that were swapped in these trees (Fig. 4), and their position was poorly supported. This artifact can probably be explained by the high evolutionary rates in the clade of *Vexillifera* spp. and the fact that presumably vexilliferan environmental sequences were included for the first time in the current phylogenetic analyses. These could have a destabilizing effect on the tree topology, as some of these sequences are also incomplete. This effect could be overcome by the reduction of the number of outgroup sequences (Fig. 5). When the dataset was reduced to only *Vexillifera* spp. with *Cunea*

spp. added as outgroup, the position of the two clades stabilized against the algorithm of analysis, but still was poorly supported with bootstrap/Bayesian posterior probability. Anyway, all three clades are fairly well-supported, and based on these reconstructions, a scenario suggesting a single evolutionary transition between marine and freshwater biotopes seems to be reasonable.

A similar tree topology is not reproduced by the COI gene analysis (Fig. 6). This situation was noticed before (Kudryavtsev *et al.*, 2018), and it can be explained by the short length of the COI sequences and their high evolutionary rates combined with a small number of sequenced species. Therefore, COI gene may not be a suitable marker for reconstruction of the evolutionary relationships between species of *Vexillifera*, but it can be used to determine the species identity.

Analysis of the SSU rRNA gene sequences shows that the clade corresponding to the genus *Vexillifera* comprises, besides named species, four sequences of the uncultured amoebozoans. These are Ivry07 (FJ577811) that branches as sister to *V. bacillipedes*, Joinv04 and Joinv13 (FJ577828 and FJ577830, respectively), sisters to *V. fluvialis*, and Sey078 (AY605213) that occupies the most basal position in the clade of freshwater *Vexillifera* spp. The former three sequences originate from the study of microbial communities of the water treatment plants in France (Poitellon *et al.*, 2009), while the latter, from the Seymaz River in Switzerland (Berney *et al.*, 2004). Although these sequences are quite divergent from those of the named species and form long branches in the tree, they share several nucleotide patterns in the conserved sites, typical of the other species of *Vexillifera* that is additional evidence that these sequences belong to the genuine members of this genus.

Our data indicate that members of the revealed *Vexillifera* clades demonstrate different morphological characters that make it difficult to outline morphological boundaries between these clades. This problem was also faced by Kudryavtsev *et al.* (2018), however, with accumulation of the amount of investigated species evolutionary trends in morphology may become

evident. We can currently mention that the possible common feature of the freshwater species of *Vexillifera* is the presence of refractile inclusions in the cytoplasm (Bovee, 1985; Mascaro *et al.*, 1985; Page, 1988; Dyková *et al.*, 2011; Van Wichelen *et al.*, 2016). One of the marine branches (*V. minutissima* + *V. kereti*) shares nucleus with peripheral nucleoli (Page, 1983; Kudryavtsev *et al.*, 2018). The cell coat structure is variable among branches, but we can mention that generally, it is either an uneven amorphous layer, or it consists of separate distinct units first described as “T-shaped glycostyles” (Mascaro *et al.*, 1985) in the species of freshwater origin (Page, 1979b, 1988; Dyková *et al.*, 2011). It is the same in the species of marine origin (Dyková *et al.*, 2011; Pizzetti *et al.*, 2016; Kudryavtsev *et al.*, 2018), or they may possess hexagonal glycostyles. Interestingly, the latter are recorded in two independent evolutionary branches: *V. minutissima* + *V. kereti* (in *V. minutissima*), and in a branch probably related to *V. armata* (in *V. armata* Page, 1979a and *V. cf. armata* strain VO16.21I.5.1) (Page, 1979a, 1983). The cell coat consisting of “T-shaped structures” also occurs in the other clades of the Dactylopodida, in particular, in *Cuneia* spp. (Kudryavtsev, Pawlowski, 2015; Kudryavtsev, Volkova, 2020). Based on this data, we can suggest that this type of a cell coat structure is probably basal for *Vexillifera*, while hexagonal glycostyles may have evolved several times independently in different clades. This is similar to the hypothesis of the evolution of pentagonal glycostyles in the order Vannellida (Smirnov *et al.*, 2007).

Marine species of *Vexillifera* and their relations to environmental salinity.

Few attempts to determine ranges of salinity tolerance in marine members of the genus *Vexillifera* have been made before. In particular, Schaeffer (1926) reported that *Vexillifera aurea* did not tolerate the seawater dilution below 25% (i.e. ca. 8‰), but did not show any significant changes in 50% dilution (15–16‰). Sawyer (1975) investigated the subject in two other species of *Vexillifera* – *V. browni* and *V. otto*

isolated from the Chincoteague Bay (Atlantic coast, USA). He found that these species neither survived nor grew at salinities of 15‰ and below. Unfortunately, no experimental data on salinity tolerance were collected for *V. armata* or *Vexillifera* sp. K9 (Pizzetti *et al.*, 2016). However, the data we obtained during this study indicate a relative stenohalinity of strain *Vexillifera cf. armata* described here: the growth and survival of the studied strain could only be maintained at salinity values of 15–50‰, and no further dilution or increase in the salinity was tolerated. This remarkable feature is in accordance with the conditions in the original biotope where the strain was isolated: sublittoral benthos of the Sea of Japan, at the depth of 70 m. Probably, no significant salinity oscillations in this biotope occur, therefore, the studied amoeba is not adapted to the higher and lower salinity values. It remains open, however, whether this strain can tolerate a broader range of salinities once exposed to the marginal values for a prolonged period of time.

List of data and materials derived from this study

The type material designated for *V. armata* Page, 1979 is stored at the Natural History Museum (London, UK) as a series of permanent slides (Page, 1979). The strain *Vexillifera cf. armata* VO16.21I.5.1 is preserved in the form of a culture (accession No RC CCMAM 0466), Epon embedding for transmission electron microscopy (accession No F140) and two purified samples of the total genomic DNA (accession Nos A566, A571), maintained at the resource center “Culture Collection of Microorganisms” of the St. Petersburg State University Research Park, Russia. Accession numbers for the molecular sequence data are: MT228921–MT228922 (SSU rRNA gene), MT228923–MT228925 (COI gene). Video record of living amoebae is publicly available at: <https://youtu.be/ErsePXUikCo>.

Acknowledgements. We dedicate this publication to the memory of the late Andrey Alexandrovich Dobrovolskij, a gentle, reliable and

supportive teacher to all of us who passed away last year. The authors are grateful to Prof. A.V. Chernyshov, Prof. V.V. Yushin (Laboratory of Embryology, National Scientific Center of Marine Biology, Far-Eastern Branch of RAS) for the possibility to use laboratory rooms at the Vostok marine station of the NSCMB; staff of the Vostok station, in particular, Nikolay Kashenko for help with sampling and initial processing of the material. Supported by the ZIN RAS budgetary grant AAAA-A19-119031200042-9 to AK and RFBR grant 18-34-00726-mol-a to EV (electron microscopic study and salinity tolerance investigations).

Compliance with ethical standards

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with animals performed by any of the authors.

Supporting Information

Additional Supporting Information may be found online:

Supplementary video S1. Living amoebae on the glass surface.

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Responsible editor V.V. Aleshin