School of Doctoral Studies in Biological Sciences

University of South Bohemia in České Budějovice
Faculty of Science

Diversity, phylogeny and phylogeography of free-living amoebae

Ph.D. Thesis

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České Budějovice 2016

This thesis should be cited as:
Tyml, T. 2016. Diversity, phylogeny and phylogeography of free living amoebae. Ph.D. Thesis Series, No. 13. University of South Bohemia, Faculty of Science, School of Doctoral Studies in Biological Sciences, České Budějovice, Czech Republic, 135 pp.

Annotation

This thesis consists of seven published papers on free-living amoebae (FLA), members of Amoebozoa, Excavata: Heterolobosea, and Cercozoa, and covers three main topics: (i) FLA as potential fish pathogens, (ii) diversity and phylogeography of FLA, and (iii) FLA as hosts of prokaryotic organisms. Diverse methodological approaches were used including culture-dependent techniques for isolation and identification of free-living amoebae, molecular phylogenetics, fluorescent *in situ* hybridization, and transmission electron microscopy.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

České Budějovice, 6.10. 2016

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Tomáš Tyml

This thesis originated from a partnership of Faculty of Science, University of South Bohemia, and Institute of Parasitology, Biology Centre of the ASCR, supporting doctoral studies in the Parasitology study programme.

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Financial support

The author of this thesis was supported by funding from the Czech Science Foundation (505/12/G112), Ministry of Education, Youth and Sports (LM2010009, LM2015078), Grant Agency of University of South Bohemia (140/2013/P), and European Social Fund Project (CZ.1.07/2.2.00/28.0190). Czech Research Arctic Infrastructure (University of South Bohemia), J. G. Mendel Antarctic station (Masaryk University), and Argentine Antarctic Institute are gratefully acknowledged for having facilitated this work in many ways.

Acknowledgements

It is a great pleasure for me to thank all people who supported me during my studies. My deepest gratitude goes to my supervisors, Martin Kostka and Iva Dyková. Iva supervised me since my first steps in research and provided guidance in all aspects of my academic development. Without her comprehensive support and help during the whole time I surely would not be where I am now. I am also infinitely grateful to Oleg Ditrich for never ending fruitful discussions covering a broad range of topics and for making my life of field biologist so adventurous. I am thankful to Josef Elster for the opportunity to be involved in eight expeditions to the High Arctic, Svalbard and to Milan Gelnar for taking me into his team. My thanks also go to Isaure de Buron for a great opportunity of staying in her laboratory at College of Charleston, South Carolina. My thanks belong also to Vincent A. Connors and William A. Roumillat for a tremendous amount of support during my stays in South Carolina. I am also grateful to Matthias Horn who enabled me to work in his group at the Division of Microbial Ecology, University of Vienna and to Frederik Schulz who introduced me to the “bacterial symbiont topic”. I also extend my thanks to all who collected environmental samples for me. I am grateful to Vladimír Bukva, Keith Edwards and Jason Dean who corrected the English texts. A great debt of gratitude is due to all my friends and family, particularly Lenka, for their support and patience.

Thank you!

List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

Dyková, I. & **Tyml, T.** 2016. Testate amoeba *Rhogostoma minus* Belar, 1921, associated with nodular gill disease of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish. Dis.*, 39:539–546.

- IF_{2015} = 2.053; *Q1 in Veterinary sciences rank (15/138)*

Tomáš Tyml performed the molecular identification of *Rhogostoma minus* and contributed to the final draft of the manuscript.

Schulz*, F., **Tyml*, T.**, Pizzetti, I., Dyková, I., Fazi, S., Kostka, M., & Horn, M. 2015. Marine amoebae with cytoplasmic and perinuclear symbionts deeply branching in the *Gammaproteobacteria*. *Sci. Rep.*, 5:13381.

**these authors contributed equally to this work*

- IF_{2015} = 5.228; *Q1 in Multidisciplinary sciences rank (7/63)*

Tomáš Tyml participated in sampling, conceiving and conducting the experiments, analysing the results, preparing figures, and writing the manuscript.

Tyml, T., Kostka, M., Ditrich, O. & Dyková, I. 2016. *Vermistella arctica* n. sp. nominates the genus *Vermistella* as a candidate for taxon with bipolar distribution. *J. Eukaryotic. Microbiol.*, 63:210–219.

- IF_{2015} = 2.738; *Q2 in Microbiology rank (53/123)*

Tomáš Tyml isolated the strains of *Vermistella arctica*, conceived the study, carried out the morphological characterisation and the phylogenetic analysis, and drafted the manuscript.

Pizzetti, I., Schulz, F., **Tyml, T.**, Fuchs, B. M., Amann, R., Horn, M. & Fazi, S. 2016. Chlamydial seasonal dynamics and isolation of the "*Candidatus* Neptunochlamydia vexilliferae" from a Tyrrhenian coastal lake. *Environ. Microbiol.*, 18:2405–2417.

- IF_{2015} = 5.932; *Q1 in Microbiology rank (16/123)*

Tomáš Tyml was responsible for identification of the natural host of *Neptunochlamydia vexilliferae* and morphological characterisation of the two partners, i.e., dactylopodid amoeba *Vexillifera* sp. strain K9 harbouring *N. vexilliferae*. He participated on writing the manuscript.

Tyml, T., Lares-Jiménez, L. F., Kostka, M. & Dyková, I. 2016. *Neovahlkampfia nana* n. sp. strengthening an underrepresented subclade of Tetramitia, Heterolobosea. *J. Eukaryotic. Microbiol.*, DOI: 10.1111/jeu.12341

- IF_{2015} = 2.738; *Q2 in Microbiology rank (53/123)*

Tomáš Tyml prepared sequences of culture clones, performed the phylogenetic analysis, and wrote the manuscript.

Kostka, M., Lares-Jiménez, L. F., **Tyml, T.** & Dyková, I. 2016. *Copromyxa laresi* n. sp. (Amoebozoa: Tubulinea) and transfer of *Cashia limacoides* (Page, 1967) to *Copromyxa* Zopf, 1885. *J. Eukaryotic. Microbiol.*, DOI: 10.1111/jeu.12349

- IF_{2015} = 2.738; *Q2 in Microbiology rank (53/123)*

Tomáš Tyml prepared a part of sequences and contributed substantially to the final draft of the manuscript and revisions/rebuttals.

Tyml, T., Skulinová, K., Kavan, J., Ditrich, O., Kostka, M. & Dyková, I. 2016. Heterolobosean amoebae from Arctic and Antarctic extremes: 18 novel strains of *Allovahlkampfia*, *Vahlkampfia* and *Naegleria*. *Eur. J. Protistol.*, 56:119–133.

- IF_{2015} = 2.553; *Q2 in Microbiology rank (60/123)*

Tomáš Tyml participated in sampling, strain isolation and their morphological characterisation. He prepared a part of sequences, performed the phylogenetic analysis, and drafted the manuscript.

Contents

Section I

General introduction

In the contemporary view of evolution, free-living amoebae (FLA) are considered a heterogeneous group of eukaryotic microorganisms that share some morphological and biological characteristics but differ substantially in many others. To understand the current state of knowledge of FLA diversity including trends in their research, the diversity of these organisms should be considered in the context of general knowledge of biodiversity and the methodological basis of its study available in a specific period.

Biodiversity in general

The term 'biodiversity' in a widely accepted interpretation can be used to describe diversity at multiple scales of biological organization (genes, populations, species and ecosystems) and can be considered at any geographic scale (local, regional, global). However, it is always important to specify the level of organization and the geographic scale concerned (Magurran 2004). The most frequently quoted is the definition by Pedrós-Alió (2006) who stated that "biodiversity is the total, specific, taxonomic or genetic richness contained in nature or in any local or taxonomic part of it".

Biodiversity studies typically focus on species, which is the fundamental unit of taxonomic classification and a basic unit in ecological studies. In a metaphorical sense, Agapow et al. (2004) presented species as a currency of biology and together with other authors (e.g., Magurran 2004) drew attention to species delineation. The latter problem remains one of the most challenging issues in biodiversity studies due to the application of different concepts of species and resulting inconsistencies in biodiversity data. At least 24 concepts of species and even more associated definitions are in use today (Mayden 1997). Mayr's biological species concept is one of the most famous. Mayr (1966) defined species as groups of interbreeding natural populations that are reproductively isolated from other such groups. The weakest points of this concept are its low applicability in practice and its inapplicability to asexual organisms. The search for an 'ideal species concept' is still ongoing, being stimulated by discussions about practical and theoretical viewpoints of "species problem" including the lack of rules for determination of taxonomic ranks of unicellular protists where boundaries defining species are somewhat arbitrary (Patterson 1999). Fitzhugh (2005) recommends to treat species as a hypothesis and thus not to describe taxa as facts but testable hypotheses about the structure of biodiversity (Pante et al. 2015). As a solution, phylogenetic species-concept (PSC) based on DNA sequence information has been introduced in three different versions. Common to all versions of PSC is an attempt to identify the

smallest biological entities that are diagnosable and/or monophyletic (Mayden 1997). This makes this concept readily applicable in practice, however, Mayden (1997) does not consider it appropriate to use any version of PSC as the primary concept. The need for a simple and reliable species identification or delineation has led to introduction of DNA barcoding (e.g., Hebert et al. 2003; Schindel and Miller 2005), which is a DNA sequence-based approach using one or few reference genes (usually part of it/them) in order to assign undetermined specimens to known species or facilitate the discovery of unknown species.

Comparative examination of the diversity of organisms present in different spatial units (ecosystems, biomes or continents) requires quantifying species diversity/heterogeneity. Although 'quantifying nature' can hardly be complete, many indices of biodiversity have been established, as e.g., Shannon index, Simpson index and Species-area relationship. Following the terminology of Whittaker (1972), biodiversity may be expressed by the concept of α, β, and γ diversity. A sum of species richness within sites or samples (α diversities) and the dissimilarity between these sites or samples (β diversities) determine the global/total diversity (γ diversity). Migrations of species between these sites decrease the β component of diversity but at the same time increase the α component of diversity (Loreau 2000).

In the last two decades, DNA sequence information has increasingly been used in ecologically oriented research of free-living eukaryotic microorganisms including also total diversity studies of selected ecosystems. Biodiversity has been measured alternatively using DNA sequences for deriving molecular operational taxonomic units (MOTUs) (e.g., Blaxter 2004; Schloss and Handelsman 2005) or operational taxonomic units (OTUs) (Caron et al. 2009) instead of identification of species.

Diversity of microeukaryotes

As compared with diversity of macroorganisms, current knowledge of microeukaryotic diversity is limited due to difficulties in identification and enumeration of these organisms. Any ecological study focusing on diversity requires identification and (ideally) enumeration of microorganisms despite the fact that they have small body size, high rates of population growth, high rates and extent of dispersal, immense abundance, capacity for clonal reproduction, diverse and often cryptic sexual reproduction, ability of social/multicellular lifestyle, some of them also form resting stages and in general they have a limited range of morphological features. At the end of the 19th century, Koch's school

of microbiologists introduced culture techniques facilitating the study of microbial diversity and elaborated model systems that influenced formation of biological theories dealing with specific aspects of the microbial realm including the ecological theories (e.g., Jessup et al. 2004). Pure culture had become the most frequent way to describe and determine a substantial part of microorganism diversity until an introduction of more advanced methods. Although the isolation of microorganisms is undoubtedly time-consuming and laborious, for over a century it was the almost exclusive approach in the studies of microbial diversity. Among other approaches to study microorganisms, culturing still has a privileged status, despite of the Amann et al.'s (1995) estimation that approximately only 1 % of microorganisms can be cultivated using standard and alternative techniques (Kamagata and Tamaki 2005; Thomas et al. 2006). The indications of the vast diversity of uncultured life in all kinds of environments (Hugenholtz 2002; Rappe and Giovannoni 2003) have substantially stimulated the development of culture-independent studies of microorganism communities. An enormous diversity of microorganisms has been uncovered in the last decades thanks to molecular tools. Most of the studies are based on analyses of ribosomal RNA genes (rDNA), especially 16S rDNA for Bacteria and Archea and 18S rDNA for Eukaryota, and on using environmental DNA (eDNA) extracted from bulk environmental samples such as soil, water, and even air. The eDNA approach has offered an opportunity to explore ecological patterns of microbial diversity. Initially, the predominant approach was the 16S/18S rDNA amplification followed by cloning of the obtained amplicons and conventional Sanger sequencing of the molecular clones (e.g., Giovannoni et al. 1990). PCR-based approach, however, may fail to capture a significant portion of diversity because the so-called 'universal' primers are not universal and do not amplify the targeted DNA segment of all taxa (Sipos et al. 2007). Nowadays, cost-efficient high-throughput sequencing (HTS) technologies have progressed rapidly and enabled more comprehensive analyses of microbial communities through the study rDNA amplicons, shotgun metagenomic sequence data, and shotgun metatranscriptomic sequence data (Green and Neufeld 2016). Although the amplicon cloning and conventional sequencing strategies are being replaced by the high-throughput ones, a lot of limitations and challenges still remain in the microbial diversity surveys.

Regardless of the shift to the PCR-independent HTS methods, fundamental problems persist, i.e., the lack of the 16S/18S rDNA reference sequences for a substantial number of microbial taxa including a few high-level taxa (Hug et al. 2016) and numerous taxonomically mislabelled sequences in public databases (Kozlov et al. 2016). Moreover, DNA extraction protocols are not fully efficient (Stach et al. 2001; Vishnivetskaya et al. 2014) and also

16S/18S rRNA regions of various microorganisms can possess different accessibilities to primers and enzymes owing to RNA secondary structure (Geisen et al. 2015). Finally, from the very beginning, environmental HTS studies revealed a huge number of low-abundance populations of microorganisms (Sogin et al. 2006), the so-called 'rare biosphere'.

The rare biosphere (RB) represents a part of a microbial community which is found in a given environmental sample at a specific time point and in an extremely low abundance defined arbitrarily as 0.1 % or 0.01 % (Galand et al. 2009; Pedrós-Alió 2012) or using empirical threshold (Gobet et al. 2010; Haegeman et al. 2013). However, these low-abundance organisms affect both α and β diversity significantly. Lynch and Neufeld (2015) classified the RB microorganisms into five categories based on their temporal abundance profiles, i.e., (1) periodic recruitment from the RB, (2) occasional recruitment from the RB, (3) periodic distribution but permanent rareness, (4) permanent rareness, and (5) transient rareness. The abundance profiles 1–3 belong to RB taxa with an ability to exploit optimal growth conditions and increase their abundance. This very feature provides to the 1–3 RB categories a greater role in microbial communities than would be expected in view of their temporal contribution to the total abundance and biomass. The conditionally rare taxa are often those that exhibit seasonal turnover or can rapidly respond to environmental changes (e.g., nutrient over-enrichment, decreased predation/competition). Although the ecological role of the RB is largely unknown, it seems these taxa are essential for understanding microbial community changeovers (Shade et al. 2014). The RB probably serves as a reservoir of diversity, a 'seed bank' for eventual colonization under optimal conditions (Lynch and Neufeld 2015). An ability of microorganisms to switch from a dormant stage to an active stage depending on environmental conditions favours them. The dormant stage (a state of reduced metabolic activity and usually protected against unfavourable environmental conditions) allows them to survive harsh conditions and still be present. Following a rapid switch to the active stage, these microorganisms may exploit an appropriate opportunity and become abundant.

Critical ecological roles of RB members have been confirmed in some ecosystems, e.g., diazotrophs in the ocean (LaRoche and Breitbarth 2005), ammonia-oxidizing prokaryotes in soils (Leininger et al. 2006) or methanogenic archaea in guts (Saengkerdsub and Ricke 2014). Importantly, RB members may be unrecognized using traditional low-throughput approaches, e.g., culturing or molecular cloning libraries: they either go undetected or their relative abundance is underestimated. However, it should be noted here that the concept of enormous RB importance has also its opponents (e.g., Reeder and Knight 2009) who point

to high risk of technical bias in recognition of the RB, i.e., erroneous and chimeric sequences resulting in the HTS pipelines.

Biogeography and phylogeography

The birth of phylogeography as a scientific discipline with deep roots in historical biogeography and population genetics is ascribed to Avise et al. (1987). In the special issue of Molecular Ecology (Bermingham and Moritz 1998), phylogeography was presented as a bridge linking the study of micro- and macroevolutionary processes. Hickerson et al. (2010) interpreted the term phylogeography as a phylogenetic analysis of organismal data in the context of the geographic distribution of organism. Whereas biogeography deals with the geographic distribution of organisms in general, phylogeography relies on greater resolution of genetic data and focuses on the spatial arrangements of genetic lineages, especially within and among closely related species (Riddle et al. 2008).

Although microorganisms form an important part of the biodiversity and are widely recognized as essential participants in ecosystem processes, they are still poorly understood from biogeography points of view. According to Sutherland et al. (2013), one of the fundamental ecological questions, i.e., whether the same macro-ecological patterns apply to microorganisms and macroorganisms, and whether they are caused by the same processes, had long been beyond the interest. In comparison with macroorganisms, which have been studied since von Humboldt (1805) and Wallace (1876), biogeography of microorganisms was neglected until quite recently when the new methodological approaches have enabled more extensive surveys of microbial diversity (Schloss and Handelsman 2004).

There are two main competing hypotheses concerning phylogeography of microorganisms. During the last century, the Bass Becking classic dictum: ‘everything is everywhere, but, the environment selects’ (Baas Becking 1931; de Witt and Bouvier 2006) was the prevailing view in the microbial biogeography debate. This tenet claims that microorganisms have cosmopolitan distribution and no real biogeography. Some microbial species proliferate only in habitats fitting their needs, however, their geographical distribution does not reflect history at all or at least not to the extent seen among macroorganisms. Large local population sizes and remarkable dispersion potential were perceived as main reasons why microorganisms do not have limited distribution. In other words, the global diversity (γ diversity) of a given microbial group could be ascertained

by exploring a handful of habitats since these distinct habitats should harbour the worldwide microbial diversity. This hypothesis, i.e., the ubiquitous dispersal hypothesis, was advocated mainly by protistologists, especially by Tom Fenchel, Bland J. Finlay, and their collaborators (e.g., Finlay and Clarke 1999; Finlay 2002; Finlay and Fenchel 2004). Furthermore, Wilkinson (2001) suggested that for testate amoebae cosmopolitan distribution became common in species of size below 100–150 μm. Similarly, Fenchel and Finlay (2004) found small organisms (bellow 1 mm) tend to have a cosmopolitan distribution. The ubiquitous dispersal hypothesis was not universally accepted and received considerable criticism (Lachance 2004). Wilhelm Foissner belongs to the leading opponents demonstrating alternative hypothesis for protists, especially ciliates. Foissner (1999, 2006) proposed a "moderate endemicity model" claiming that protists are somewhat similar to macroorganisms in their distribution patterns. This hypothesis is bolstered by the existence of many conspicuous species ('flagship species') observed only in certain specific geographic regions despite intensive sampling efforts elsewhere. The flagship species/taxa were introduced by Tyler (1996) and promoted by Foissner (2006) as the way to prove local endemism of microorganisms, i.e., to focus on showy or unusual taxa that are unlikely to be overlooked in other localities.

Interestingly, the proponents of both hypotheses underline under-sampling as an important factor hindering our understanding of the distribution patterns of microorganisms. Both groups expect that modern methods or more detailed studies can validate their point of view. Indeed, one can easily imagine that at least some of the newly discovered microorganisms claimed to be endemic "flagship" species can be found elsewhere, once we look for them harder (Finlay et al. 2004), as well as more truly endemic species can be identified once we look for them harder (Chao et al. 2006). Indeed, the support for the moderate endemicity model has gradually increased owing to steady growth of molecular data on microorganisms around the world.

One of the first examples of a significant non-random distribution of microorganisms was reported by Cho and Tiedje (2000). Among 248 fluorescent Pseudomonas strains isolated from 38 soil samples from 10 sites on 4 continents they did not observe any overlapping genotypes between the sites as well as between the continental regions, which therefore indicates a strict site endemism of fluorescent *Pseudomonas*. Using a population genetic approach, Whitaker et al. (2003) demonstrated that hyperthermophilic archaea (hotspring *Sulfolobus* assemblages) possess endemic populations isolated by geographic barriers. Bass et al. (2007) mapped global diversity and distribution of three different clades of cercomonads (*Eocercomonas* and *Paracercomonas*) using ITS1 rDNA

sequences and revealed a restricted distribution for some of these. Finally, Bates et al. (2013) proved geographical patterns for different groups of eukaryotic microorganisms from across the eukaryote tree and strongly supported what Foissner (1999; 2006) proposed as the moderate endemicity model.

The ubiquitous dispersal hypothesis has recently been abandoned and 'finally and irrevocably' rejected by van der Gast (2015). Despite this, or perhaps precisely because of this, microbial phylogeography is far from being a dead issue. Further study of issues concerning the microbial phylogeography, such as dispersion of microorganisms, phylogeography of microbial communities, micro- and macroorganisms associations with respect to their geographic distribution, is stimulated by the recent refute of the ubiquitous dispersal hypothesis.

As a part of research focused on phylogeography of prokaryotes, micro- and macroeukaryotes, localities with extreme environmental conditions as well as far distant localities with similar conditions are studied. The Polar regions are no exception. The presence of identical taxa in the polar and subpolar provinces of the Northern and Southern hemispheres without evidence for connecting populations found in the tropics was reported many times, however, it was only molecular confirmation of this phenomenon that enabled to define the term bipolar distribution and bipolar species. Koufopanou et al (1999) defined bipolarity as an extreme case of distribution, in which the disjunctive components are limited to Arctic and Antarctic regions. As an alternative to this term, Shi and Grunt (2000) and Burridge (2002) used the term antitropical distribution of species and recognized three types of this distributions: bipolar (high-latitude, generally ≥50° latitude in each hemisphere), bitemperate (approx. between 30° and 50° latitudes in each hemisphere) and a combination of both. Truly bipolar/antitropical distribution of life forms that are confined to the high-latitude regions of both hemispheres is considered rare by these authors; best known are examples at the generic level, although increasingly apparent also at the species level (see Sections V, VI). The recognition of high genetic similarity between Arctic and Antarctic populations of some microeukaryotes, e.g., planktonic and deep sea foraminifera and dinoflagellates, raised the fundamental question of whether gene flow occurs between bipolarly distributed organisms that are separated by the tropical barrier (Darling et al. 2000, 2004; Montresor et al. 2003; Pawlowski et al. 2007). The quest for the right answer is in the progress taking into account not only the mechanisms but also timing of events leading to trans-equatorial transit and subsequent genetic exchange, i.e., the role of cooling periods associated with glacial cycling in the quaternary period (Darling et al. 2000, 2004). Cold environments were identified as promising targets for

diversity studies of microorganisms in general and emerged as one of the richest sources of data on protistan species (Stoeck et al. 2007).

Diversity of FLA

The early pioneering studies on diversity of FLA were conducted by naturalists of the 18th to early 20th centuries (among others, Gruber 1881, 1894; Schardinger 1899; Cash and Hopkinson 1905; Vahlkampf 1905; Alexeieff 1912). These studies dealt primarily with large/giant amoebae considered evolutionary most important. Necessity to revise taxonomy of the known amoebae became obvious with the establishment of the genera *Hyalodiscus* and *Cochliopodium* by Hertwig and Lesser (1874) but it was Schaeffer (1926), who – with deep respect to the previous attempts – published the important "Taxonomy of the amebas" in which he included about 100 species of FLA classified in 24 genera and 5 families.

Until the end of millennium, the advances in the knowledge of FLA diversity basically reflected the gradual improvement of light microscopes, introduction of culturing methods and, most important, implementation of transmission electron microscopy. Quality of taxonomic work increased as documentation of diagnostically important ultrastructural details became an integral part of species descriptions. In that period, Frederic C. Page was the leading personality in FLA studies. He deserves deepest respect and admiration for the wide scope of his work and a great number of truly fundamental articles and monographs that have remained an indispensable reference till these days despite that some were published almost 50 years ago (e.g., Page 1967). The accumulation of new information on ultrastructure of eukaryotic microorganisms brought about “A Newly Revised Classification of the Protozoa” (Levine et al. 1980) dividing these organisms into seven phyla with FLA assigned to Sarcomastigophora Honigberg et Balamuth, 1963 (Subphyllum Sarcodina Schmarda, 1871). FLA “survived” in the same systematic position for a long time.

With the advent of molecular methods the scope of diversity studies extended and substantially influenced the taxonomy of eukaryotes. Among other attempts to improve the classification of eukaryotes, Tautz et al. (2003) proposed “a scheme in which DNA would be the scaffold of a taxonomic reference system, whilst maintaining the importance of the morphological information associated with whole specimens”. Similarly to other eukaryotes, morphological characters of FLA became increasingly supplemented by molecular and phylogenetic data

that facilitate to derive evolutionary relationships between organisms. Calls for changes of eukaryote classification have intensified with the loss of interest in recognition of morphological determinants available for alpha taxonomy of FLA. As a temporary solution, "The New Higher Level Classification of Eukaryotes with Emphasis on the Taxonomy of Protists" was proposed on behalf of The International Society of Protistologists in 2005 (Adl et al. 2005). An updated version, i.e., "The Revised Classification of Eukaryotes" (Adl et al. 2012) maintained the system of "supergroups" introduced in the former version, incorporated advances of recent widespread use of phylogenomic-scale phylogenetic analyses and massively increased taxon sampling in rRNA-based phylogenies. Furthermore, the revision placed a focus on clustering genera without creating superfluous ranks (subphylum, class, subclass, order, suborder, etc.).

That revised classification of eukaryotes (Adl et al. 2012) reflects the current state of knowledge on FLA and demonstrates their complexity and heterogeneity in that they are members of most of the supergroups of eukaryotes. The ever accelerating development of the knowledge basis of FLA along with the rapid influx of innovative ideas about eukaryotic relationships in general that occurred in the last ten years cannot be meaningfully presented in one coherent summary. Instead, presented below are examples of novel data and major steps in building up our understanding of FLA diversity. Prospective fields of future research are also indicated.

To date, the comparatively most detailed information has been collected on the diversity within the supergroup Amoebozoa Lühe, 1913 (specifically, Tubulinea Smirnov et al., 2005; Discosea Cavalier-Smith et al. 2004/Eudiscosea Tekle et al., 2016; Archamoebae Cavalier-Smith, 1983) and within the supergroup Excavata Cavalier-Smith, 2002, which encompasses Discoba Simpson in Hampl et al., 2009 with Discicristata Cavalier-Smith, 1998 superior to Heterolobosea Page et Blanton, 1985, which all has attracted a considerable attention. The enormous volume of data that has been accumulated on the diversity of FLA belonging to the two supergroups mentioned above is undoubtedly due to the fact that in both these supergroups were described genera containing species pathogenic for humans (*Acanthamoeba* Volkonsky, 1931; *Balamuthia* Visvesvara, Schuster et Martinez, 1993; *Sappinia* Dangerad, 1896 and *Naegleria* Alexeieff, 1912). Some examples of gradual accumulation of new findings and emendation of the basis for comparative studies on diversity of FLA are worth mentioning:

Serious studies on diversity of acanthamoebae date back to the isolation of the first standard *Acanthamoeba* strain by Castellani (1930). Non-

morphological markers were introduced to *Acanthamoeba* diversity research in the 1970s (Visvesvara and Balamuth 1975). In the 1980s, sequences of nuclear 18S rRNA gene supplemented morphometry of cysts [considered more distinctive than morphology of trophic stages (Pussard and Pons 1977; Visvesvara 1991)] and became the standard for characterization of individual strains and a basis for genotype classification system (Gunderson and Sogin 1986). This methodological approach proved to be with considerable forethought. A total of 20 sequence types (T1–T20) have been recognized to date, facilitating classification of isolates and enabling searches for relationships between the nominal species and genotypes (Fuerst 2014). The description of a new species which is pathogenic for humans, *Acanthamoeba byersi* Qvarnstrom, Nerad et Visvesvara, 2013, provides an excellent example. A recent analysis of the whole genome assembly of *Acanthamoeba castellani* ATCC 30010 (Clarke et al. 2013) has further expanded possibilities of *Acanthamoeba* diversity studies and facilitates functional genomic studies.

In the vannellid amoebae, currently classified in the group Flabellinia (Eudiscosea) (Smirnov et al. 2005; Tekle et al. 2016), three important stages in diversity research can be traced: The first stage encompassed light microscopical studies, followed by acquisition of ultrastructural data on the cell surface, i.e., recognition of different types of glycocalyx organization and the use of this character for the generic diagnoses of *Vannella* Bovee, 1965 and *Platyamoeba* Page, 1969. The next step revealed an incongruence of phylogeny with ultrastructurtural markers of *Vannella* and *Platyamoeba*. The presence/absence of glycostyles on the cell surface was declared an invalid generic distinction and, based on the principle of priority, *Platyamoeba* spp. were formally transferred into *Vannella* (Smirnov et al. 2007). Four well-separated single-genus (*Vanella*, *Ripella*, *Lingulamoeba*, and *Clydonella*) clades of vannellids were presented in this article. The third stage in diversity studies of vannellids is associated with the effort to find a safe molecular tool for species distinction in the whole group of naked lobose amoebae. Nassonova et al. (2010) selected six species of vannellid amoebae representing two independent clades in the 18S rRNA gene tree and came to the conclusion that 18S rRNA gene and ITS are inappropriate for species differentiation, while distinctive and homogeneous sequences of COI gene (obtained for each well-defined vannellid morphospecies) are good candidates for barcoding of amoebae.

In Himatismenida Page, 1987 (Discosea sensu Adl et al. 2012), the utility of COI gene for DNA barcoding was tested in *Cochliopodium*, the species of which are morphologically well defined. The study by Tekle (2014) resulted in a compelling recommendation of COI as a barcode marker in *Cochliopodium*

and also poses a challenge to unveil the true diversity of Amoebozoa (their species discovery and strain identification).

Diversity of Dactylopodida, another group of Flabellinia, has attracted a lot of attention due to pathogenicity of some species for aquatic organisms including farmed fish, e.g., Atlantic salmon (*Salmo salar*) and turbot (*Scophthalmus maximus*). The search for true agent of Amoebic Gill Disease (AGD) among phylogenetically related strains of *Paramoeba* Schaudinn, 1896 and *Neoparamoeba* Page, 1987 isolated from gills of Atlantic salmon took almost 20 years (Kent et al. 1988; Dyková et al. 1998; Dyková and Novoa 2001; Young et al. 2007, 2008). Ambitious attempts to fulfil Koch's postulates for infection trials failed many times due to difficulties in culturing specific strains and most probably also because the factors that characterise opportunistic infections are extremely complex and difficult to imitate experimentally. Crosbie et al. (2012), however, described the first known successful culture of *Neoparamoeba perurans* and fulfilled Koch's postulates for *N. perurans* as a causative agent of AGD. The current research on the control of AGD deals also with a remarkable endosymbiotic association of *Paramoeba* and *Neoparamoeba* species with the eukaryotic organism *Perkinsela amoebae* (Hollande, 1980). A strict congruence in the phylogeny of this endosymbiont and its amoeba host (Dyková et al. 2008; Young et al. 2014) has revealed new insights into co-evolution of organisms belonging to different supergroups of eukaryotes.

Search for phylogenetic position of less well-known aggregatively fruiting amoebae (colloquially called "cellular slime molds") supported former doubts about the monophyly of this group of organisms (e.g., Baldauf et al. 2000; Schaap et al. 2006; Brown et al. 2009). Phylogenetic analysis based on sequences of 18S rRNA gene placed *Copromyxa protea* in the eukaryotic supergroup Amoebozoa together with the Tubulinea, in which there had been no previous report of an aggregative fruiting habit (Brown et al. 2011, see also Section IV).

The research of diversity within Heterolobosea Page and Blanton, 1985 has been influenced by medically important issues in the same way as research of acanthamoebae. Since the first cases of primary meningoencephalitis in humans and the diagnosis of the agens as *Naegleria fowleri* (Carter 1970), the main interest focused, for a long time, on sources of its infection, closely related species and their differences. Many species of *Naegleria* have been separated using ITS-5.8 S rDNA sequences. A total of 47 *Naegleria* spp. described to date represent a large percentage of 140 nominal species estimated as an approximate total for Heterolobosea (Pánek and Cepicka 2012; Pánek et al. 2014). Based on the 18S rRNA gene and ITS sequences, some morphologically similar amoebae

assigned formerly to *Vahlkampfia* Page, 1988 were transferred to other genera and renamed as *Tetramitus*, *Neovahlkampfia* and *Paravahlkampfia* (Brown and De Jonckheere 1999). A remarkable diversity of Heterolobosea has become apparent only in the last decade, as series of species and new genera have been described from extraordinary habitats (e.g., those with low pH, high salinity or extreme hydrothermal conditions, rich in minerals, etc.). Phylogenetic analyses based on 18S rRNA gene or ITS sequences place species traditionally listed within Heterolobosea, i.e., acrasid slime molds and species of newly established genera (e.g., *Euplaesiobystra*, *Tulamoeba*, *Selenaion*, *Marinamoeba*, *Allovahlkampfia*, *Fumarolamoeba*, *Parafumarolamoeba*) into Tetramitia Cavalier-Smith, 1993, a strongly supported heterolobosean clade with seven subclades (Pánek et al. 2012; Park et al. 2012) and into a less represented clade Pharyngomonadidae (Cavalier-Smith and Nikolaev 2008). A recent description of *Neovahlkampfia nana* (Tyml et al. 2016) has supported one of two poorly represented subclades of Tetramitia (see Section III).

Cercozoa Cavalier-Smith, 1998 [belonging to Rhizaria Cavalier-Smith, 2002 in the supergroup SAR (Burki et al. 2008)] together with Amoebozoa are most abundant groups of soil protists (Urich et al. 2008) and with a high hidden species diversity predicted by examining environmental DNA (Bass and Cavalier-Smith 2004). Cercozoa are highly diverse in morphology (cercozoan species exhibit body forms of flagellates, amoeboflagellates, filose testate amoebae, naked/testate reticulose amoebae) as well as in ecological means. In addition to bacterivorous representatives, Cercozoa includes also algivorous and mycophagous amoebae (Dumack et al. 2016), endobiotic parasites of plants, diatoms, brown algae, and oomycetes (Neuhauser et al. 2014) or species associated with nodular gill disease of rainbow trout (Dyková and Tyml 2016; see Section II).

Modern methods of sequence data generation, re-evaluation of the phylogeny of eukaryotes and the broadened scope of FLA diversity research have brought into question the traditional theory that these organisms are asexual. The reproductive strategies of individual phylogenetic lineages of amoeboid organisms evidence (directly or indirectly) substantial differences. Mapping sexuality onto the eukaryotic tree of life, Lahr et al. (2011) emphasized that the majority of amoeboid lineages were anciently sexual, while multiple independent losses of sex occurred only in the later evolution. They mentioned a direct evidence of sexual life cycles in free-living thecamoebids, the sorocarpic slime mould *Copromyxa* and testate lobose amoebae (Arcellinida), and a "possible sexuality" in another three lineages of FLA. A comprehensive description of non-meiotic "parasexual activity" in *Cochliopodium* spp. by Tekle

et al. (2014) that includes plasmogamy and karyogamy, formation of plasmodial stage and fragmentation of the latter into uninucleate amoebae will undoubtedly initiate revision of the fusion behaviour observed in other amoebae and intensify clonality versus sexuality debates on FLA.

FLA as hosts of other organisms

In FLA, which are important components of soil and water ecosystems that play the role of predators controlling bacterial populations, a temporary presence of bacteria in their cytoplasm is a well-known phenomenon (Rodriguez-Zaragoza 1994). Interactions between bacteria and amoebae (whether transient or long-term) have recently been summarized several times (e.g., Greub and Raoult 2004; Horn and Wagner 2004; Molmeret et al. 2005). Transient association of amoebae with the bacterium *Legionella pneumophila* (facultative pathogen of humans, agent of Legionnaire's disease) is known in detail and about 20 cases have been recorded in which the facultative symbionts belonged to Alpha-, Beta-, and Epsilonproteobacteria; Actinobacteria; Firmicutes; Chlamydiae and Fungi. Obligate endosymbionts of FLA were found within Alpha- and Betaproteobacteria, Chlamydiae and Bacteroidetes. Schulz et al. (2015) supplemented the list of obligate symbionts of amoebae with a member of Gammaproteobacteria (see Section VII). Identificatin of novel acanthamoebal endocytobionts of the family Parachlamydiaceae extended the knowledge of the phylogenetic diversity of chlamydiae and turned the attention from the public health issue also to distribution of chlamydiae in the environment (Everett et al. 1999; Greub and Raoult 2002; Horn et al. 2000; Horn & Wagner 2004; see also Section VIII).

An unexpected pattern of symbiotic associations of FLA with prokaryotes was disclosed by means of transmission electron microscopy. In addition to the common intracytoplasmic localization, symbionts were detected also in the nucleoplasm (*Nucleicultrix amoebiphila* in '*Hartmannella*' sp., Schulz et al. 2014), arranged around the nucleolus (chlamydial symbiont 'Pn' in *Naegleria clarki*, Michel et al. 1999; Walochnik et al. 2005), and the perinuclear cisterna (*Nucleophilum amoebae* in amoebozoan JAMX8 strain, Schulz et al. 2015; see Section VII). A discovery of *Nucleicultrix amoebiphila* in the nucleus of '*Hartmannella*' sp. and the fact that this symbiotic association can be reproduced experimentally provides a unique possibility to better understand mechanisms underlying endonuclear symbiosis (Schulz et al. 2014; Schulz and Horn 2015).

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Section II

Nodular gill disease of rainbow trout associated with testate amoeba

Journal of Fish Diseases 2016, **39**, 539–546 doi:10.1111/jfd.12384

Testate amoeba *Rhogostoma minus* Belar, 1921, associated with nodular gill disease of rainbow trout, *Oncorhynchus mykiss* (Walbaum)

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Abstract

The case study targeted to determine the aetiology of nodular gill disease (NGD) of farmed rainbow trout. The methods included microscopical examination of gill material in fresh, culturing of isolated organisms, histology, transmission electron microscopy and molecular biology identification. The results revealed an intravital colonization of fish gills by the testate amoeba *Rhogostoma minus* Belar, 1921. *Rhogostoma* infection was found in all fish examined microscopically (15/15); in contrast, naked amoebae related to fully developed NGD lesions were found in minority of these fish (5/15). They belonged to four genera, *Acanthamoeba*, *Vermamoeba*, *Naegleria* and *Vannella*. Results presented in this study contribute to the mosaic of findings that contrary to amoebic gill disease of marine fish turn attention to the possibility of the heterogeneous, multi-amoeba-species and multifactorial aetiology of NGD.

Keywords: naked amoebae, nodular gill disease, *Rhogostoma minus*, testate amoebae.

Introduction

Gill disorders of freshwater salmonids associated with amoebae (*Thecamoeba hoffmani*) were first reported by Sawyer *et al.* (1974, 1975). In 1985, Daoust and Ferguson described a nosological entity 'nodular gill disease' (NGD) of rainbow trout and defined it as multifocal hyperplasia of gill epithelium associated with amoebae (*Cochliopodium* sp.). Unfortunately, none of these reports included a detailed diagnosis or an adequate documentation of the agent. As to regards of the amoeba species involved, subsequent studies of NGD brought different results. Dyková *et al.* (2010) proved the attachment of *Naegleria* trophozoites to hyperplastic epithelium of rainbow trout by *in situ* hybridization; however, isolation attempts revealed a co-occurrence of other species of naked amoebae in the affected gill tissue, not allowing simplification of NGD aetiology to any one amoeba species. To date, naked amoebae belonging to five genera, *Acanthamoeba*, *Vermamoeba* (formerly *Hartmannella*), *Naegleria*, *Protacanthamoeba* and *Vannella*, have been reported as potential agents characterized by morphological and molecular data (Dyková *et al.* 2010). As other studies of NGD in freshwater salmonids reported just a general amoebic aetiology (Speare 1999; Buchmann *et al.* 2004; Antychowicz 2007; Tubbs, Wybourne & Lumsden 2010), a broader spectrum of agents is conceivable. To better understand the aetiology of NGD, data from outbreaks should more effectively characterize the amoebae by culture, morphology, and genetic studies.

This study is based on an outbreak of rainbow trout gill disease in a farm in north Moravia, Czech Republic, referred to us for diagnosis in June of 2014. According to the farm history provided, NGD had been repeatedly diagnosed in rainbow trout of market size, but the amoeba agent had not been identified. To make an aetiological diagnosis

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and understand what component factors could be involved in causing disease, we collected both fish material and essential data on water supply system and environmental events.

Materials and methods

Sampling for aetiological diagnosis

Fifteen rainbow trout *Oncorhynchus mykiss* of market size (28–35 cm) manifesting the most severe clinical signs of gill disease were selected from a dense stock and transported to the laboratory with water from the tank and adequate aeration. Another 15 random fish from the same stock were examined macroscopically for gill lesions on the spot.

Laboratory examination of fish (n = 15)

Post-mortem gross examination of gills and organs of the body cavity was followed by microscopic examination of gill lesions and isolation attempts targeted at free-living amoebae as potential agents of grossly visible gill alterations. Light microscopy of scraped gill mucus, squashed samples of gill tissue and material lavaged from gill arches (using Page 1988 amoeba saline) concentrated by centrifugation was carried out in translucent light and with the aid of an Olympus BX60 microscope equipped with Nomarski differential interference contrast (DIC) optics. The procedure used for isolation of organisms from samples of gill tissue followed that routinely used for isolation and enrichment of gymnamoebae (Page 1988; Dyková & Kostka 2013). To evaluate histopathological lesions, tissue samples of gills and parenchymatous organs of the body cavity were duplicated. These were fixed both in Davidson fixative and in neutral buffered formalin and processed using routine histological techniques (dehydration with ethanol series, embedding in Histoplast, staining with H&E).

Establishment and maintenance of cultures

The same three types of samples as collected from gills for examination in fresh (i.e. mucus, tissue, and lavaged material) were inoculated on 1.5% agar containing trace amounts of yeast and malt extracts and moistened on the surface with a few drops of Page amoeba saline (Page 1988). Six agar plates were used per fish, two for each type of gill material to perform a simultaneous incubation at 10 and 20 °C. Due to a bacterial load and contamination with fungi, primary isolates required daily observation. As we were not able to predict the progress of isolation attempts, all primary isolates, including contaminated ones, were stored for DNA extraction in cases of need.

Morphological identification of isolated organisms

The observations of cultured cells of interest were performed with an Olympus BX 60 microscope using Nomarski DIC optics. For transmission electron microscopy, cultures were fixed on agar plates *in situ* with cacodylate- or phosphate-buffered 2.5% glutaraldehyde, washed off after 30 min, pelleted and post-fixed with 1% osmium tetroxide for 1 h. Pellets were dehydrated in acetone series and embedded in Spurr resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a JEOL JEM 1010 electron microscope operating at 80 kV.

Molecular identification of isolated organism

DNA extraction from homogeneous cell culture of ZER3 strain was done using DNeasy Blood and Tissues Kit (Qiagen) according to the manufacturer's instructions. Eukaryotic SSU rRNA gene was amplified by PCR, using universal primers Erib1 and Erib10 complementary to the conserved ends of the eukaryotic SSU rRNA genes (Barta *et al.* 1997). PCR (25 μL) included 12.5 μL 2 × concentrated PPP Master Mix (Top-Bio), 0.1 μM of each primer and 1 μL of genomic DNA. PCR was performed in Mastercycler eP (Eppendorf) under the following conditions: after initial denaturation at 95 °C for 10 min, 35 cycles of denaturation at 94 °C for 1 min and annealing at 44 °C for 1.5 min followed by final extension at 72 °C for 10 min. PCR product was purified using the High Pure PCR Product Purification Kit (Roche Diagnostics) and cloned into the pCR™4 vector using TOPO TA Cloning Kit (Invitrogen) following the manufacturers' instructions. Plasmid DNA from three clones was purified using the High Pure Plasmid Isolation Kit (Roche Diagnostics) and sequenced by universal M13 primers at Macrogen Inc. (Amsterdam). Multiple alignment comprising all available sequences of *Rhogostoma* was prepared using the MAFFT 7 (Katoh 2013), and sequence identities were determined.

Results

Findings common to all fish examined

Grossly visible lesions were observed on gills of all 15 fish specimens selected for laboratory examination as well as on the gills of 15 randomly checked specimens from the same stock. The most severe lesions were consistent with dramatic clinical signs that aggravated while fish were given food. Fish competing for food changed their swimming pattern and position in the water column, gulping at the water surface with flared opercula, that is, presenting signs of oxygen depletion. The most severe lesions consisted of a diffuse hyperplastic response of gills; flared opercula gave the impression that gills could not fit into the opercular cavity. Invariably, distal parts of gill filaments, mostly up to the middle of their length, were pale or white. They were covered with an enormous amount of mucus, which gave the gills a glistening appearance. Grossly visible swellings of gill filaments with focal discolouration and pronounced hyperproduction of mucus were present in all fish examined.

Microscopic observations of fresh material. Gill samples from the 15 fish examined microscopically shared a uniform finding. Multiple small cells of non-host origin were present in mucus as well as squashed gill tissue (Fig. 1a). Observed in translucent light under the pressure of coverslip, these foreign cells had a rounded outline. They were characterized by a transversal strip of dark, tiny bodies localized in the cytoplasm. In Nomarski optics, the cells appeared as slightly ovoid or irregular spheres with numerous refractile bodies in their cytoplasm.

Microscopic observations of cultured organisms. In 12 h past transfer of the three types of material (i.e. gill mucus, gill tissue, lavaged material) to agar plates, cells of our interest (those with the same light microscopical characteristics as observed in fresh material) started to multiply together with bacteria and other microorganisms (yeasts, fungi, algae, etc.). Homogeneous population of organisms representing a novel strain (ZER3) of our interest was obtained by subculturing of a primary isolate that was not contaminated by fungi. To assure that a representative culture of ZER3 was studied, ZER3 cells were repeatedly compared with cells from other (contaminated) isolates.

When observed in translucent light on the surface of agar plates, most cells were separate individuals even in dense cell populations (Fig. 1b). In well-established cultures, a growing zone was observed on the periphery, where conspicuous groups of cells were present that resembled flower petals by their arrangement. These cells were of ovoid shape with a slightly conical part pointing to the group centre. Such 'petal formations' consisting of four up to 12 cells, as well as piles of more cells formed in the course of subsequent multiplication, could be seen even through agar plates (Fig. 1c). Similar cell groupings were observed in hanging drop preparations (Fig. 1d). Separate individual cells in cultures were predominantly spherical or slightly ovoid (Fig. 1e). The diameter of spherical cells ($n = 20$) was 8.5 (7.3–9.7) μm, whereas average size of ovoid cells ($n = 20$) was 7.9 × 10.7 μm. Some cells were cordiform with vacuoles discernible on one side only (Fig. 1f, arrow). The average size of the latter cells ($n = 10$) was 8.8 × 10.5 μm. A faint indication of pseudopodia formation was noted in cultures *in situ* on agar plates and was conclusively confirmed in hanging drop preparations where also division of cells was observed (Fig. 1g). Due to spherical or ovoid shape of cells, filose pseudopodia attached to coverslip could only be seen out of the focus of cell bodies. They were thin, hyalin, sometimes branching or projecting from a broad hyalin base but never anastomosing (Fig. 1g). Filose pseudopodia were observed in separate individuals as well as cell unions ('petal formations'). The adhesion of cells on the surface of agar and on coverslips was considerably firm. We did not succeed in observation of true active locomotion of cells. Sedentary type of culture containing a few cells slowly moving on the spot predominated in our observations.

Ultrastructure of isolated organism. A key finding of electron microscopical observations of ZER3 amoeba strain was the fine structure of 'ventral' part of trophozoites adhered to substrate with oral aperture (pseudostome) (Fig. 2a) lined by oral (appertural) collar being a modified part of the organic shell (Fig. 2b). This finding unambiguously confirmed the determination of ZER3 strain as a testate amoeba. Other ultrastructural features of special interest were a thin, single-layered flexible organic shell that adhered to the whole surface of cell (Fig. 2a), a single rounded nucleus with a centrally located nucleolus, mitochondria with

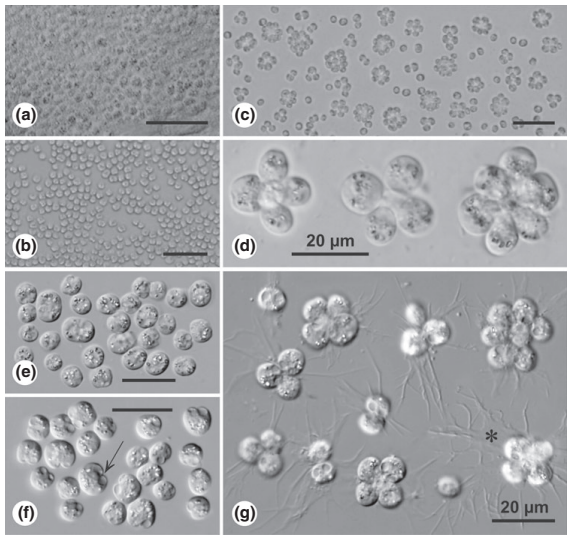

Figure 1 Light microscopy of *Rhogostoma minus*, strain ZER3 isolated from gills of farmed *Oncorhynchus mykiss*. (a) Trophozoites as observed in fresh material taken from fish gills. (b) Well-established agar plate culture as observed through Petri dish in centric part around agar blocks transferred from previous passage. (c) Aggregates of cells resembling flower petals as observed through Petri dish on the surface of agar. (d) Aggregates of cells formed in Page amoeba saline-based suspension on hanging drop coverslip (24-h-old preparation). (e) Usual appearance of amoebae from a well-grown agar plate culture, observed in hanging drop preparation. Refractile bodies are well visualized by Nomarski DIC optics. (f) Trophozoites with conspicuous intussusception on their periphery (arrow), seen in hanging drop preparation. (g) Individual and fused cells attached to coverslip, exhibiting filose pseudopodia emerging from pseudostome (out of focus). Note the broad hyaloplasmic adhesive region (asterisk) from which some filopodia extend. Hanging drop preparation image is focused primarily on pseudopodial complexes seen in Nomarski DIC optic. Scale bars (a) = 100 µm; (b, c) = 50 µm; (e, f) = 20 µm.

tubular cristae (Fig. 2c), Golgi apparatus with cisternae arranged in dictyosome-like formation (Fig. 2d), numerous vesicles, digestive vacuoles and symbiotic bacteria that were constantly present in the cytoplasm. On the periphery of bacteria-containing vacuoles were observed yet unidentified electron-dense structures.

Based on correlated light and electron microscopy studies, we concluded that the newly isolated ZER3 amoeba strain shares most characters with *Rhogostoma minus* Belar, 1921 (as described also in Mylnikova & Mylnikov 2012) and with F-20 strain of *Difflugiella* sp. described in a detail ultrastructural study by Griffin (1972).

Molecular identification. To identify the obtained sequences, we used the BLAST search against the DDBJ/EMBL/GenBank databases and unambiguously confirmed the cercozoan affiliation of ZER3 strain. The closest BLAST hits belonged to five *Rhogostoma* and several environmental sequences. Pairwise distances determined between SSU rRNA gene sequences of ZER3 and *Rhogostoma* spp. indicated sequences AJ514867 (*R. minus*), HQ121431 (*R. minus*) and HQ121436 (*R. micra*) as the most similar (identities 97.7%, 97.6% and 96.7%, respectively). Thus, ZER3 strain was identified with *R. minus* (Belar 1921), which has recently been assigned to Cercozoa, Cryomonadida and Rhizaspididae into a well-supported clade consisting of six sequences (Howe *et al.* 2011). The three clone sequences of the SSU rDNA obtained from this strain were identical; therefore, one sequence only has been deposited in DDBJ/EMBL/GenBank databases (Accession Number LC032468).

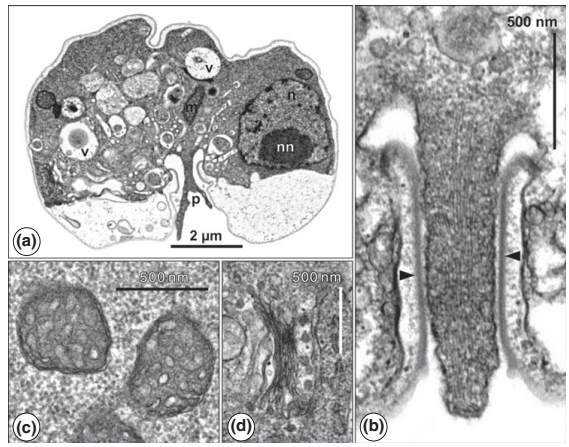

Figure 2 Fine structure of *Rhogostoma minus*, strain ZER3. (a) Electron micrograph of an entire trophozoite sectioned in diagnostically important plane through oral region with pseudopodial stem emerging through oral opening. Structures labelled: m mitochondria; n nucleus; nn nucleolus; p pseudopodium; v vacuoles with bacteria. (b) Detail of oral aperture. Main pseudopodial strand containing microtubules is lined with a distinct collar (arrowheads). (c) Mitochondria with tubular cristae. (d) Golgi apparatus with cisternae arranged in dictyosome-like formation.

Other findings

In addition to the testate amoeba *R. minus*, which was present in all rainbow trout examined, another six strains belonging to four genera of naked amoebae were isolated from gill samples of five fish. They were *Vannella* (ZER10, ZER12, ZER14), *Naegleria* (ZER2V), *Vermamoeba* (ZER2M) and *Acanthamoeba* (ZER15) (Fig. 3). All of these were isolated with a 14- to 16-day delay relative to *R. minus* (ZER3 strain), from repeatedly moistened gill tissue samples maintained at 20 °C. Such a long delay in obtaining the primary isolates suggests that only a low number of cells were present in tissue samples. A careful subsequent histological examination of gill tissue samples revealed several small groups of naked amoebae scattered throughout the gill tissue in four of 15 fish examined (Fig. 4).

Source of infections

Data collected from the farm owner helped us to better understand the NGD outbreak studied. Three weeks before fish manifested severe clinical signs of NGD, a heavy rainfall occurred in the region. Due to this event, in the dam that supplies the rainbow trout farm, the turbidity of water increased substantially by presumably soil particles, brook and river sediments and other eroded material washed off from an extensive area upstream. No exact data regarding saprobic water classification were obtained; however, the increased turbidity was observed even in the depth of water column from which the farm draws water. No environmental screening of the presence of free-living amoebae in water-contaminating materials is available; however, it is concluded that both the testate and naked amoebae had been introduced into the farm system via water supply (either in a large number or under circumstances favourable for their fast multiplication).

Discussion

Compared with previous reports on confirmed and potential agents of NGD of salmonids, the finding of *R. minus* infection presented in this paper is exceptional in several respects. First, there are only two previous reports on an incidental finding of testate amoebae in fish: one from a not specified tissue of trout (Simitzis & Le Goff 1981) and the other one from gills of *Cyprinus carpio* (Lom & Dyková 1992). As regards presence of free-living amoebae in altered gill tissue, previous findings were confined to naked amebae, the generic affiliation of which is not difficult to assess at the level of light microscope. This is not the case of testate amoebae with soft shell. The main characters traditionally used in the identification of testate amoebae are the shape, structure and composition of the shell and the type of pseudopodia they emit. Unlike identification of testate amoebae with agglutinated shells (with xenosomes

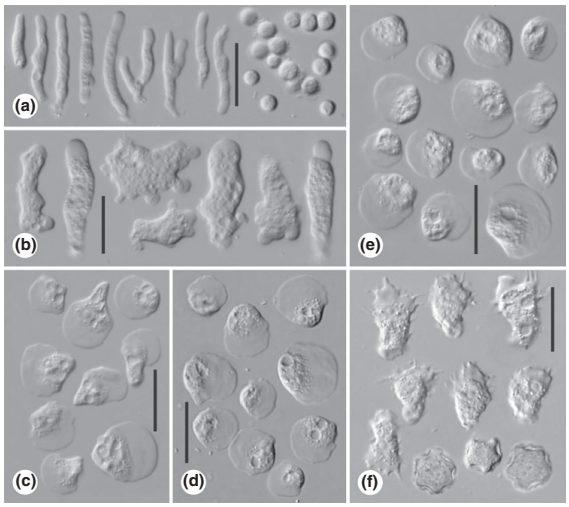

Figure 3 Trophozoites of naked amoebae isolated from gills of rainbow trout under study. (a) ZER2M strain of *Vermamoeba* sp. (b) ZER2V strain of *Naegleria* sp. (c) ZER10 strain of *Vannella* sp. (d) ZER12 strain of *Vannella* sp. (e) ZER14 strain of *Vannella* sp. (f) ZER15 strain of *Acanthamoeba* sp. Scale bar (joint for a to f) = 20 μm.

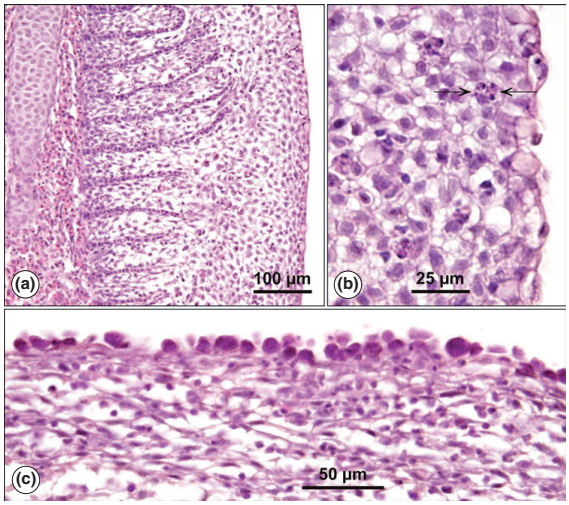

Figure 4 Histopathological lesions in gills of rainbow trout examined (H&E). (a) Extensive hyperplasia of epithelium on the tip of gill filament that is transformed into club-shaped formation. (b) Trophozoites of *Rhogostoma minus* (arrows) scattered among hyperplastic epithelium of gill filament. (c) Trophozoites of naked amoebae attached to the surface of hyperplastic epithelium.

or idiosomes), in the identification of thecate amoebae that form thin flexible shells closely adherent to cell membrane, light microscopical study is not sufficient: a detailed study of the fine structure is required to find the ventral pseudostome lined with collar. Trying to identify our novel ZER3 strain, we faced the problem of a limited number of studies available for comparison. Most of the electron microscopic characters observed in our ZER3 strain were consistent with those described in Le-2 strain of *R. minus* Belar, 1921 by Mylnikova & Mylnikov (2012) and in F-20 strain of *Difflugiella* sp. of Griffin (1972). However, the organism studied by Griffin (1972) as a model of locomotion was assigned to *Difflugiella* only tentatively (Griffin

himself admitted the possibility of revision of this generic affiliation). In relation to the top quality of description and documentation of ultrastructural details presented by Griffin (1972), it is regrettable that F-20 strain evidently has not been maintained in culture collection for sequencing. Anyway, thanks to molecular data collected in the recent phylogenetic study focused on Cercozoa (Howe *et al.* 2011), the testate amoeba isolated in our study could be unambiguously determined as *R. minus*.

The affected rainbow trout displayed typical NGD lesions containing *R. minus* as the predominating amoeba. However, the interpretation of aetiology of lesions and, specifically, of the role of this testate amoeba cannot be conclusive. A possible scenario of the development of these lesions might have included a primary fast-course infection by naked amoebae with only a subsequent infection by *R. minus* that played the dominant role only in the late phase studied. Our findings accentuate the need to study NGD in its whole course and complex circumstances so that both the principal and component causes of the disease may be elucidated.

In the light of a recent recognition of phylogenetically diverse lineages of amoebae/amoeboid organisms within Amoebozoa and Cercozoa (Adl *et al.* 2012; Howe *et al.* 2011), determination of agents within lesions has become more demanding but it remains an essential prerequisite for a correct aetiological diagnosis.

The previous data and the present study clearly show that NGD exemplifies a fish disease problem that is closely related to the environment. We fully identify ourselves with the opinion formulated by Cameron (2002) that it is not possible to consider either the aquatic animals or the environment in isolation but that it is necessary to study the state of health of the entire farm system.

Acknowledgements

This study formed part of the ECIP project No. 505/12/G112 supported by the Grant Agency of the Czech Republic.

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Received: 15 March 2015
Revision received: 1 April 2015
Accepted: 1 April 2015

Section III

Heterolobosean clade Tetramitia I and its new species

The Journal of Eukaryotic Microbiology

ISOP Published by the International Society of Protistologists

Journal of Eukaryotic Microbiology ISSN 1066-5234

ORIGINAL ARTICLE

Neovahlkampfia nana n. sp. Reinforcing an Underrepresented Subclade of Tetramitia, Heterolobosea

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Keywords
18S rRNA; Discicristata; free-living amebas; sequence variability; taxonomy; ultrastructure.

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Received: 30 March 2016; revised 16 June 2016; accepted June 16, 2016.

doi:10.1111/jeu.12341

ABSTRACT

The study provides robust genetic evidence that a newly isolated naked ameba with morphological and ultrastructural features indicative of Heterolobosea is a new species. *Neovahlkampfia nana* n. sp. associates with the yet underrepresented subclade of Tetramitia I. Considerable differences found in 18S rRNA gene sequences of individual molecular clones derived from DNA of five clonal cultures, using a low fidelity DNA polymerase, raised the issue of intragenomic sequence variation, a phenomenon that has not been previously studied in Heterolobosea. However, as proved using a higher fidelity DNA polymerase, the sequence variability observed was introduced by PCR mediated by the low fidelity polymerase and fixed by molecular cloning. This points to the potentially dubious validity of some current nominal species of Heterolobosea that differ from one another in just one or two base positions.

WHEN Heterolobosea Page and Blanton 1985 was established as a class, it included schizopyrenid amoeboflagellates and acrasid slime molds. The past few decades have seen a remarkable increase in the knowledge about this class, especially by employing molecular methods. Currently, Heterolobosea is considered to be a major eukaryotic group of the supergroup Excavata (Adl et al. 2012), which includes about 150 nominal species (Geisen et al. 2015) of immensely diverse protists (Pánek and Cepicka 2012; Pánek et al. 2012, 2014a). Of those, the most attention has been paid to vahlkampfiids (Vahlkampfiidae Jollos, 1917). The most diverse group within Heterolobosea is Tetramitia (Cavalier-Smith and Nikolaev 2008), which is divided into seven major clades (Pánek et al. 2012; Park et al. 2012). A substantial number of molecular studies have been conducted on five of these seven clades (e.g. microaerophilic and anaerobic heterolobosean species of *Pseudoharpagon* and *Monopylocystis*), however, there is also a well supported clade that until recently has consisted of only two sequences: one of the type strain of *Neovahlkampfia damariscottae* (Page 1974) Brown and De Jonckheere 1999 (the type and only species of the genus) and the other one of strain AND9 of a soil ameba (Lara et al. 2007). In this paper, we present light microscopic, ultrastructural, and molecular data of a novel strain of *Neovahlkampfia* and describe it as a new species.

MATERIALS AND METHODS

Isolation and culturing

The study strain (UD12) was isolated from gill tissue of farmed rainbow trout (*Oncorhynchus mykiss*) sampled from a stock affected by nodular gill disease (NGD) in its early phase of development. A gill tissue sample was inoculated on the surface of nonnutrient agar (Page 1967) and the primary isolate obtained on day 10 post inoculation was transferred onto 1.5% agar with malt and yeast extracts and then subcultured on the basis of regular daily observation of cultures through Petri dishes. Agar plate cultures were maintained in an incubator at 20 °C. No attempt was made to completely purify the cultures from accompanying bacteria and no bacteria were added as food. In order to test salinity tolerance, trophozoites washed off agar plates and concentrated by centrifugation were resuspended and exposed to four different concentrations (75, 50, 25, and 12.5%) of synthetic (Sigma-Aldrich, Darmstadt, Germany) seawater for 2 h.

Thereafter, they were plated on standard NN agar (Page 1988) and checked up for 10 subsequent days. Passage No 12 was used for isolations of five clonal cultures (UD12/I-UD12/V). Due to the small size of the trophozoites, it took over 20 attempts to obtain five clones.

Light and electron microscopy

Light microscopy observations of the trophozoites were made in hanging drop preparations, using an Olympus BX60 microscope equipped with Nomarski optics and an Olympus DP71 digital camera. Images of 100 trophozoites captured as more or less straight limax forms were used for measurements. Because the primary isolate was contaminated with a bodonid flagellate, much attention was paid to enflagellation tests and the potential occurrence of individual flagellates in subcultures; repeated attempts were made to induce the flagellate transformation of trophozoites harvested from plate cultures as described by Page (1967) and Chang (1958).

For the ultrastructure study, cells on agar plates were fixed with 3% glutaraldehyde in ameba saline (Page 1988) for 20 min, washed off, pelleted, postfixed with 1% osmium tetroxide for 1 h, dehydrated with a series of acetones, and embedded in Spurr resin. A JEOL JEM 1010 electron microscope equipped with the Mega view III soft imaging system was used for examining and documenting ultrathin sections stained with methanolic uranyl acetate and lead citrate solutions.

DNA extraction, PCR, cloning, and sequencing

Depending on the phase of UD12 subculturing, two different series of DNA samples were prepared. For one series, homogeneous cultures of UD12 were washed off the agar plates with ameba saline, concentrated by centrifugation, and resuspended in TNES buffer (Asahida et al. 1996). DNA was obtained from these samples using the phenol-chloroform extraction method. For the other DNA series, five clonal cultures of UD12 (UD12/I–UD12/V) were prepared the same way as the previous samples and DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany).

For 18S rRNA gene amplification, the PCR protocol described in Dyková et al. (2008) was followed using Taq-Purple DNA polymerase (Top-Bio, Prague, Czech Republic) and the primers 18e (Hillis and Dixon 1991) and Erib10 (Barta et al. 1997). Furthermore, 18S rRNA gene was amplified also using the same primers and Q5 High Fidelity Master Mix (New England Biolabs Inc., Ipswich, MA) according to manufacturer's recommended protocol. The entire ITS region was amplified according to Garstecki et al. (2005) using the same Taq-Purple DNA polymerase and DNA of *Vahlkampfia signyensis* F21M strain as a positive control. The PCR products obtained were purified with the High Pure Purification kit (Roche Diagnostics GmbH, Mannheim, Germany). Sequencing of the first group was done at Macrogen Europe Inc. (Amsterdam, the Netherlands) in forward and reverse direction with the aforementioned primers and with the internal primer 620F (5′-GCCAGCACCCGCGGTAATTCC-3′) while those from the five clonal cultures (UD12/I–UD12/V) were cloned into pCR™4-TOPO® vector using TOPO® TA kit (Invitrogen, Paisley, UK). Plasmid DNA was purified from independent transformed colonies of *Escherichia coli* One Shot® TOP10 using the High Plasmid Isolation kit (Roche Diagnostics GmbH, Mannheim, Germany). A total of 65 molecular clones, 25 from the UD12/I culture clone and 10 from each of the UD12/II–V culture clones, were sequenced bidirectionally with the M13-pUC primers and the internal 620F primer (as above) at Macrogen Europe Inc. The sequences obtained were manually assembled, checked for unclear basecalls and edited using Geneious R9.0.5 software (Geneious, Auckland, New Zealand). The sequences were deposited in the DDBJ/EMBL/NCBI databases under Acc.Nos. LC126917–LC126981.

Phylogenetic analysis

Sequence identities were checked against the DDBJ/EMBL/NCBI databases using BLAST search. To infer the phylogenetic position of the novel strain UD12, we generated a dataset composed of 18S rRNA gene sequences of 78 heterolobosean representatives with *Pharyngomonas* sequences as the outgroup. The dataset was aligned by the MAFFT online version using the Q-INS-i strategy taking secondary RNA structure into consideration (Katoh and Toh 2008) and trimmed to 1,233 unambiguously aligned nucleotide positions. Maximum likelihood (ML) and Bayesian inference (BI) gene trees were constructed using RAxML (Stamatakis 2006) and MrBayes (Huelsenbeck and Ronquist 2001), both implemented in Geneious. ML analysis was computed under the GTR GAMMA I model and rapid bootstrapping (1,000 replicates). Bayesian analysis was performed using four simultaneous Markov–Monte Carlo chains (temperature 0.2) under $10{\cdot}10^6$ generations and sampled every 2,000 generations with the covariance GTR + I + G substitution model. The first 25% of the computed generations were discarded as the burn-in. The −lnL was plotted against generation to check that burn-in was sufficient. For all the culture clones of the UD12 strain, general genetic diversity patterns were evaluated using DnaSP 5.10 (Librado and Rozas 2009).

RESULTS

Light microscopy of UD12

On the surface of agar plate cultures observed under the light microscope, a broad annular growing zone could be seen with densely accumulated, small, more or less cylindrical trophozoites. The growing zone of trophozoites gradually expanded peripherally, while globular aggregates appeared in the center of this zone. In the hanging drop preparation, the trophozoites from the growing zone displayed features typical of the "limax" monopodial heterolobosean amebas with eruptive cytoplasmic flow (Fig. 1A). Moving trophozoites were mostly cylindrically

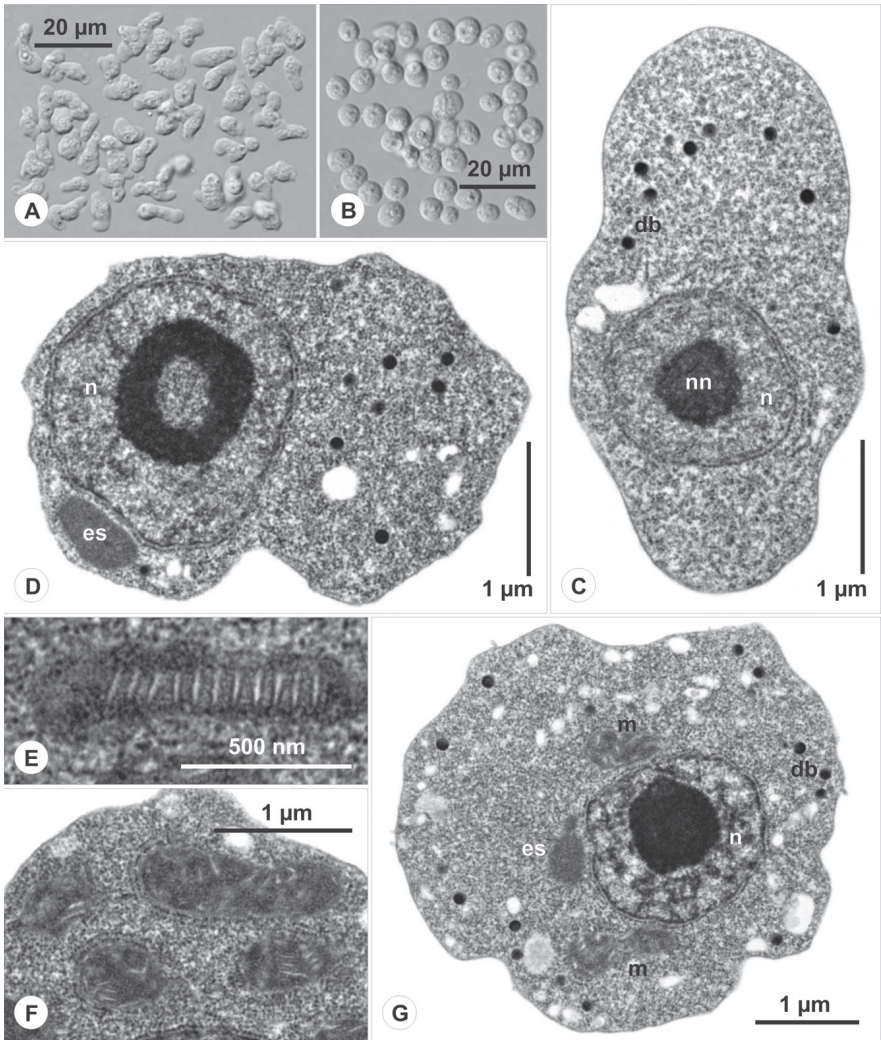

Figure 1 Light microscopy and ultrastructure of *Neovahlkampfia nana* n. sp., strain UD12. (**A**) Locomotive forms and (**B**) globular forms as seen in hanging drop preparations (Nomarski DIC). (**C**) Anterior part of cytoplasm of locomotive form rich in ribosomes, with vesicular nucleus (n), prominent nucleolus (nn), and dense bodies (db). (**D**) Locomotive form with enigmatic electron-dense structure (es) in the vicinity of nucleus (n). (**E**) Mitochondrion with parallel flattened/discoid cristae arranged in a distinct pattern. (**F**) Mitochondria with discoid cristae arranged in a less organized pattern, surrounded by rough endoplasmic reticulum. (**G**) Globular form with nucleus (n), mitochondria (m), electron-dense structure (es), and dense bodies (db) in cytoplasm.

symmetrical (the cytoplasmic eruptions appeared at the anterior part as rounded hyaline caps); occasionally, however, the shape and direction of locomotion of the trophozoites changed by eruptions directed toward the sides. The granuloplasm contained a single vesicular nucleus with a prominent nucleolus. The posterior part of the trophozoites was more or less rounded with no projections visible in the light microscope and contained a contractile vacuole perceptible in both the hanging drop preparations and the conventional covererslip covered preparations. The locomotive forms ($n = 100$ cells from the 2-d culture) were 8–15 (mean 12) μm long; the cylindrical "limax" forms were 3–6 (mean 4.5) μm broad; the length: breadth ratio was 2.2–3.2 (mean 2.7). The globular forms agglomerated in the central zones of well-established agar plate cultures were 6–8 (mean 7) μm in diameter (Fig. 1B). If suspended in ameba saline or distilled water, young trophozoites from the growing zone rounded up almost immediately or in up to 5 min (2–5 min) and formed globular floating forms 7 μm in diameter. Within another approximately 20 min, these globules attached and regained all the characteristics of the locomotive form (Fig. 1A). The trophozoites remained active for at least 24 h in both ameba saline and distilled water; however, the proportion of active cylindrical trophozoites was lower in distilled water than in ameba saline. The amebas failed to transform into flagellates in the enflagellation tests using either ameba saline or distilled water.

When several drops of a cell suspension were washed off the surface of a parent agar plate culture and then subcultured, the multiplication of cells intensified: a confluent layer of mutually adherent globular cells covered the surface of the agar plates within 48 h while cylindrical trophozoites were less prominent. The liquid medium (ameba saline with trace amounts of yeast and malt extracts) did not enhance the growth of the strain under study. The response of trophozoites exposed to 75 and 50% synthetic seawater for 2 h was negative: the cells shrank immediately and irreversibly. The concentrations of 25% (which corresponds to the upper limit of brackish water salinity, 3–10 ppm) and 12.5% were tolerated, an extended exposure time, however, was not tested.

Ultrastructure of UD12

The cell surface of the locomotive forms was smooth, the cell membrane was thin, and no cell coat was discernible. The cytoplasm of the trophozoites was rich in ribosomes, the nucleus of the vesicular type contained a single central nucleolus (Fig. 1C, D), and the mitochondria with discoid cristae (Fig. 1E, F) were frequently surrounded by a closely associated rough endoplasmic reticulum (Fig. 1F). An ultrastructurally defined contractile vacuole was not observed. Nonmotile globular and aggregating cells showed various stages of nuclear division characteristic of closed orthomitosis. Along with mitochondria with discoid cristae, as many as 40% of the globular cells contained in ultrathin sections an irregularly outlined microbody/organelle (i.e. electron-dense material limited by a close-fitting membrane that was located in the vicinity of the nucleus (Fig. 1C, 2A–C). These microbodies slightly varied in density and outline; some manifested signs of disintegration or internal degradation (Fig. 2D, E). Some were closely associated with the rough endoplasmic reticulum (Fig. 2C). These microbodies were exceptionally present also in locomotive forms (Fig. 1D). Although the uniform globular shape of the nonmotile form seen in the light microscope implied that cysts might be formed, cyst formation was not observed either in fresh or aging cultures regardless of whether on agar plates or in liquid medium. Moreover, in ultrathin sections, the outline of the globular cells was irregular due to an evident lack of firm envelopes.

Based on the results of light and transmission electron microscopy, we were not able to identify strain UD12 with any previously described ameba species. Its placement in Heterolobosea results from the eruptive morphotype of the trophozoites and the type of mitochondria with discoid cristae. However, molecular data were required for identification at lower taxonomic levels.

Sequence data and phylogeny

All the 18S rRNA gene sequences obtained matched the soil ameba AND9 (AY965861) and *N. damariscottae* (AJ224891) as the closest sequences in Blast searches (90% and 86–87% identity, respectively). Besides these, few other sequences with 85–90% identity scores were found, however, those belonged to uncultured eukaryotes (from activated sludge or rice field soil sources) and were represented by relatively short fragments (400–700 bps only). For phylogenetic analysis of the molecular clone sequences obtained, the sequence of clone 1 was used as the representative, because of the constant results of all molecular clones in the blast searches. The 18S rRNA gene-based phylogenetic reconstruction (Fig. 3) couples UD12 with the soil ameba AND9 (100% ML bootstrap and 1.00 Bayesian probability). This pair branches with *N. damariscottae* (again, 100% ML bootstrap and 1.00 Bayesian probability). The three sequences mentioned above form a fully statistically supported Tetramitia I subclade. The phylogenetic reconstruction splits Tetramitia into six subclades (Tetramitia I–VI) and places *Selenaion koniopes* as the earliest branching representative of Tetramitia (Tetramitia VII) (Fig. 3). The overall topology of the tree corresponds well with those published elsewhere (e.g. Pánek et al. 2012, 2014b). The subclades I–V are fully supported (100% ML bootstrap and 1.00 Bayesian probability). Only subclade VI reaches a lower bootstrap support (69%), which is again consistent with the results of Pánek et al. (2012). The internal transcribed spacer region of UD12 was not successfully amplified and therefore the sequence of *N. damariscottae* (AJ698839) in the DDBJ/EMBL/NCBI databases remains the only one of a cultured representative of the Tetramitia I group.

The direct 18S rRNA gene sequencing of DNA samples of nonclonal as well as clonal cultures resulted in chromatograms containing mixed signals in several positions.

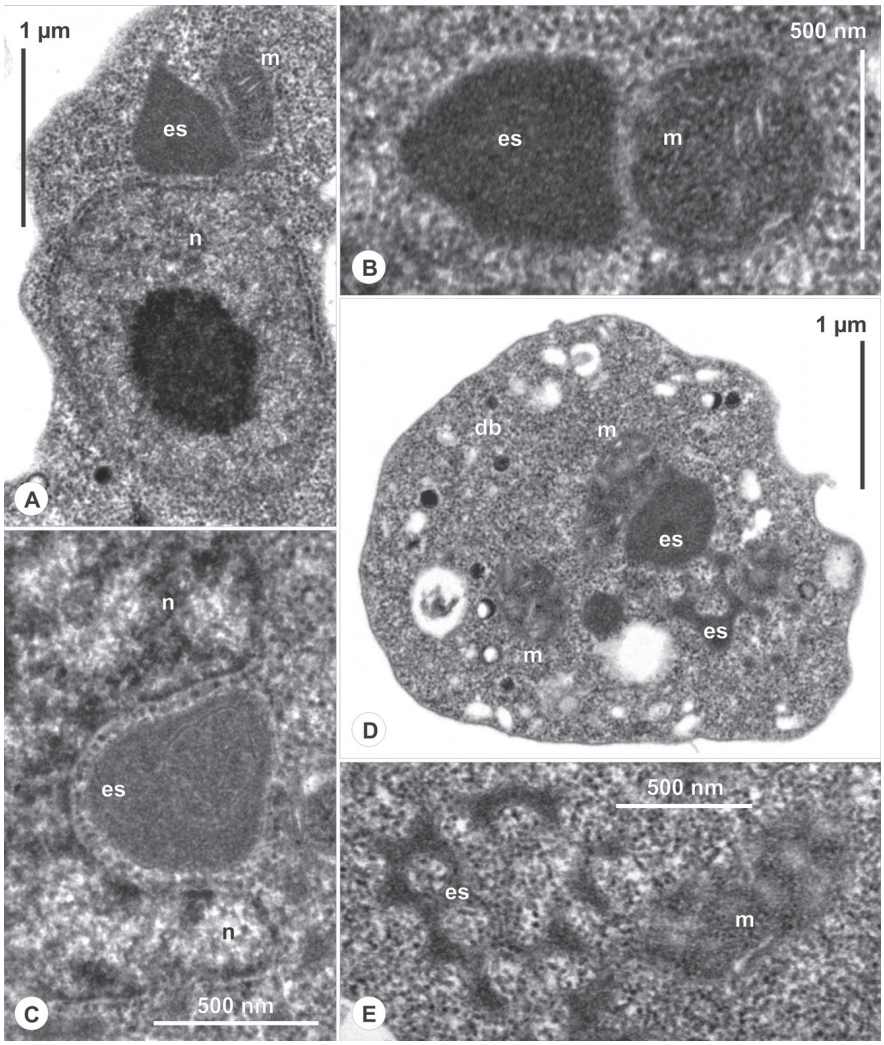

Figure 2 Ultrastructure of *Neovahlkampfia nana* n. sp., strain UD12, globular form. (**A**) Electron-dense structure (es) located to the side of mitochondrion (m), in the vicinity of nucleus (n). (**B**) Detail of electron-dense structure (es) and mitochondrion (m). (**C**) Electron-dense structure (es) closely apposed to nucleus (n) is surrounded by rough endoplasmic reticulum and contains a not clearly delimited substructure. (**D**) Overview of cell with mitochondria (m), dense bodies (db), partly disintegrated electron-dense structure (es), and vacuoles in cytoplasm. (**E**) Detail of partly disintegrated electron-dense structure (es) and mitochondrion with signs of alteration (m).

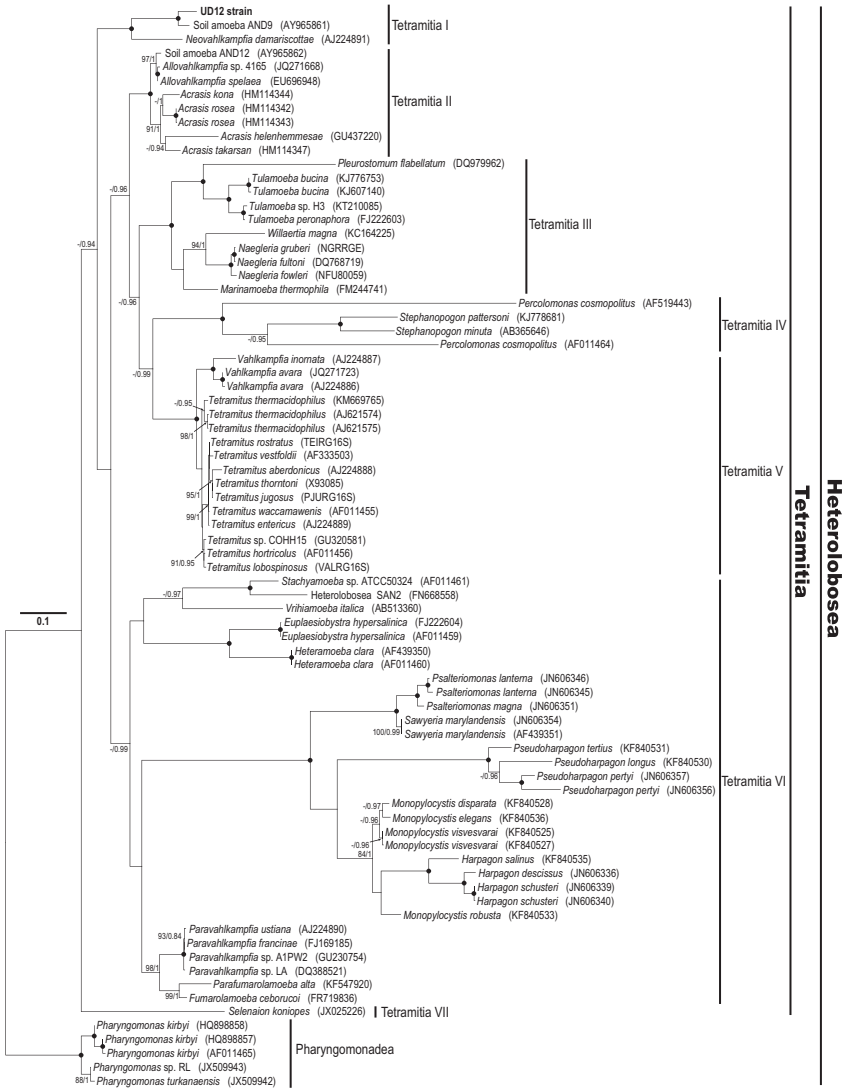

Figure 3 Maximum likelihood tree of 78 18S rRNA gene sequences inferred under the GTR GAMMA I model in RAxML. The novel UD12/I sequence (molecular clone 1) is in bold. Branch support values for both different reconstruction methods are shown (ML bootstraps/BA posterior probabilities) at the nodes. Only values higher than 0.8 or 80 are presented. Black dots indicate 100/1 support values. Pharyngomonadea serves as the outgroup. DDBJ/EMBL/NCBI accession numbers of sequences are listed next to the taxon/strain names.

Table 1. DNA sequence polymorphism values

Culture clone(s)	# mc	S	h	Hd	π
UD12/I	25	75	25	1	0.00298
UD12/II	10	25	9	0.978	0.00254
UD12/III	10	34	10	1	0.00326
UD12/IV	10	25	10	1	0.00240
UD12/V	10	36	10	1	0.00353
UD12/I–V	65	187	63	0.999	0.00295

mc, number of molecular clones; S, number of variable sites; h, number of haplotypes; Hd, haplotype diversity; π, nucleotide diversity per site.

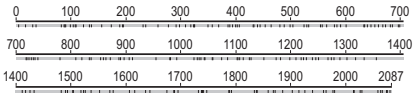

Figure 4 Distribution of single nucleotide polymorphisms of UD12/I–V culture clones along the 18S rRNA gene generated by Taq DNA polymerase-catalyzed PCR.

This fact and an observation of PCR products as a single band by agarose-gel electrophoresis led us to favor the molecular cloning approach. The lengths of the obtained sequences were 2,087 bps with the exception of five molecular clones (found among UD12/I, III–V) with a singleton deletion and the final length of 2,086 bps. Furthermore, all sequences possessed single nucleotide polymorphisms (SNPs) reaching up to 0.86% of the sequence divergence (up to 18 bps). General genetic diversity patterns (number of variable sites, S; number of haplotypes, h; haplotype diversity, Hd; nucleotide diversity per site, π) are presented in Table 1. SNPs were distributed nearly equally along the whole 18S rRNA gene as shown in Fig. 4. Using the same primers, but a high-fidelity DNA polymerase in the repeated PCR, the purported variability of 18S rRNA gene was not confirmed. All nine molecular clones gained from UD12/II and UD12/IV werre identical with each other as well as with three identical molecular clones (LC126917, LC126944, and LC126950) gained by Taq-Purple DNA polymerase.

DISCUSSION

Our new heterolobosean ameba is reported more than 40 yr after the description of *Vahlkampfia damariscottae* Page 1974. The type and only strain of the latter species was later studied by molecular methods (Brown and De Jonckheere 1999). They transferred this species into a newly named genus, *Neovahlkampfia* Brown and De Jonckheere 1999. To date, *V. damariscottae* has not been recorded again and no other *Neovahlkampfia* species has been since described. This suggests that *Neovahlkampfia* species are rare but, at the same time, it contradicts the widely held assumption that heterolobosean amebas (in general) are ubiquitous. We suppose that, as far as *Neovahlkampfia* species are concerned, their apparent rareness is partly related to the enormous difficulties encountered in attempting to separate these tiny amebas from mixed primary isolates on agar plates. In view of this, it is worth mentioning that the most closely related strain (AND9) was isolated from liquid media by the sampling dilution method (Lara et al. 2007).

The eventual role of *Neovahlkampfia nana* in etiology of nodular gill disease that started to develop in rainbow trout when gill tissue samples were collected cannot be specified in this study. The material received for examination was decomposing and therefore unsuitable for histology. In addition, other, larger species of free-living amebas were isolated from the same material.

When determining newly isolated and subcultured ameba strains, light microscopic characters and details of ultrastructure often point to a candidate genus. Molecular tools then provide confirmation/rejection of the genus and decisions at the species level. Heterolobosean amebas, however, are notorious for their high morphological similarity under light microscopy (Brown and De Jonckheere 1999; Geisen et al. 2015) while ultrastructure data are missing for many of them. Consequently, the analysis of molecular data had priority in the determination of UD12. Nonetheless, the comparison of UD12 with the available data on the type strain of *N. damariscottae* (Page 1974) resulted in recognition of a congruence in the morphotype of the locomotive stages, the globular form of floating, dividing and resting stages, the discoid type of mitochondrial cristae, the nonexistence of cysts and of flagellate transformation, and the absence of an ultrastructurally defined dictyosome and contractile vacuole. On the other hand, the uroidal filaments and amorphous cell coat on the plasma membrane mentioned in *V. damariscottae* by Page (1974, 1980) were not observed in UD12.

Among the details of UD12 ultrastructure, the conspicuous electron-dense organelle located in the vicinity of the nucleus deserves particular attention. This structure has not been reported for the type strain of *N. damariscottae* (Page 1974) nor, to the best of our knowledge, has the simultaneous presence (in the same cell) of this organelle and mitochondria displaying the characteristic pattern of organization been reported for any other free-living ameba cultured under aerobic conditions. Irregular aggregations of amorphous material ("A") and dense black bodies ("B") observed in the cytoplasm of the halophilic heterolobosean *Selenaion koniopes* (Park et al. 2012) resemble structures that we detected incidentally in *Naegleria* strains in our other studies but they differ substantially from those documented in our strain UD12. Pánek et al. (2012, 2014a,b) described "acristate mitochondria (hydrogenosomes)" in obligate heterolobosean anaerobes or microaerophils. For structures similar to hydrogenosomes in which putative cristae could be recognized only exceptionally they coined the term "mitochondrion-related organelles (MROs)". This, together with recently published genomic data on *Naegleria gruberi* (Fritz-Laylin et al. 2010), led Pánek et al. (2014a) to consider existence of a complex metabolism in some anaerobic heteroloboseans that they described as new species.

The enigmatic electron-dense structures that were found in the cytoplasm of the UD12 cells, along with mitochondria with well defined discoid cristae, had no internal membranes and thus looked similar both to the acristate organelles described in anaerobic heteroloboseans (Pánek et al. 2014a) and to some inclusions documented by Fawcett (1966). Comparison of dozens of ultrathin sections through the globular UD12 cells revealed certain signs of transformation of the organelle in question. This led to considerations of the principal hypotheses on mitochondriogenesis including the origin of mitochondria from nonmitochondrial structures as well as data on regressive changes of mitochondria including pyknosis (Brandt and Papas 1959; Cheville 2009; Fawcett 1966; Ghadially 1988; Hoffman and Grigg 1958; Rouiller and Bernhardt 1956). However, we failed to prove the existence of a sequence of events leading to the production of discicristate mitochondria from acristate organelles; similarly, regressive changes of discicristate mitochondria also could not be clearly proven in our study. Even though a conclusive interpretation of the conspicuous electron-dense organelles that were found in the cytoplasm of UD12 is currently impossible, we document those in this paper because they may be of great interest in future comparative studies.

The need to collect more data on the ultrastructure of Heterolobosea and to also document rare characters is evidenced by, for example, recent findings of nuclei without central nucleoli in *Selenaion koniopes* and of mitochondria that lack the rim of cisternal profiles of RER in *Pharyngomonas turkanaensis* (Park and Simpson 2016; Park et al. 2012; respectively). Both these findings are inconsistent with the corresponding ultrastructural characters considered typical of Heterolobosea (i.e. single nucleus with a central nucleolus, mitochondria with discoidal/flattened cristae surrounded by cisternae of RER). Moreover, surprising details of the ultrastructure of microaerophilic or anaerobic heteroloboseans (Pánek and Cepicka 2012; Pánek et al. 2014a,b) advocate revision of heterolobosean characteristics.

The combined molecular evidence, pairwise distance, and phylogenetic position bolstered by full statistical supports, reliably places UD12 into *Neovahlkampfia*, at the same time showing its clear difference from the only described species of this genus (*N. damariscottae*). UD12 and *N. damariscottae* also differed morphologically (see above), however, in common with De Jonckheere and Brown (2005), we consider molecular data decisive for vahlkampfiid taxonomy. For species delimitation in the vahlkampfiids, De Jonckheere and Brown (2005) introduced the ITS region as the molecular marker capable of discerning genera (5.8S) as well as species (ITS lengths and sequences). The ITS sequence is not available for UD12. Although the 18S rRNA gene has been proposed as the universal eukaryotic prebarcode followed by a group-specific barcode (Pawlowski et al. 2012), our molecular data undoubtedly warrant description of UD12 as a new *Neovahlkampfia* species. Strain UD12 and *N. damariscottae* share only 86–87% 18S rRNA gene sequence identity, however, we rejected this to propose a new genus because of the limited taxon sampling available for Tetramitia I.

As the sequence variability observed presently consisted of singleton deletions and mutations, technical errors in PCR, sequencing, and/or assembly might be suspected as the cause. On the one hand, this explanation would be consistent with that given by Weber and Pawlowski (2014), who studied the intragenomic polymorphism in Foraminifera. On the other hand, the extent of present variation (up to 0.86%) is far beyond that explicable by technical error. Admittedly, DNA polymerases do produce errors during the PCR and Taq polymerase produces the highest error rate (McInerney et al. 2014). However, single-base substitution errors are produced at a low frequency of $1–20 \times 10^{-5}$ (see McInerney et al. 2014 and references therein). The intragenomic variation in rRNAs has been recognized to be also growth-stage (Mashkova et al. 1981; Wegnez et al. 1972) or life-stage specific (Gunderson et al. 1987). Taking the molecular clones obtained using the high-fidelity DNA polymerase into account, we are confident that the sequence variability observed was caused by technical errors, namely using the low-fidelity PCR polymerase. Such extensive errors introduced by low-fidelity DNA polymerases and fixed by cloning can have a significant impact on our assessment of heterolobosean diversity: validity of some current nominal species of Heterolobosea that differ from one another in just one or two base positions thus becomes potentially dubious.

TAXONOMIC SUMMARY

Excavata Cavalier-Smith 2002, emend. Simpson 2003
Discoba Simpson in Hampl et al. 2009
Discicristata Cavalier-Smith 1998
Heterolobosea Page and Blanton 1985
Tetramitia Cavalier-Smith 1993, emend. Cavalier-Smith in Cavalier-Smith & Nikolaev 2008
Neovahlkampfia Brown and De Jonckheere 1999

NEOVAHLKAMPFIA NANA N. SP

Diagnosis Monopodial amebas with longitudinal cylindrical symmetry and eruptive cytoplasmic flow in locomotion; locomotive forms 8–15 (mean 12) μm long, 3–6 (mean 4.5) μm broad, L/B ratio 2.2–3.2 (mean 2.7); a single vesicular nucleus with a prominent nucleolus; floating, dividing, and resting stages globular, 6–8 (mean 7) μm in diameter; cysts are not formed; flagellated stages were not observed.

Type material Type strain denominated UD12 is deposited in the CCAP; several passages are cryopreserved in the Collection of the Institute of Botany and Zoology, Masaryk University, Czech Republic; eight DNA samples and eight blocks of Spurr-embedded amebas are stored in the principal author's collection.

Origin Freshwater, gill-isolate from rainbow trout, *Oncorhynchus mykiss* (Walbaum).

Etymology The specific name "*nana*" (Latin, noun, f.) meaning "a female dwarf" refers to the small size of the ameba.

Gene sequence The nearly complete 18S rRNA gene sequences were deposited in DDBJ/EMBL/NCBI databases under Acc. Nos. LC126917–LC126981. The LC126917 sequence (UD12/I molecular clone 1) was used in the phylogenetic analyses.

ACKNOWLEDGMENTS

Prof. Marco Galeotti of Università degli Studi di Udine, Italy is gratefully acknowledged for sharing with us gill tissue samples. This research was financially supported by the Czech Science Foundation (project 505/12/G112) and the Ministry of Education, Youth and Sports (LM2015078). We are grateful to Ass. Prof. Keith Edwards who corrected the English text. We also appreciate suggestions of anonymous reviewers toward improvement of the manuscript.

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Section IV

Revision of *Copromyxa* genus

The Journal of Eukaryotic Microbiology

ISOP Published by the International Society of Protistologists

Journal of Eukaryotic Microbiology ISSN 1066-5234

ORIGINAL ARTICLE

Copromyxa laresi n. sp. (Amoebozoa: Tubulinea) and Transfer of *Cashia limacoides* (Page, 1967) to *Copromyxa* Zopf, 1885

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Keywords
Euamoebida; free-living amoebae; limax amoebae; phylogeny; ultrastructure.

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Received: 16 December 2015; revised 21 June 2016; accepted June 22, 2016.

doi:10.1111/jeu.12349

ABSTRACT

Five amoeboid organisms of different origin (isolated from fish organs, soil and digestive tract of earthworm) that shared light microscopical and ultrastructural features including type and arrangement of mitochondrial cristae were subjected to phylogenetic analyses based on sequences of SSU rDNA and protein coding genes (actin, cytochrome oxidase I, and eukaryotic elongation factor 2). The reconstruction of multigene phylogeny of the strains studied (i) revealed that they belong to the same single-genus *Copromyxa* clade; (ii) strongly supported position of *Copromyxa cantabrigiensis* (syn. *Hartmannella cantabrigiensis*) within the genus; (iii) together with comparisons of light and electron microscopy data justified reclassification of *Cashia limacoides* (syn. *Vexillifera expectata*) to *Copromyxa limacoides* n. comb., and (iv) justified description of a new species, *Copromyxa laresi* n. sp.

IN the 1960s and 1970s, Frederick C. Page published several important works (esp. Page 1967, 1974) dealing with limax amoebae including the genus *Hartmannella* Alexeieff, 1912. These amoebae are rather small (approx. 20 μm long) freshwater/soil amoeboid protists moving slowly but steadily in the form of monotactic amoeba. When Page described a new species of the genus, *Hartmannella cantabrigiensis* Page, 1974, he did not consider cellular slime molds as possible candidates for careful comparison, although myxamoebae of some genera are actually limax amoebae closely resembling those studied by him. Especially myxamoebae of *Copromyxa* Zopf, 1885, documented by Fayod (1883) or Nesom and Olive (1972), fit nicely Page's (1974) emended diagnoses of both Hartmannellidae and *Hartmannella*. This fact was appreciated neither by protozoologists nor mycologists, although Cavalier-Smith (1993) realized that there is a link between *Copromyxa* and lobose amoebae. Only recently Brown et al. (2011) have shown that *H. cantabrigiensis* and *Copromyxa protea* (Fayod) Zopf (1885) are sister taxa. They were so close in their analysis that they were considered congeneric and *H. cantabrigiensis* was transferred to the genus *Copromyxa*. Smirnov et al. (2011) did not support this interpretation and preferred to retain the two genera separate. The ability to form fruiting bodies, never observed in *C. cantabrigiensis*, justifies the separation in their view. It should be noted that the fruiting habit of *C. protea* is readily lost or absent in culture conditions (Brown et al. 2011; Smirnov et al. 2011, p. 561). At that time, no other species belonging to *Hartmannella*/*Copromyxa* clade was available for more detailed analysis. *Hartmannella vermiformis* and *Hartmannella abertawensis* are phylogenetically distinct: they are in fact representatives of the genera *Vermamoeba* Cavalier-Smith and Smirnov, 2011 and *Nolandella* Page, 1983, respectively. Yet, in the very same year, a paper on *Vexillifera* Schaeffer, 1926 was published (Dyková et al. 2011) showing that strain 4730 previously described as *Vexillifera expectata* (Dyková et al. 1998) did not cluster with other *Vexillifera* species, but rather with *C. cantabrigiensis* (*C. protea* was not included). In a later study (Corsaro et al. 2013), strain 4730 branched as a sister group to the duo of *C. cantabrigiensis* and *C. protea*. Very recently (Van Wichelen et al. 2016) two new species of *Copromyxa* were described,

C. microcystidis Van Wichelen and Vanormelingen, in press and *C. vandevyveri* Van Wichelen and Vanormelingen, in press, indicating that the clade is more species rich than previously known.

In our long-term studies of fish-associated free-living amoebae (FLA), some strains of environmental origin completed our cryopreserved collection. The individual strains were documented at the light and electron-microscopical levels in Dyková and Kostka (2013). Based on small subunit rRNA gene sequences (SSU rDNA), five strains introduced in the latter publication were assigned to the genus *Copromyxa* and three of those were identified with named species. Sequences of our *Copromyxa* strains contributed substantially to the formation of our opinion on the *Copromyxa/Hartmannella* clade. Analysis of phylogenetic relationships among the strains based on sequences of SSU rDNA and three protein-coding genes showed that one strain within this group has a sufficiently clear position as an independent species. Uncovered phylogenetic relationships support the opinion that all four lineages belong to a single genus, *Copromyxa*. Moreover, careful reconsideration of morphological data presented here and in previous works (Dyková and Kostka 2013; Dyková et al. 1998) leads us to the conclusion that the aforementioned "*Vexillifera*" *expectata* strain 4730 represents in fact *Cashia limacoides* (Page, 1967) Page, 1974 originally described as *Harmannella limacoides*. We transfer it into the genus *Copromyxa*.

MATERIALS AND METHODS

Strains included in the study

Five strains (ZEB1, ZEB4, LUM, ALC3, and 4730 strain) of limax amoebae included in the study were selected based on their morphological features documented previously by Dyková and Kostka (2013). ZEB strains were isolated from gills of two specimens of zebra danio, *Danio rerio* (Hamilton) from stock kept in an experimental facility in Vigo, Spain. ZEB4 strain was used only in morphological part of this study due to an identical SSU rDNA sequence (GenBank Acc. No JQ271679) with the ZEB1 strain. LUM strain belonging to *C. protea* was isolated from an undetermined earthworm collected in Haklovy Dvory, South Bohemia, Czech Republic; ALC3 strain of *C. cantabrigiensis* from wet soil collected along the Almaciga carretera, Tenerife, Canary Islands, Spain and 4730 strain from the liver of European perch, *Perca fluviatilis* L. from Černovický potok brook, South Bohemia, Czech Republic.

Methods of isolation, culturing, and morphological studies of strains

The essential steps of isolation and culturing procedures followed the recommendations of Page (1988) and Kalinina and Page (1992). They are summarized in Dyková and Kostka (2013) where our *Copromyxa* strains included in the present study were briefly presented for the first time as items of the Collection. The methods of morphological and ultrastructural studies were also summarized in the above mentioned publication. Strain 4730 was originally presented in Dyková et al. (1998). An extensive documentation of our previous observations made using a BX51 light microscope equipped with Nomarski differential interference contrast (DIC) (Olympus, Tokyo, Japan) and with a JEM 1010 electron microscope (JEOL, Tokyo, Japan), partly presented in Dyková and Kostka (2013), was revised in the context of data from contemporary phylogenetic analyses.

Amplification of target genes, cloning, and sequencing

Actin, cytochrome oxidase I (COI), eukaryotic elongation factor 2 (eEF2) genes and SSU rDNA were included in the study. SSU rDNA sequences of the studied amoebae were amplified with Erib1 and Erib10 primers (Barta et al. 1997) and are already published (Dyková and Kostka 2013). All the remaining gene sequences of ZEB1, LUM, ALC3, and 4730 strain were amplified by PCR carried out in a 25 μl volume in buffer with 1.5 mM $MgCl_2$ containing 0.2 mM of each dNTP an 1.25 U of Taq-Purple DNA polymerase (Top-Bio, Vestec, Czech Republic), 0.5 μM of each primer and 1 μl of template DNA. Actin and COI genes were amplified using the primers and conditions described by Tekle et al. (2007) and Folmer et al. (1994), respectively. For eEF2 genes, a nested PCR was performed using newly designed primers EF2a1 (GAA GTC ACT GCT GCN CTN CGN GTN ACN GA) and EF2a4 (AAA TCT CCA GGT GNA GYT CNC CNG CNC C) for the first step and EF2a2 (GGT GTT TGC GTC CAA ACN GAR ACN GTN CT) and EF2a3 (CGC CCG AAG GCA TAG AAN CGN CCY TTR TC) for the second one. Amplification of genes consisted of an initial denaturation at 95 °C for 3 min and (i) 5 cycles of 94 °C for 1 min, 45 °C for 1 min and 72 °C for 1.5 min; (ii) 25–30 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 1.5 min and then a final extension at 72 °C for 10 min. Successfully amplified products were then gel isolated or directly purified depending on the condition of the desired bands using the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany). In the case of the actin gene, given its variability (Gallwitz and Seidel 1979), the series of plasmids were produced from each strain using the TOPO® TA Cloning® Kit for Sequencing, with One Shot® TOP10 Chemically Competent *Escherichia coli* and pCR™4-TOPO® Vector (Invitrogen, Paisley, UK) following manufacturer's instructions. Transformed *E. coli* were seeded on LB plates containing 50 μg/ml ampicilin (Biotika, Slovenská Ľupča, Slovakia) and incubated at 37 °C. Colonies were then screened by PCR and plasmid DNA was extracted using the High Pure Plasmid Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany. Purified plasmids and PCR products were sequenced at Macrogen Inc. (Amsterdam, Netherlands).

Phylogenetic analyses

Phylogenetic analyses of datasets composed of SSU rDNA and protein coding gene sequences (actin, COI,

eEF2), plus a concatenated dataset containing all of the above mentioned were used to reveal and test both the phylogenetic position of strains of *Copromyxa* clade among tubulinea and relationships among them.

We prepared two SSU rDNA datasets, a larger (taxon richer) and a smaller one. The former contains SSU rDNA sequences of representatives of all important subgroups of Tubulinea, while the latter one concentrates on *Copromyxa* clade and its closest relatives – this allows less stringent trimming of the alignment and thus more molecular data for reconstructions of *Copromyxa* evolution. The resulting SSU rDNA dataset contained 30 taxa for the large dataset and 19 for the small dataset; actin dataset contained 37 sequences, COI dataset contained 15 taxa and eEF2 dataset contained 17 taxa. A concatenated dataset was also prepared. It contained only two outgroup taxa (*Saccamoeba* and *Chaos carolinense*) to avoid excessive deletion of informative positions. Among the four different gene sequences we used, COI sequence is unavailable for these amoebae. The COI sequence of MSED6 strain (very close to *S. lacustris* in SSU rDNA phylogeny; see Fig. S1) was used in the concatenate. The alignments were prepared in ClustalX v. 2.1 (Larkin et al. 2007) and checked in BioEdit 7.2.5 (Hall 1999) for ambiguously aligned positions. After their deletion, the lengths of large SSU, small SSU, actin, COI, eEF2, and concatenate datasets were 1,464, 1,764, 796, 534, 797, and 3,810 bps respectively.

All datasets were analyzed with RAxML 8.0.20 (Stamatakis 2014) using maximum likelihood (ML) approach. GTRGAMMAI model suggested as best by MrModeltest2 (Nylander 2004) was used for all the analyses; rapid bootstrapping was employed to compute the support of the most likely topologies (1,000 replicates). Bayesian inference analysis of all datasets was run in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) with default options, GTR invgamma model specified and 2×10^6 generations; burnin was default: 25% of the sampled trees (-lnL vs. generation plot was visually checked to assure -lnL plateau was reached).

RESULTS

Morphology of strains

Trophozoites of the five strains included in this study (ZEB1, ZEB4, LUM, ALC3, and 4730) share the same monotactic morphotype (Fig. 1A–F). They are limax-shaped with a hyaloplasmic cap formed in the direction of movement. In their posterior part, trophozoites have a single contractile vacuole. The rounded or tapering uroid has no differentiation visible in the light microscope. Vesicular nucleus with centrally located nucleolus is a prominent structure in the granuloplasm. Morphometric data are summarized in Table 1. Trophozoites of strain 4730 are smaller (in the culture they do not reach 20 μm), their length/breadth ratio does not exceed 3, anterior hyaloplasmic cap is less developed, and formation of spores was not observed. Morphometric similarity of LUM, ALC3, ZEB1, and ZEB4 strains is evident as shown in Table 1. The maximum average length and breadth was observed in trophozoites of strain ZEB1 (length/breadth ratio > 4). Spherical or slightly ovoid cysts were observed in all strains of this subgroup. Morphological observations of LUM, ALC3, ZEB1, and ZEB4 are consistent with the generic diagnosis of *Copromyxa* Zopf, 1885 as emended by Brown, Silberman and Spiegel, 2010, however, sorocarps were not observed under the conditions routinely used in culturing of free-living amoebae on agar plates.

Ultrastructure of strains

Same as at cellular level, strains under study share features also in their ultrastructure; several details, however, divide this group of strains. Strain 4730 differs from the other ones by having glycocalyx differentiated in cylindrical glycostyles, whereas cell membrane in the other strains lacks this feature (Fig. 3A–E). Hyaloplasmic cap is less pronounced in strain 4730 than in the other strains. The cytoplasm in LUM, ALC3, ZEB1, and ZEB4 is clearly divided into a granuloplasmic part surrounding vesicular nucleus and a hyaloplasmic part on the periphery of trophozoites. Ribosome-rich granuloplasm contains numerous, mostly bean-shaped mitochondria, endoplasmic reticulum, phagosomes, vesicles, and aggregates of microfilaments on the border with hyaloplasm (Fig. 2, 3F). Golgi apparatus has a dictyosome-like arrangement of cisternae (Fig. 3G). Spongiome can be observed in the vicinity of contractile vacuole (Fig. 3H). Mitochondria randomly distributed in granuloplasm are seen as variable, mostly rounded or elliptical profiles (depending upon orientation to the plane of sectioning). The profiles of tubular cristae in all studied strains as well as their arrangement within mitochondrial matrix are specific, not seen in other FLA amoebae (Fig. 4A–H). Nonbranching twisted/curved profiles are those most common; in a small proportion of sectioned mitochondria are seen straight parts of cristae (Fig. 4C). In all these strains was observed a most peculiar helical pattern of cristae (Fig. 4A–H). Cysts (Fig. 3I) with a single-layer wall were observed in all strains except for strain 4730.

Sequences

We obtained a total of 18 partial actin gene sequences from *Copromyxa* strains: three clones were derived from both ZEB1 and ALC3 strains and six clones from LUM and 4730 strains. The length of the sequences was 795 or ca. 835 base pairs; the latter value was reached by 4 of the 6 sequences belonging to 4730 strain. They contained a putative intron near their 5′ end. From all four strains, sequences of COI gene (ca. 630 bps) and eEF2 gene (ca. 650–700 bps, shorter fragment of ca. 500 bps for ZEB1) were also obtained. Moreover, to improve the taxon sampling of COI (and concatenated) dataset, we sequenced *Saccamoeba* sp. strain MSED6 (which is represented by sequence Nr. JQ271718 in SSU rDNA dataset; Fig. S1).

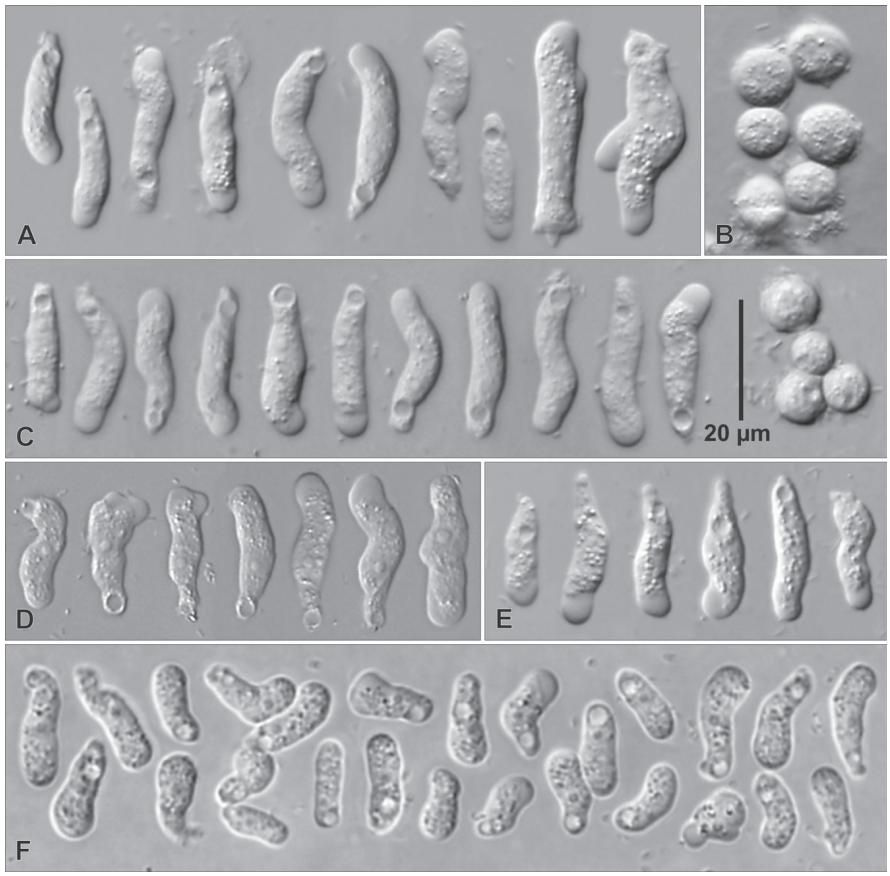

Figure 1 Trophozoites and cysts of *Copromyxa* strains studied. (**A, B**) *Copromyxa laresi* strain ZEB1; (**C**) *C. laresi* strain ZEB4; (**D**) *Copromyxa protea* strain LUM; (**E**) *Copromyxa cantabrigiensis* strain ALC3; (**F**) *Copromyxa limacoides* strain 4730. Scale bar in C indicates magnification of all figures. Images from Dyková and Kostka (2013).

Table 1. Measurements of trophozoites (µm)

Strain	Length	Breadth	L/B ratio
4730	15.4 (13.0–17.6)	4.9 (4.1–5.6)	L/B = 3
LUM	23.7 (20.9–24.9)	5.6 (5.2–6.5)	L/B = 3.8
ALC3	24.2 (22.3–26.0)	5.2 (4.6–6.5)	L/B = 4.4
ZEB1	27.5 (21.3–35.3)	5.5 (4.6–6.5)	L/B = 4.9
ZEB4	25.3 (21.4–27.9)	5.6	L/B = 4.4

The accession numbers of newly obtained actin, COI and eEF2 gene sequences are LC102260–LC102286.

The uncorrected pairwise distances between each of the six pairs formed from LUM, ALC3, ZEB1, and 4730 strain SSU rDNA sequences (Table 2) were similar: they were never lower than 0.10 and reached up to 0.16. The distances were lower for COI sequences: 0.09–0.13 (Table S1).

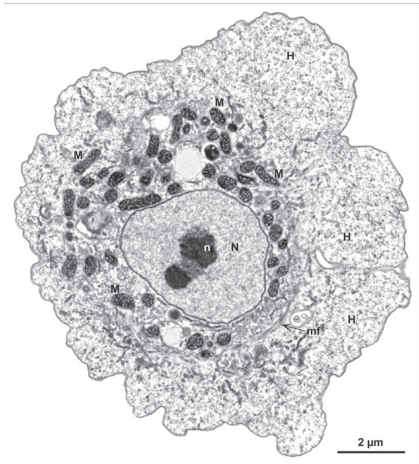

Figure 2 *Copromyxa laresi* strain ZEB1, overview of trophozoite ultrastructure. H, hyaloplasm; M, mitochondria; N, nucleus; n, nucleolus; mf, bundles of microfilaments at the interface of granuloplasm and hyaloplasm.

Phylogenetic analyses

The analysis of the larger dataset of SSU rDNA sequences (Fig. 5) shows a very well-supported group of *Saccamoeba*, *Glaeseria*, *Copromyxa* and an uncultured hartmannellid (the sequence with Acc. Nr. GQ330602). In this group, the *Copromyxa* clade has a moderate bootstrap and posterior probability support (72/0.99), but the relationships within it are not so clearly revealed: the statistic support for internal nodes is rather poor. *C. protea* forms a group with strain 4730 + *C. microcystidis*, *C. cantabrigiensis* is their sister group followed by more basal *C. vandevyveri* and ZEB strains that are sister to the rest. The smaller SSU rDNA dataset (Fig. S1) allowing for less strict alignment trimming shows a different, but also poorly supported topology, where *C. cantabrigiensis* and *C. protea* are sister taxa, 4730 strain groups with them, *C. microcystidis* is more basal and ZEB1 strain + C. *vandevyveri* together form the basalmost lineage.

The analysis of actin gene (Fig. S2), shows basal 4730 strain and *C. protea* + *C. cantabrigiensis* being sister taxa. Even though the support is not high because of short branch lengths, the multiple clones of each strain always cluster together. The analysis of the eEF2 gene (Fig. S3) shows a different topology than the ones shown above (4730 + ZEB strains, *C. protea* basal), even though it still groups all the *Copromyxa* clade members together in a clade with a very good support. In COI phylogeny (Fig. S4), *C. protea* + *C. cantabrigiensis* and 4730 + ZEB strains are shown to form sister taxa, respectively.

The analysis of the concatenate of *Copromyxa* genes (SSU rDNA, actin, COI, and eEF2 genes) outgrouped by *Chaos* and *Saccamoeba* sequences (Fig. 6) resulted in a topology, where strain 4730 is the basalmost lineage and the grouping of *C. protea* and *C. cantabrigiensis* obtained an enhanced support (97/1). In all cases, the topology of *Copromyxa* group reconstructed with Bayesian inference did not differ from topology in ML trees.

DISCUSSION

Strain 4730 was presented by Dyková et al. (1998) as *Vexillifera expectata*. The decision to classify it as *Vexillifera* sp. was based predominantly on the absence of cysts, presence of hexagonal glycostyles and also on light microscopy observations made under nonstandard conditions (i.e. observed immediately after the preparation, without sufficient time for the trophozoites to settle down and demonstrate standard behavior). Note that the trophozoites illustrated in Fig. 1 in Dyková et al. (1998) were documented after short osmium fixation. Phylogenetic analysis of its SSU rDNA sequence (Dyková et al. 2011) suggested it is in fact a member of Tubulinea closely related to *Copromyxa* sp. included in the analysis. Strain 4730 is listed as *Copromyxa expectata* in Dyková and Kostka (2013), but this was not a validly published new combination according to article 41 of the International Code of Nomenclature for algae, fungi, and plants (McNeill et al. 2012). After critical reexamination of all data available, we identify strain 4730 with *Cashia limacoides* (Page, 1967). We found a number of correspondences, most importantly (citations from Page's diagnosis; Page 1967): (i) "limax form broader anteriorly, often clavate", which is also true for strain 4730; see Fig. 1F for comparison; (ii) "ectoplasmic zone at anterior end limited, often only a crescent"; (iii) "nucleus [. . .] located in posterior half of majority" – as seen in Fig. 1F; (iv) "several coarse, dark, slightly refractile granules in endoplasm" again visible in many trophozoites in Fig. 1F; (v) "median length of 14 µ and a length:breadth ratio averaging 3.3", which is in a very good accordance with our measurements (see Table 1); (vi) "no cysts produced in culture"; (vii) Page further mentions in the same paper contractile vacuoles at the posterior end, and (viii) floating forms with up to 8 hemispherical mounds resembling single pseudopods of the locomotive form – such floating forms of strain 4730 are documented in Fig. 2 in Dyková et al. (1998). In Page (1985), ultrastructure of *Cashia limacoides* is studied and further correspondences between *Cashia* and 4730 strain can be noticed: (ix) glycostyles of the same form in both organisms (see Fig. 3D, E) and finally (x) the presence of helical cristae, which we show to be a common feature of all *Copromyxa* strains presented here.

Unfortunately, the culture of *Cashia* deposited in CCAP by Frederik C. Page is no longer available, so no molecular data can be obtained from the type culture. Nonetheless, we failed to find any difference between *Cashia* and 4730 strain, while similarities are plentiful. We thus consider

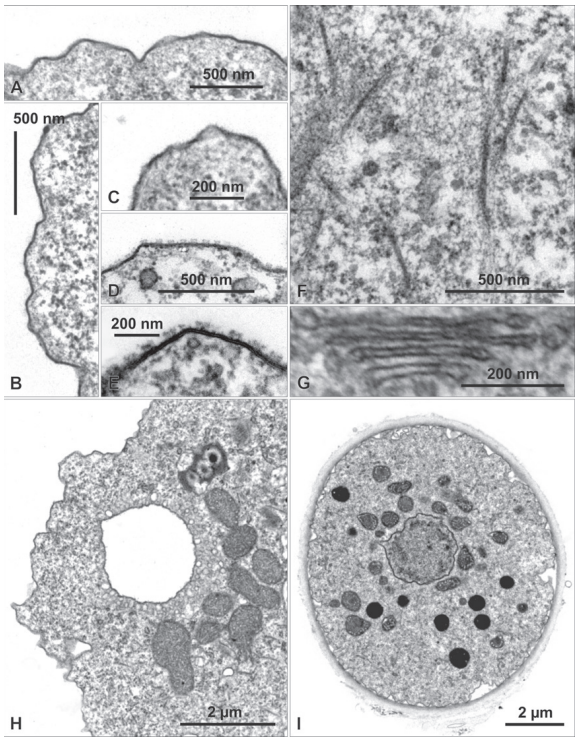

Figure 3 Strains compared for details of ultrastructure. (**A**–**E**) Cell surface of *Copromyxa* strains: *Copromyxa laresi* strain ZEB1 (A), *Copromyxa cantabrigiensis* strain ALC3 (B), *Copromyxa protea* strain LUM (C), and *Copromyxa limacoides* strain 4730 (D, E); (**F**) Bundles of microfilaments on the periphery of granuloplasm (*C. laresi* strain ZEB4); (**G**) Golgi apparatus with dictyosome-like arrangement of cisternae (strain ZEB1); (**H**) Spongiom and group of mitochondria surrounding vacuole (strain ZEB4); (**I**) Cyst with a relatively thin wall (strain ZEB4).

the two organisms conspecific: *Vexillifera expectata* is a junior synonym of *Cashia limacoides*, which in turn is to be transferred into *Copromyxa* based on our data.

Based on morphology and comparisons of SSU rDNA sequences, we consider strains ALC3 and LUM representatives of *Copromyxa cantabrigiensis* and *C. protea* respectively. There is also an actin gene sequence of *C. cantabrigiensis* available (Fahrni et al. 2003), positioned next to sequences of *C. cantabrigiensis* strain ALC3 in the actin tree, as expected. Strain 4730 is the type strain of *Vexillifera expectata*, which we identified as (synonymized with) *Cashia limacoides* in the current study; it is transferred to the genus *Copromyxa*. Strains ZEB1 and ZEB4 represent a yet undescribed species, *C. laresi*. All *Copromyxa* strains form a well-supported monophyletic group in analyses of all sequenced genes and of the concatenate. The clade is well-nested in Euamoebida group in SSU rDNA phylogeny. The internal topology of *Copromyxa*/*Hartmannella* clade is not resolved well, but with exception of eEF2 gene and the larger SSU rDNA datasets, the data mostly support sister position of *C. protea* and *C. cantabrigiensis*, a grouping that is strongly favoured by concatenated alignment. Depending on the dataset, *C. laresi* or *C. limacoides* are then either basal to the rest or cluster together. In SSU rDNA phylogenies the picture is further complicated by two additional taxa, *C. microcystidis* and *C. vandevyveri* (Van Wichelen et al. 2016) with variable positions. Either way, we believe this arrangement supports inclusion of all four studied species in a single genus, *Copromyxa*, in agreement with Brown et al. (2011)

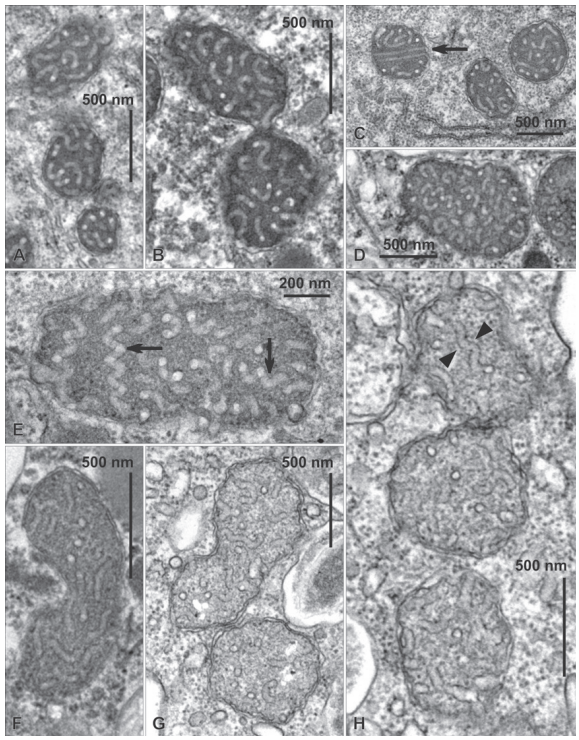

Figure 4 Mitchondria of trophozoites showing a highly characteristic pattern of organization. (**A**–**D**) *Copromyxa laresi* strains ZEB1 and ZEB4: sections demonstrate profiles of tubular nonbranching cristae that are mostly shown twisted. In optimum plains of section (especially in D) are seen long helical segments. Occasionally, profiles of cristae are straight (arrow in C). (**E**) Several helical profiles seen in optimum plain of section markd with arrows (*Copromyxa cantabrigiensis* strain ALC3). (**F**) *Copromyxa protea* strain LUM. (**G, H**) Mitochondria of *Copromyxa limacoides* strain 4730. Note helical segments (arrowheads) among numerous twisted profiles.

and contrary with the view of Smirnov et al. (2011). Otherwise, retaining holophyletic genera would be difficult as we would need a new genus for *C. laresi* morphologically undistinguishable from either *C. cantabrigiensis* or nonfruiting strains of *C. protea*. The two *Copromyxa* species described by Van Wichelen et al. (2016) would also request generic reassignments. This seems to be an unnecessary inflation of the number of generic names. The helical mitochondrial cristae seem to be a good character uniting *Copromyxa* species.

Let us emphasize that at this moment there is no sequenced *Hartmannella* species remaining: every single *Hartmannella* species studied by Page in the last century was transferred to another genus either by Page himself or by his followers. Without molecular data, the generic status of the several *Hartmannella* species described since cannot be reliably checked. Given the dubious status of the poorly described type species, *H. hyalina* Dangeard, 1900, the future fate of the genus remains contentious.

TAXONOMIC SUMMARY

Based on the presented data we feel justified in describing a new *Copromyxa* species and transferring *Cashia limacoides* to the genus *Copromyxa*.

Table 2. Uncorrected pairwise distances between *Copromyxa* SSU rDNA sequences

	ALC3	ZEB1	4730
LUM	0.11	0.12	0.16
ALC3		0.12	0.15
ZEB1			0.16

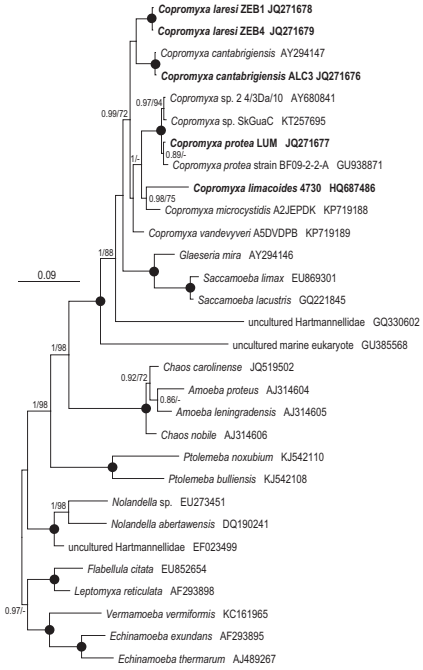

Figure 5 Maximum likelihood (ML) constructed tree for SSU rDNA gene sequences showing the phylogenetic position of the five strains of *Copromyxa* under study (in bold) within the Tubulinea. GenBank accession numbers of sequences used in analysis are listed next to the taxon names. The scale bar represents evolutionary distance in change/site. ML bootstrap values and Bayesian posterior probabilities (PP) are presented at each node. Bootstrap values and PP of 100%/1.0 are represented by a black dot. Support values less than 70% or 0.70 for the respective method are not presented or represented as a hyphen.

Amoebozoa Luhe, 1913
Tubulinea Smirnov et al., 2005
Euamoebida Lepsi, 1960
Copromyxa Zopf, 1885

Copromyxa laresi n. sp.

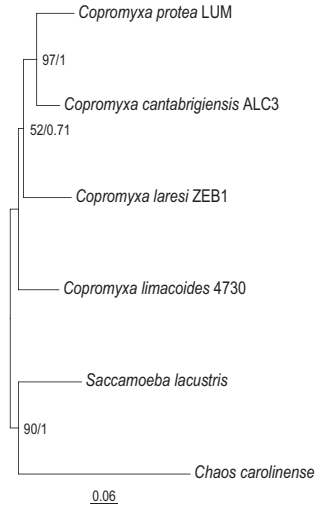

Figure 6 Maximum likelihood (ML) constructed tree based on four concatenated gene sequences (SSU rDNA, actin, cytochrome oxidase I and eukaryotic elongation factor 2) of the four strains of *Copromyxa* under study plus two outgroup taxa. The scale bar represents evolutionary distance in change/site. ML bootstrap values and Bayesian posterior probabilities (PP) are presented at each node.

Diagnosis

Species with characteristics of the genus. Freshwater-inhabiting monotactic naked amoeba considered amphizoic due to the origin of type strain. Length in locomotion is 27.5 (21.3–35.3) μm, breadth 5.5 (4.6–6.5) μm, L/B ratio > 4 (4.9). SSU rDNA variable region V4 shorter than 235 bps (233 bps in the type sequence: bases in positions from 642 to 874). The first four bases in the 5′ end of V4 region are followed by a short AT-rich region (ATs from position 646 to 652 of the type sequence).

Type material

Strain ZEB1. Cryopreserved culture of the type strain has been deposited in Culture Collection of the Institute of Parasitology, Czech Academy of Sciences, České Budějovice, Czech Republic; TEM blocks are available from the authors upon request. The type strain was isolated from gills of zebra danio, *Danio rerio* (Hamilton) from stock kept in the experimental facility of Instituto de Investigaciones Marinas, Vigo, Spain.

Gene sequence data

The nearly complete SSU rDNA sequence has been deposited in GenBank under Acc. No JQ271678.

Etymology

The species name refers to the family name of Dr. Fernando Lares Villa in recognition of his effort to attract young scientists to the field of free-living amoebae.

Note. The trophozoites of *C. protea* and *C. cantabrigiensis* are very similar to those of *C. laresi*. Unlike *C. protea*, *C. laresi* was never observed to form fruiting bodies. In can be distinguished from both species on the basis of SSU rDNA sequence (*C. cantabrigiensis* and *C. protea* have longer V4 region and its 5′ end is rich in Gs in both species).

Copromyxa limacoides n. comb.
Syn.:
Hartmannella limacoides Page, 1967
Cashia limacoides (Page, 1967) Page 1974
Vexillifera expectata Dyková et al. 1998

Notes. See esp. Page's original description (Page 1967), Page (1974, 1985) for morphology information. Note that morphometrics in Dyková et al. (1998) and the mentioned observation of eruptive lobopodia were based on trophozoites in nonstandard conditions. The measurements are updated here (Table 1). The type culture is lost. Strain 4730 has been deposited in Culture Collection of the Institute of Parasitology, Czech Academy of Sciences, České Budějovice, Czech Republic; TEM blocks are available from the authors upon request. The nearly complete SSU rDNA sequence has been deposited in GenBank under Acc. No HQ687486, sequences of 4 protein coding genes are also available.

ACKNOWLEDGMENTS

The authors are grateful to the Czech Science Foundation for financial support (project 505/12/G112).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Maximum likelihood (ML) constructed tree for SSU rDNA gene sequences showing the phylogenetic position of the five strains of *Copromyxa* under study (in bold) within Hartmannellidae.

Figure S2. Maximum likelihood (ML) constructed tree for actin gene sequences showing the phylogenetic position of the four strains of *Copromyxa* under study (in bold) within the Tubulinea.

Figure S3. Maximum likelihood (ML) constructed tree for eukaryotic elongation factor 2 gene sequences showing the phylogenetic position of the four strains of *Copromyxa* under study (in bold) within the Tubulinea.

Figure S4. Maximum likelihood (ML) constructed tree for cytochrome oxidase I gene sequences showing the phylogenetic position of the four strains of *Copromyxa* under study (in bold) within the Amoebozoa.

Table S1. Uncorrected pairwise distances between *Copromyxa* COI sequences.

SUPPORTING INFORMATION

***Copromyxa laresi* n. sp. (Amoebozoa: Tubulinea) and transfer of *Cashia limacoides* (Page, 1967) to *Copromyxa* Zopf, 1885** by Martin Kostka, Luis Fernando Lares-Jiménez, Tomáš Tyml, Iva Dyková

Supplementary Table 1. Uncorrected pairwise distances between *Copromyxa* COI sequences.

	ALC3	ZEB1	4730
LUM	0.09	0.13	0.10
ALC3		0.13	0.11
ZEB1			0.12

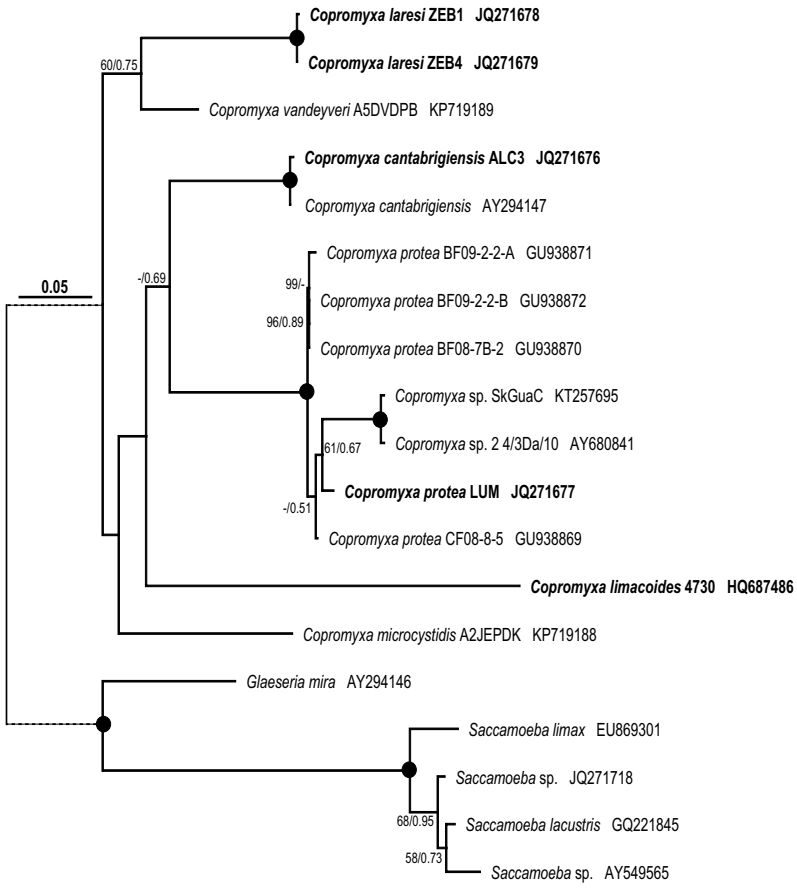

Fig. S1. Maximum likelihood (ML) constructed tree for SSU rDNA gene sequences showing the phylogenetic position of the five strains of *Copromyxa* under study (in bold) within Hartmannellidae. GenBank accession numbers of sequences used in analysis are listed next to the taxon names. The scale bar represents evolutionary distance in change/site. ML bootstrap values and Bayesian posterior probabilities (PP) are presented at each node. Bootstrap values and PP of 100%/1.0 are represented by a black dot. Support values less than 70% or 0.70 for the respective method are not presented.

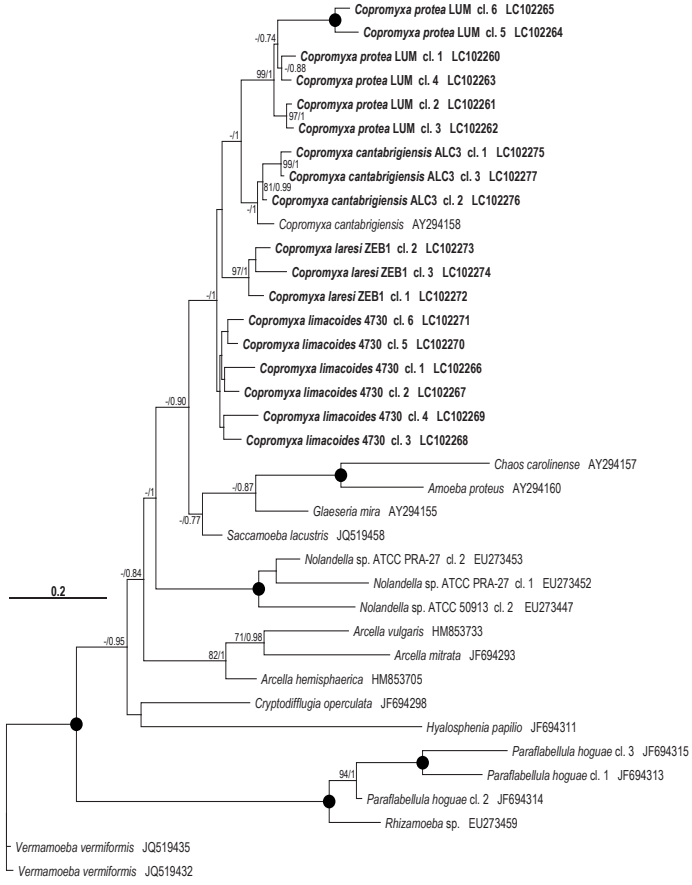

Fig. S2. Maximum likelihood (ML) constructed tree for actin gene sequences showing the phylogenetic position of the four strains of *Copromyxa* under study (in bold) within the Tubulinea. GenBank accession numbers of sequences used in analysis are listed next to the taxon names. The scale bar represents evolutionary distance in change/site. ML bootstrap values and Bayesian posterior probabilities (PP) are presented at each node. Bootstrap values and PP of 100%/1.0 are represented by a black dot. Support values less than 70% or 0.70 for the respective method are not presented.

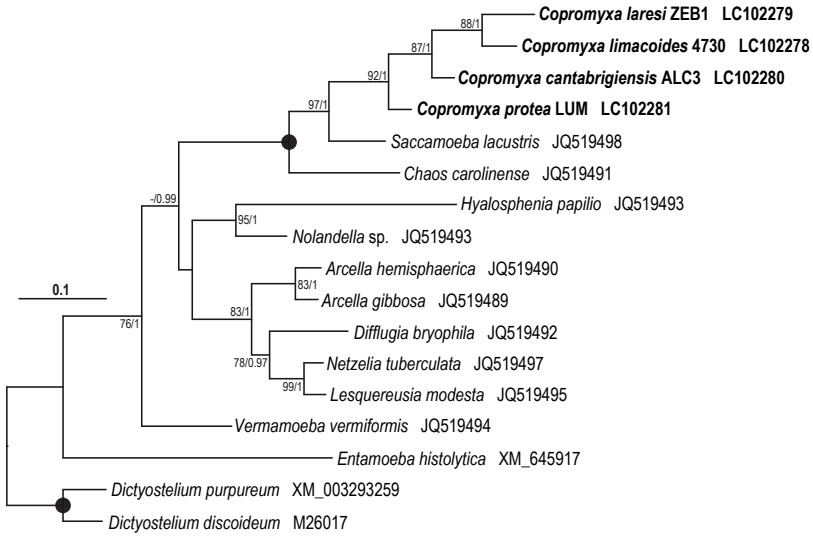

Fig. S3. Maximum likelihood (ML) constructed tree for eukaryotic elongation factor 2 gene sequences showing the phylogenetic position of the four strains of *Copromyxa* under study (in bold) within the Tubulinea. GenBank accession numbers of sequences used in analysis are listed next to the taxon names. The scale bar represents evolutionary distance in change/site. ML bootstrap values and Bayesian posterior probabilities (PP) are presented at each node. Bootstrap values and PP of 100%/1.0 are represented by a black dot. Support values less than 70% or 0.70 for the respective method are not presented.

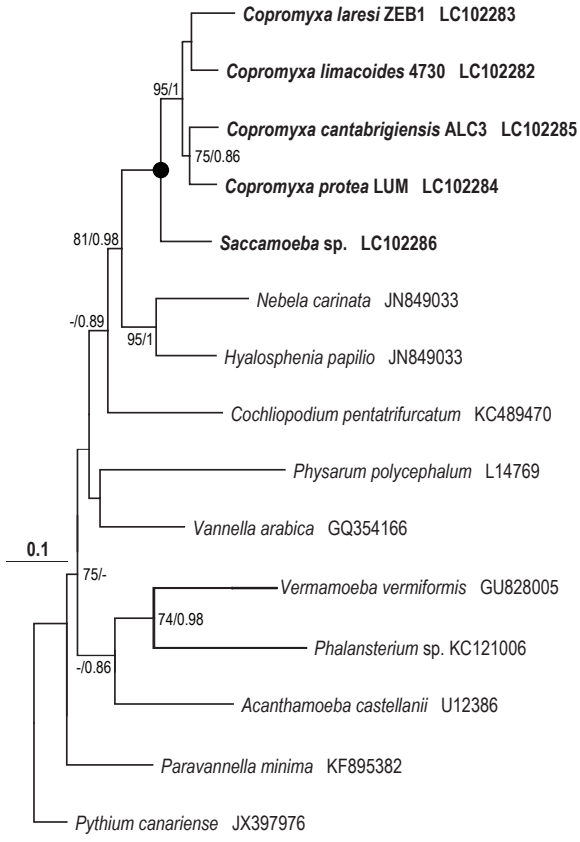

Fig. S4. Maximum likelihood (ML) constructed tree for cytochrome oxidase I gene sequences showing the phylogenetic position of the four strains of *Copromyxa* under study (in bold) within the Amoebozoa. GenBank accession numbers of sequences used in analysis are listed next to the taxon names. The scale bar represents evolutionary distance in change/site. ML bootstrap values and Bayesian posterior probabilities (PP) are presented at each node. Bootstrap values and PP of 100%/1.0 are represented by a black dot. Support values less than 70% or 0.70 for the respective method are not presented.

Section V

Vermistella **as a candidate for taxon with bipolar distribution**

The Journal of Eukaryotic Microbiology

Published by the International Society of Protistologists

Journal of Eukaryotic Microbiology ISSN 1066-5234

ORIGINAL ARTICLE

Vermistella arctica n. sp. Nominates the Genus *Vermistella* as a Candidate for Taxon with Bipolar Distribution

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Keywords
actin; biogeography; bipolar distribution; free-living amoebae; phylogeny; polar regions; SSU.

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Received: 29 December 2014; revised 28 August 2015; accepted August 29, 2015.

doi:10.1111/jeu.12270

ABSTRACT

A new amoebozoan species, *Vermistella arctica* n. sp., is described from marine habitats in the central part of Svalbard archipelago. This is the first report on Arctic amoebae belonging to the genus *Vermistella* Moran and Anderson, 2007, the type species of which was described from the opposite pole of the planet. Psychrophily proved in the new strains qualifies the genus *Vermistella* as a bipolar taxon. Molecular phylogenetic analyses based on 18S rDNA and actin sequences did not show any affinity of the genus *Vermistella* to *Stygamoeba regulata* ATCC® 50892™ strain. A close phylogenetic relationship was found between *Vermistella* spp. and a sequence originating from an environmental sample from Cariaco basin, the largest marine permanently anoxic system in the world. Possible mechanisms of bipolar distribution are discussed.

FREE-LIVING amoebae (FLA) are currently considered to be a heterogeneous assemblage of microeukaryotes belonging to five supergroups (Adl et al. 2012). For a long time they have been studied using traditional culture-dependent methods based on the morphology of trophozoites and eventual cyst stages. Until the discovery of FLA as agents of human infections (Culbertson et al. 1958; Fowler and Carter 1965), the sampling sites for isolation of amoebae were selected rather accidentally. Ecological aspects were studied due to the search for sources of human infections caused by species of *Naegleria* and *Acanthamoeba*. Broader ecological studies of the early period of free-living amoeba research are listed in the key publications by Page (1983, 1991). More recent studies introduced a modified culture-dependent approach that integrates morphological study with molecular methods and phylogenetic analyses of selected molecular markers. Thanks to this approach the accuracy of generic and species delineation of FLA improved, the assemblages of amoebae that share evolutionary history could be defined, and the significance of data from a variety of ecosystems increased. Nonetheless, despite the amount of data collected on the diversity of FLA, exact data on their global distribution (biogeography) are largely missing. This fact has been repeatedly noted in the last few decades when characterization of free-living microeukaryote communities in specific microhabitats has become a topic of primary interest (Finlay and Fenchel 2004; Gast et al. 2004; Stoeck et al. 2007) and new DNA-based methodological approaches have been developed for studies of the total diversity of ecosystems (Bik et al. 2012; Caron et al. 2009). Among amoebozoans the best explored distribution patterns are known for testate lobose amoebae, the arcellinids (see esp. Foissner 2006, 2007 and Smith et al. 2008 for references). Their shell allows identification of morphospecies even at fossil level. The distribution appears to be limited to the Southern Hemisphere and the tropics in *Nebela vas* (Smith and Wilkinson 2007); in *Nebela ansata*, the distribution range is remarkably narrow (Heger et al. 2011). The potential of arcellinid species to inhabit both polar regions negatively correlates with their size (Yang et al. 2010).

The exploration of extreme habitats on the planet has become a challenge for this type of study including FLA. The Arctic and Antarctic offer a unique opportunity to test amoeba species distribution/biogeography in the high Northern and Southern latitudes. They share numerous environmental characteristics which are absent in the

latitudes between, of which low temperature and extreme seasonal fluctuations of light supply predominate (Barnes 2012; Kuklinski and Barnes 2010). Furthermore, some taxa are distributed in both polar zones only, thus showing an extreme disjunct distribution or so-called bipolar distribution. Such distribution pattern is known across various groups of life (e.g. Crame 1993; Koufopanou et al. 1999; Kuklinski and Barnes 2010; Shi and Grunt 2000) including the eukaryotic microorganisms (Darling et al. 2000; Montresor et al. 2003; Pawlowski et al. 2007).

Free-living amoebae records from the Arctic are limited due to very few studies. De Jonckheere (2006) performed isolation attempts from Arctic freshwater samples at room temperature (RT). He tested strains with vahlkampfiid morphology for growth at 30 and 37 °C and identified six isolates of *Naegleria*. Three recently described *Naegleria* species (*N. arctica*, *N. neopolaris* and *N. spitzbergensis*) are of Arctic origin, whereas two other (*N. polaris* and *N. neodobsoni*) were diagnosed from Arctic as well as sub-Antarctic samples (De Jonckheere 2006).

There is one report from Antarctica and sub-Antarctic regions on *Acanthamoeba* and *Naegleria* identified in Antarctic soil and water samples by screening at 30 °C (Brown et al. 1982). Five *Naegleria* species (*N. polaris*, *N. neodobsoni*, *N. neoantarctica*, *N. neochilensis* and *N. paradobsoni*) were described from the Crozet archipelago in sub-Antarctica by De Jonckheere (2006). In the latter study, screening of the sampled materials was also performed at RT. An overview of previous reports on Antarctic gymnamoebae was presented by Moran et al. (2007) along with a description of the type species of a newly established genus, *Vermistella* Moran and Anderson, 2007 and new marine *Platyamoeba* species, *P. oblongata* and *P. contorta*, subsequently reclassified to *Vannella* genus (Smirnov et al. 2007).

Vermistella was established to accomodate a naked, psychrophilic amoeba strain isolated from deep-sea sediment collected near the Ross Ice Shelf (76°53′S, 154°14′W), Antarctica. *Vermistella antarctica*, the type and only species of this genus, has been described in detail (Moran et al. 2007). The authors of the description deposited the type strain (S-241) in the ATCC® (acc. no. PRA-216), generated its complete 18S DNA sequence (GenBank acc. no. DQ229956) and found that *V. antarctica* was not related to any described family of gymnamoebae.

Smirnov (2009) considered *Vermistella* a junior synonym of *Stygamoeba* Sawyer 1975 that was listed as an *incertae sedis* genus within Rhizopoda. In a revised classification of naked lobose amoebae (Smirnov et al. 2011), Smirnov and Cavalier-Smith resurrected *Vermistella* and established a new family Stygamoebidae for *Stygamoeba* and *Vermistella* within a newly established order Stygamoebida. All known members of the Stygamoebida possess unique combination of features at both morphological and ultrastructural level; in particular, flattened and ribbon-like mitochondrial cristae, presence of distinctive dictyosomes and very similar locomotive forms (Smirnov et al. 2011).

The high quality of taxonomic information on *V. antarctica* Moran and Anderson, 2007, supplemented with physiological and ecological data, provides a reliable base for comparison with other marine gymnamoebae and offers an exceptional opportunity to introduce *Vermistella* species from the opposite part of our planet.

MATERIALS AND METHODS

Amoeba strains isolation and culturing

The three amoeba strains presented in this study originated from Billenfjorden in the central part of the Svalbard archipelago, Arctic, where they were obtained from marine invertebrates collected by scuba diving at a depth of 10–20 m in three different localities where temperature was almost exclusively 4 °C. One strain (SV198) was isolated from gills of the hermit crab *Pagurus pubescens* collected in Skansbukta (78°31′N, 16°4′E) in August 2009. The other two strains, DX2 and DC17C, were isolated from the body surface of the tubeworm *Circeis spirillum* sampled in Petuniabukta (78°40′N, 16°28′E) and Brucebyen (78°39′N, 16°42′E), respectively, in July 2012. The methods of isolation, culturing and cloning followed those described elsewhere (Dyková and Lom 2004). Gill samples or worms were placed onto 2.5% MY75S agar medium moistened regularly with sterile seawater and kept at ambient temperature (0–8 °C) during stay at the Czech field station until transferred to the laboratory. Then the culturing conditions were stabilized at 10 °C and fluctuation of humidity was reduced by Parafilm® (Bemis Europe, Soignies, Belgium) sealing of Petri dishes. Due to their slow growth, the strains were subcultured at an interval of 1–5 wk. Several attempts of enhancing trophozoite multiplication were made, raising the temperature to 15 or 20 °C. Strains DX2, DC17C and SV198 are deposited in the Culture Collection of Algae and Protozoa (Scottish Marine Institute, Oban, Argyll, UK) under accession codes CCAP 2581/1, CCAP 2581/2 and CCAP 2581/3 respectively.

Microscopy

To observe live amoebae, agar plates were washed with cold seawater (4 °C) and the suspension of trophozoites was placed onto coverslips and kept at 1 °C. After 2 h, amoebae were observed in hanging drop preparations using a BX53 microscope (Olympus, Tokyo, Japan) equipped with Nomarski differential interference contrast. Observations and documentations were carried out as fast as possible to avoid increasing the temperature above 2 °C. For comparison, the procedure was also performed at RT. For transmission electron microscopy (TEM), two protocols were followed: trophozoites were fixed in situ (i) with cacodylate buffered 3% glutaraldehyde, washed off after 30 min, pelleted and postfixed with 1% osmium tetroxide for 1 h or (ii) exposed to osmium tetroxide vapours for 30 min, washed off with 0.1 M cacodylate buffer and pelleted. Pellets of tropho-

zoites were dehydrated in an acetone series and embedded in Spurr resin (EMS, Fort Washington, PA). Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a JEOL JEM 1010 (JEOL, Tokyo, Japan) electron microscope operating at 80 kV. Knowing that the apparent structure of mitochondria (e.g. poorly defined cristae and matrix) can be related to specific conditions of culturing and fixation, we compared the fine structure of mitochondria under different conditions. Passages at different age, held at different conditions during culturing, i.e. various temperatures or humidity, were repeatedly fixed and prepared for TEM. This study also included the *Stygamoeba regulata* ATCC® 50892™ (ATCC, Manassas, VA) strain cultured on MY75S agar medium at 20 °C and processed for TEM in the same manner as the *Vermistella* strains.

DNA isolation, amplification and sequencing

DNA was extracted using JETquick Tissue DNA Spin Kit (GENOMED GmbH, Lohne, Germany) and Genomic DNA Tissue Mini Kit (Geneaid, Tao-Yuan, Taiwan) for the SV198 strain and DX2 plus DC17C strains, respectively, according to manufacturers' protocols. Primer pairs Erib1 + Erib10 (Barta et al. 1997) or 460F + 1700R (Dyková et al. 2008) were used to amplify the 18S rRNA gene. Sequences of the gene for actin were obtained according to Tekle et al. (2007). The PCRs and cloning (in the case of the 18S and actin genes) were performed as described previously (Dyková et al. 2008, 2010). Three and five clones of each strain were sequenced from 18S rRNA and the actin gene, respectively, at Macrogen Inc. (Amsterdam, the Netherlands). Sequences of 18S rRNA and actin genes were obtained from all new *Vermistella* strains. Both genes were cloned because of a mixed signal in some positions after direct sequencing of PCR products. In the case of DX2 strain, a shorter fragment of 18S rDNA sequence compensates for the failed full length amplification. Only one clone of each strain and gene was used in all final analyses (labelled as clone 1).

Phylogenetic analyses

Phylogenetic analyses of data sets composed of either 18S rDNA or actin gene sequences were used to reveal and test both the phylogenetic position of *Vermistella* among amoebozoans and relationships between Arctic and Antarctic strains. The resulting 18S rDNA data set contained 77 amoebozoan taxa plus 10 amorphean outgroups. It was prepared in ClustalX v. 2.1 (Larkin et al. 2007) and checked in BioEdit 7.2.5 (Hall 1999) for ambiguously aligned positions – after their deletion, its length was 1,328 bp. The same software was also used to prepare a data set based on actin gene sequences: it contained 35 amoebozoan sequences plus four opisthokont outgroup; its final length was 254 amino acids (AAs). All data sets were analysed with RAxML 8.0.20 (Stamatakis 2014), where GTRGAMMAI model suggested as best by MrModeltest2 (Nylander 2004) was used for the 18S rDNA analysis and PROTGAMMAILGF for actin; rapid bootstrapping was employed to compute the support of the most likely topologies (1,000 replicates). Bayesian inference analysis of 18S rDNA data set was run in MrBayes 3.1.2 (Ronquist and Huelsebeck 2003) with a GTR + I + gamma model specified and 2×10^6 generations; burnin was default: 25% of the sampled trees (−lnL vs. generation plot was visually checked to assure −lnL plateau was reached). The best fit model of protein evolution was selected using Prottest (Abascal et al. 2005). To test the proposed sister position of *Vermistella* and *Stygamoeba*, AU test implemented in Consel 0.1i (Shimodaira and Hasegawa 2001) was performed on the main 18S rDNA data set: most likely unconstrained and constrained topologies were compared along with 100 trees computed by RAxML from bootstrap replicates.

RESULTS

Culturing

The upper limit temperature for culturing Arctic *Vermistella* strains was 10 °C. Although we tried the subculturing at higher temperatures several times to increase its growth rate, the culture always stopped multiplying immediately. All isolates were distinctly unstable during several first generations and were constantly threatened by loss. The growth rates were extremely variable so that the subculturing intervals ranged from 1 to 5 wk.

Morphology

Under simulated natural conditions, i.e. low culturing temperature (10 °C) and adhesion to coverslips at 1 °C/2 h followed by a fast observation and documentation, trophozoites of all strains under study were mostly stick-like or spineolate, mostly with a broader rounded or knob-like posterior end. In SV198 strain, mean cell size ($n = 52$) during locomotion at 1–2 °C was 29.8 (20.9–40.3) μm in length, 4.4 (2.9–7.9) μm in breadth of a rounded posterior; and 7.0 (3.8–10.7) length/breadth ratio. Cells of all strains possessed a single vesicular nucleus with a mean diam. 2.5 μm ($n = 14$), a central nucleolus (mean diam. 1.2 μm). In a locomotive form the nucleus was centrally positioned in a rounded posterior end. Branched, sinusoidal, waved and slightly flattened cells, consistent with those described in the type species of *Vermistella* Moran and Anderson, 2007, were also observed (Fig. 1A). Dividing cells were gradually tapering towards the middle of an intercellular bridge (Fig. 1A). Floating forms formed short pseudopodia (Fig. 1A). Trophozoites from cultures that were maintained at 10 °C, but incubated at 20 °C for 2 h prior to study, revealed pronounced changes of morphology that were identical in all strains (Fig. 1B). The same situation occurred when the temperature increased over 2 °C during documentation. These trophozoites transformed into flattened, irregularly rounded cells with an uneven wrinkled surface resembling the contracted forms described

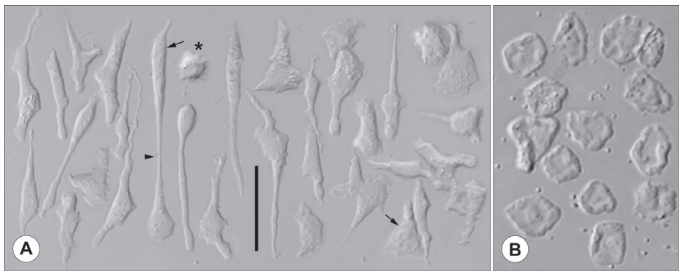

Figure 1 **A, B.** Type strain (SV198) of *Vermistella arctica* n. sp. as seen in hanging drop preparation using Nomarski differential interference contrast. **A.** Trophozoites left to adhere to a coverslip at 10 °C (temperature of culturing) for 2 h; vermiform locomotive form of trophozoites with a broader posterior part of the body (predominantly to the left of the bar), floating form with short blunt pseudopodia (*), dividing cells connected with intercellular bridge (arrowhead) and cells in nondirected movement (predominantly to the right of the bar). Note two trophozoites with visible nuclei (arrows). **B.** Trophozoites incubated at 20 °C for 2 h. Scale bar (20 μm) applies also to (B).

in *V. antarctica* by Moran et al. (2007). These cultures did not recover when returned to 10 °C. Repeated tests for thermotolerance revealed these identical results and thus they prove the psychrophily of the amoebae studied. Although all three strains were exposed to various extreme conditions (e.g. keeping in higher thermal or lower humid conditions, long-term maintaining on an agar plate), they never formed cysts or resting stages.

Ultrastructure

The fine structure of trophozoites resulting from routine as well as modified fixation was relatively well preserved. Cytoplasm of the trophozoites contained a prominent nucleus with a centrally located nucleolus, numerous vesicles, cisternae of Golgi apparatus in a parallel arrangement and feeding vacuoles with bacterial and sometimes also apparently nonbacterial content (Fig. 2A, D). The glycocalyx was electron-dense with faint signs of regular differentiation observable only at a magnification above 200,000× (Fig. 2B, C). Contrary to other details of ultrastructure, the type of mitochondria was extremely difficult to classify. They were bounded by well-preserved membranes but the matrix of some was lucid or barely discernible. Long profiles of mitochondrial cristae that were interconnected with inner bounding membranes (Fig. 2E, F) predominated in ultrathin sections from trophozoites fixed in glutaraldehyde and postfixed in osmium tetroxide as well as in those fixed in osmium tetroxide only. Such profiles corresponded to those described in the type species of *Vermistella* Moran and Anderson, 2007 as flattened, slightly curved cristae. The mitochondria of all *Vermistella arctica* strains examined had, in addition to long profiles, small irregular and circular profiles resembling transverse sections through tubular cristae (Fig. 2G). Similarly, in *S. regulata* strain ATCC® 50892™ we also observed circular profiles of mitochondrial cristae (Fig. 2H).

Molecular phylogeny

Comparison of 18S rDNA clone sequences from each of the *Vermistella* strains revealed the presence of intragenomic variability, but this was never higher than 0.7% dissimilarity. Among all *Vermistella* strains, similarity values showed a tighter phylogenetic relation between the DC17C and DX2 strains (more than 99.5% identity) than between the others. All identity values are shown in Table 1. The actin gene sequences had a higher variability than 18S rDNA within the genomes as well as among the strains. Most of the actin sequences exhibited 95.9–100% identities in AA translation. There were only three exceptional sequences among the clones which were short (189–199 AAs) and with higher values of dissimilarity (30.2% in the most extreme case). Nevertheless, the closest BLAST hits fell among the amoebozoan actin sequences and also a phylogenetic analysis of all amoebozoan actin sequences undergone in Fasttree (Price et al. 2010) assigned the sequences in the *Vermistella* cluster.

The most thorough analysis (of 18S rDNA sequences) demonstrated clearly that the newly isolated arctic *Vermistella* strains form a sister clade to *V. antarctica* among amoebae with known morphology and proved a very close relation among the arctic strains (Fig. 3). The same result was supported by analysis of actin sequences. However, the relationships of *Vermistella* to other amoebozoans remained obscured. In our analyses we never observed it to form a monophylum with *Stygamoeba* as suggested by Smirnov et al. (2011). On the 5% significance level, the AU test performed on 18S rDNA data set did not exclude either the most likely tree shown in the figure or the tree constrained to monophyletic Stygamoebida Smirnov and Cavalier-Smith, 2011. The relationships in the actin tree (Fig. S1) are generally poorly resolved but even so the placements of both taxa (*Vermistella* spp. and *S. regulata* strain ATCC® 50892™) are in agreement with the 18S rDNA analyses.

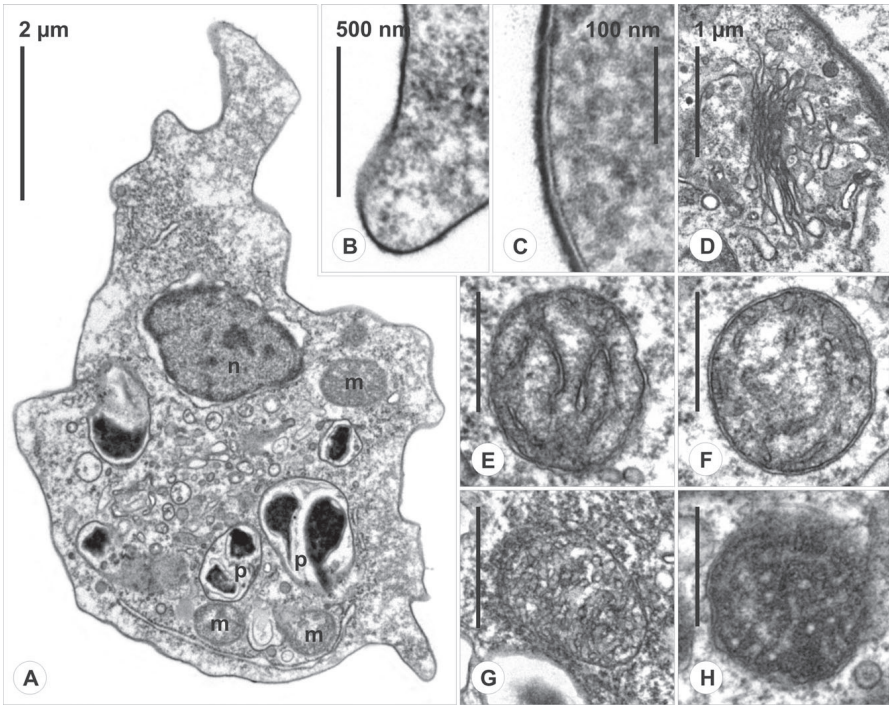

Figure 2 **A–G.** Details of the ultrastructure of the type strain (SV198) of *Vermistella arctica* n. sp. **A.** Trophozoite with nucleus (n), mitochondria (m) and phagosomes (p) in the cytoplasm. **B, C.** Cell membranes of trophozoites with thick glycocalyx. **D.** Cisternae of the Golgi apparatus in parallel arrangement. **E–G.** Mitochondria of SV198; variability in cristal profiles. Scale bars: (E, F) 500 nm; (G) 1 µm. **H.** Mitochondrion of *Stygamoeba regulata* strain ATCC® 50892™. Scale bar = 500 nm.

DISCUSSION

The arctic strains share morphological and ultrastructural features, apart from *V. antarctica*, also with the genus *Stygamoeba*. To date, two species of *Stygamoeba* have been described (Sawyer 1975; Smirnov 1996). *Stygamoeba polymorpha* Sawyer, 1975, the type species, have been characterized at light microscopy level only and neither ultrastructure nor molecular data have been obtained. Furthermore, no culture of *S. polymorpha* is maintained or cryopreserved. Nevertheless, light microscopy level provides two crucial features in differentiation between *Vermistella* spp. and *S. polymorpha*, i.e. a presence of conspicuous expanded leaf-like forms (fig. 34 in Sawyer 1975) and a dissimilar appearance of cells at division. Before an entire separation of daughter cells of *S. polymorpha*, they are connected, in contrast with *Vermistella*, by a long and evenly thin intercellular bridge and their posteriors are the widest part of the cell (fig. 35 in Sawyer 1975). *Stygamoeba regulata* differs from *Vermistella* spp. in forming cysts (Smirnov 1996). We consider all the dissimilarities between *Vermistella* spp. and *Stygamoeba* spp. to be relevant and justifying for taxonomic status of *Vermistella*. However, it is desirable to obtain new strains and enhance data on the possibly severely undersampled group of naked amoebae.

Morphological distinction between the new *Vermistella* strains and *V. antarctica* is possible especially based on the significant differences in dimensions of both locomotive and floating forms. In all compared values, cells of *Vermistella* strains isolated in the Arctic are considerably larger. No floating-form cells bearing long and fine-tapered pseudopodia were observed in our cultures, contrary to *V. antarctica*. Furthermore, the rate of locomotion of the arc-

Table 1. Sequence (18S rDNA) identity among *Vermistella* strains

Vermistella species/strain	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
(1) *Vermistella antarctica*[a]	–								
(2) SV198 strain clone1[b]	93.8	–							
(3) SV198 strain clone2	93.9	99.5	–						
(4) SV198 strain clone3	93.9	99.4	99.3	–					
(5) DC17C strain clone1[c]	93.4	97.7	97.7	97.5	–				
(6) DC17C strain clone2	93.5	97.9	97.8	97.6	99.8	–			
(7) DC17C strain clone3	93.5	97.8	97.8	97.6	99.9	99.9	–		
(8) DX2 strain clone1[d]	92.5	96.8	96.6	96.6	99.7	99.8	99.9	–	
(9) DX2 strain clone2	92.4	96.9	96.7	96.7	99.6	99.8	99.7	99.7	–
(10) DX2 strain clone3	92.4	97.0	96.8	96.8	99.6	99.7	99.7	99.7	99.6

Values are expressed in percentage.
[a]Sequence DQ229956, length 1,939 bp.
[b]*Vermistella arctica* (SV198 strain), sequences of length 1,949 bp deposited in GenBank database under acc. nos KJ874207–KJ874209.
[c]*Vermistella arctica* (DC17C strain), sequences of length 1,943 bp deposited in GenBank database under acc. nos KJ874210–KJ874212.
[d]*Vermistella arctica* (DX2 strain), partial sequences of length 1,146 bp (bases missing from both ends) deposited in GenBank database under acc. nos KJ874213–KJ874215.

tic strains was much lower than that measured by Moran et al. (2007) in *V. antarctica* strain. In consideration of this, and based on the specific morphology and adequate molecular support, we have decided to describe our *Vermistella* strains as a new species.

Ambiguities in the *Vermistella* position within Amoebozoa as well as its relationship with Stygamoebidae reflect an unexceptional existence of unstable branches in the Amoebozoa tree and a paucity of 18S for solving the deep phylogeny of Amoebozoa (Lahr et al. 2011; Smirnov et al. 2011; Tekle et al. 2008). Despite their morphological similarity, both *Vermistella* spp. and *S. regulata* ATCC® 50892™ occupy a different isolated, statistically unsupported position in the tree (Fig. 3), similarly as in fig. 3 of Lahr et al. (2011). It should be noted that during the scope of our study, we have produced a number of data set variants with different taxon sampling and more or less stringent trimming. In the resulting trees, we have never observed a closer affinity of *Vermistella* and *Stygamoeba*. The insufficiency of 18S rDNA data set to solve this question is further illustrated by the result of AU test, which was also unable to decide on either relevant topology.

Slightly inconsistent observations in as many as dozens of ultrathin sections through pelleted *Vermistella* trophozoites prevented us from making a conclusive statement regarding the types of mitochondrial cristae. The low density of the mitochondrial matrix was also not easy to interpret. Such situations were described as occasional findings and also in generalized form in diseased conditions of higher vertebrates (Cheville 2009). The quality of the image documenting the mitochondrion of *V. antarctica* (Moran et al. 2007; their fig. 49) leads to the assumption that the authors of the description faced the same problem as we did. We experienced similar difficulties when studying types of mitochondrial cristae in flabellulids and some heteroloboseans (Dyková and Kostka 2013). A satisfactory preservation of mitochondrial cristae also was not easy to obtain in the *S. regulata* studied by Smirnov (1996) (see his fig. 24–26). Generally, the type and orientation of mitochondrial cristae is a fairly regular feature in free-living amoeba species/genera (Page and Blanton 1985; Page 1987, 1988), although variations and exceptional cases of transitions from tubular to paracrystalline organization of cristae (in *Chaos carolinensis*) were also reported (Deng et al. 1999; Frey and Mannella 2000). Seravin (1993), who focused his studies mainly to protists, proposed a classification of mitochondrial cristae with three types and several subtypes/forms. Within the lamellar/flat type of cristae, he suggested to distinguish ribbon-, sheet-, bundle-like and rounded (discoidal and plate-like) subtypes; bubble-, ampule- and sack-like subtypes within the vesicular type of cristae; and the tubular cristae were considered the third type. Although some care is needed when interpreting the crystal type/form, comparison of images taken at various plains of sectioning usually provides an answer. However, in contrast to mitochondria with abundant cristae, we found it difficult to determine the type of cristae in those amoebae that contain only a few cristae in the mitochondrial matrix. In the light of these findings, we conclude that the type of mitochondrial cristae should be considered cautiously in such cases. Advanced methods, such as electron microscopic tomography, have shown that complex lamellar cristae of some mitochondria merge with the inner boundary membrane through tubular structures about 30 nm in diam. (Frey and Mannella 2000). New insight into the knowledge of lamellar/flat cristae in *Vermistella*, *Stygamoeba* and other FLA can be expected when internal structures of mitochondria are studied in the absence of fixatives, using cryo-electron microscopy.

In recent model studies, biota present both at the high Northern and Southern latitudes, but separated by a distribution gap across the tropics, have increasingly been termed to be "bipolarly," "bi-polarly" or "antitropically" distributed. Molecular methods have confirmed bipolarity in microbial eukaryotes such as planktonic (Darling et al.

Figure 3 Maximum likelihood tree based on 18S rDNA sequences showing positions of all known *Vermistella* strains and *Stygamoeba regulata*. Numbers at the nodes are bootstrap values (maximum likelihood)/posterior probabilities (Bayesian inference). Nodes with a bootstrap support of ≤ 80 and a Bayesian posterior probability of ≤ 0.80 are labelled with a dash.

2000) and deep-sea benthic foraminifera (Pawlowski et al. 2007) as well as dinoflagellates (Montresor et al. 2003). Bipolar species or genera have been reported also in bryozoans (Kuklinski and Barnes 2010), brachiopods (Shi and Grunt 2000) and molluscs (Crame 1993; Koufopanou et al. 1999). Hypotheses about the phenomenon of bipolarity in

individual groups of organisms differ regarding geological time, however, most of them agree in that the climatic changes over geological time were decisive and must have repeatedly influenced the evolution of biodiversity and formation of bipolar patterns.

To the best of our knowledge, the bipolar distribution of *Vermistella* is the first such case known in Amoebozoa. There are several hypotheses about the origin of the extreme disjunct distribution involving either dispersal or vicariance processes. The dispersion ability of *Vermistella* strains is low due to intolerance to temperatures higher than 10 °C and (apparently) nonexistent cysts or resting stages. For psychrophilic organisms, the warm equatorial belt poses a barrier that can hardly be crossed, although some may get across in deep water currents (Stepanjants et al. 2006). The latter way, however, is difficult to imagine for noncyst-forming FLA. They live at interfaces and need to adhere to a substratum. Detached, they are unable to feed and cannot survive long. It is worth mentioning, though, that in regard to the efficiency of amoeba isolation attempts in the Arctic region, the surfaces of invertebrates were superior to examination of sediments or large volumes of seawater; it was not unusual to obtain more than one isolate from one specimen of a small invertebrate. All the amoebae isolated were probably epibionts – such a life strategy may enhance their ability to spread over vast distances via phoresis.

The vicarious type of events leading to bipolar distribution may arise due to an extinction caused by global climate changes or increasing competition outside the polar regions. In that case, some populations would stay in refuges, places where the conditions remain unchanged or strong competitor pressure is lowered. Environmental sequence GU824670, the nearest hit to *Vermistella* spp. sequences in the BLAST search, comes from an anoxic zone of the Cariaco basin (Edgcomb et al. 2011). A specific habitat such as the Cariaco basin can provide a shelter from competitors and changed environmental conditions. This finding may indicate that there are yet unknown areas containing *Vermistella* in deep cold oceanic regions, resulting in this genus having a much broader distribution.

An unusual distribution can also be caused by anthropogenic transport. For example, ballast water is a possible way of spreading microorganisms over long distances (Hülsmann and Galil 2001). Introduction of microorganisms by human activities into Antarctica has been reviewed by Cowan et al. (2011).

TAXONOMIC SUMMARY

Vermistella arctica n. sp.

Diagnosis. Typically stationary or with extremely slow and steady flow movement amoeba. Monopodial locomotive form 20.9–40.3 μm in length (mean = 29.8 μm, SD = 5.8 μm), 2.9–7.9 μm in breadth of a rounded posterior (mean = 4.4 μm, SD = 1.2 μm) and with 3.8–10.7 range of length/breadth ratios (mean = 7.0, SD = 1.5) (n = 52). Nucleus difficult to observe, 2.4–2.6 μm in diam. (mean = 2.5 μm, SD = 0.1 μm) (n = 14), centrally positioned in a broader rounded posterior (in monopodial locomotive form). Spherical floating form, 6.2–8.0 μm in diam. (mean = 7.2, SD = 0.6), with radiating short blunt pseudopodia only. Mitochondrion with small irregular and circular profiles resembling transverse sections through tubular cristae.

Etymology. The specific name refers to the geographical origin of the type strain.

Type locality. Skansbukta (78°31′N, 16°4′E), marine littoral zone, central part of the Svalbard archipelago, Arctic.

Deposition of type-material. Culture of the type strain SV198 has been deposited in the Culture Collection of Algae and Protozoa (Scottish Marine Institute) under accession code CCAP 2581/3. The other strains, DX2 and DC17C, have been deposited in the same institution under codes CCAP 2581/1 and CCAP 2581/2 respectively.

Gene sequence data. Sequences of 18S rDNA have been deposited in GenBank under acc. nos KJ874207–KJ874209 and sequences of actin gene under acc. nos KJ874216–KJ874227.

Remarks. All known strains were isolated from gills or body surface of marine invertebrates (hermit crab *P. pubescens*, polychaete *C. spirillum*). To ensure data comparable with the description of *V. antarctica* (Moran et al. 2007), observation and measurements were carried out on hanging drop preparations at 1–2 °C. Sequences of other strains (DC17C, DX2) have also been deposited in GenBank under acc. nos KJ874210–KJ874215 (18S rDNA) and KJ874228– KJ874240 (actin gene).

ACKNOWLEDGMENTS

We are grateful to Keith Edwards who corrected the English text. We are also thankful to Alexey Smirnov and two anonymous referees for valuable advice and comments. The project was financially supported by the Ministry of Education, Youth and Sports (LM2010009), European Social Fund Project (CZ.1.07/2.2.00/28.0190), Czech Science Foundation (505/12/G112) and the Grant Agency of University of South Bohemia (140/2013/P).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Maximum likelihood phylogeny of amoebozoan amino acid actin sequences. The positions of *Vermistella arctica* strains and *Stygamoeba regulata* sequences are distant. Numbers at the nodes indicate bootstrap values and only values higher than 50 are shown.

SUPPORTING INFORMATION

***Vermistella arctica* n. sp. Nominates the Genus *Vermistella* as a Candidate for Taxon with Bipolar Distribution** by Tomáš Tyml, Martin Kostka, Oleg Ditrich, and Iva Dyková

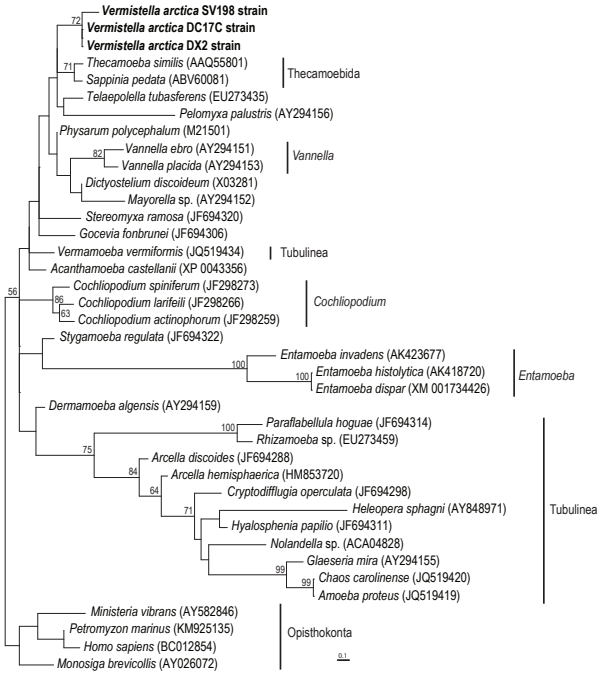

Fig. S1. Maximum likelihood phylogeny of amoebozoan aminoacid actin sequences. The positions of *Vermistella arctica* strains and *Stygamoeba regulata* sequences are distant. Numbers at the nodes indicate bootstrap values and only values higher than 50 are shown.

Section VI

Heterolobosean amoebae from Arctic and Antarctic extremes

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European Journal of Protistology 56 (2016) 119–133

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Heterolobosean amoebae from Arctic and Antarctic extremes: 18 novel strains of *Allovahlkampfia*, *Vahlkampfia* and *Naegleria*

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Received 8 February 2016; received in revised form 14 May 2016; accepted 4 August 2016
Available online 28 August 2016

Abstract

The diversity of heterolobosean amoebae, important members of soil, marine and freshwater microeukaryote communities in the temperate zones, is greatly under-explored in high latitudes. To address this imbalance, we studied the diversity of this group of free-living amoebae in the Arctic and the Antarctic using culture dependent methods. Eighteen strain representatives of three heterolobosean genera, *Allovahlkampfia* Walochnik et Mulec, 2009 (1 strain), *Vahlkampfia* Chatton et Lalung-Bonnaier, 1912 (2) and *Naegleria* Alexeieff, 1912 (15) were isolated from 179 samples of wet soil and fresh water with sediments collected in 6 localities. The *Allovahkampfia* strain is the first representative of the genus from the Antarctic; 14 strains (7 from the Arctic, 7 from the Antarctic) of the highly represented genus *Naegleria* complete the 'polar' cluster of five *Naegleria* species previously known from the Arctic and Sub-Antarctic regions, whereas one strain enriches the 'dobsoni' cluster of *Naegleria* strains of diverse origin. Present isolations of *Naegleria polaris* De Jonckheere, 2006 from Svalbard, in the Arctic and Vega Island, in the Antarctic and *N. neopolaris* De Jonckheere, 2006 from Svalbard and Greenland in the Arctic, and James Ross Island, the Antarctic demonstrate their bipolar distribution, which in free-living amoebae has so far only been known for *Vermistella* Morand et Anderson, 2007.

Keywords: Arctic; Antarctic; Heterolobosea; Molecular taxonomy; Tetramitia

Introduction

As characterised by Bell and Callaghan (2012), extreme environments are considered to be places where at least one environmental parameter (e.g., temperature, pH, salinity, and radiation) approaches values beyond those acceptable for human existence. The recognized extreme environments tend to be inhabited by specific microbial communities (Bond et al. 2000; Perreault et al. 2008; Reysenbach et al. 2000). Organisms that thrive under some kind of environmental extreme, extremophiles, are found among the Archaea, Bacteria and Eukaryota; the latter group contains also grazers of prokaryotes which play the crucial role of the top microbial predators. Molecular taxonomy approaches have recently become a powerful tool in examining organism diversity within extreme environments. Novel microbial sequences

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http://dx.doi.org/10.1016/j.ejop.2016.08.003
0932-4739/

have been obtained, often belonging to completely new lineages for which no known cultivated isolates are available (Hugenholtz et al. 1998). This also applies to some eukaryotic microorganisms (e.g., López-García et al. 2001), however, specific eukaryotic groups such as heteroloboseans have been successfully isolated and cultivated from these environments.

Heterolobosea Page et Blanton, 1985 are eukaryotic microorganisms comprising amoeboid and flagellated stages, which in the vahlkampfiids may alternate in their life cycle. Some heteroloboseans (*Acrasis* spp., some allovahlkampfiid strains) exhibit an ability to form fruiting structures, i.e., sorocarps (Brown et al. 2012). Vahlkampfiids are polyphyletic (e.g., Pánek et al. 2012). They are scattered across Tetramitia Cavalier-Smith, 1993 and contain *Naegleria*, in which *N. fowleri* is notorious for its ability to cause fatal human diseases (Carter 1970), and e.g., *Tetramitus*, *Vahlkampfia*, *Paravahlkampfia*, or *Neovahlkampfia*.

The first heterolobosean of polar origin was *Naegleria* sp. reported from Svalbard by Sandon (1927). Smith (1978) identified species of two genera (*Naegleria* and *Vahlkampfia*) in microbial communities studied in maritime Antarctic and Sub-Antarctic islands. Species of the same two genera were also reported from McMurdo Sound, Antarctica by Brown et al. (1982). However, it should be mentioned that identification of some *Vahlkampfia* spp. in the older literature has a vague significance (Page 1988). Furthermore, some formerly *Vahlkampfia* spp. are now recognised as members of different genera, e.g., *Tetramitus* (Brown and De Jonckheere 1999). More recent studies based on molecular taxonomy introduced a whole series of novel heterolobosean species from the Arctic and the Antarctic: *Tetramitus vestfoldii* isolated from the Vestfold Hills, Eastern Antarctica (Murtagh et al. 2002); *T. parangularis* isolated from Prudhoe Bay, Alaska (Robinson et al. 2007); *Vahlkampfia signyensis* isolated from Signy Island, South Orkney, the maritime Antarctic (Garstecki et al. 2005); and *Naegleria antarctica* isolated from an unspecified locality in Antarctica (De Jonckheere 2004). Later, De Jonckheere (2006) described 8 new species of *Naegleria* from high latitudes: *N. polaris* represented by three strains from the Arctic (Svalbard and Greenland) and Ile de la Possession, Crozet Archipelago, the Sub-Antarctic, *N. neopolaris*, *N. arctica*, and *N. spitzbergenensis* from Svalbard, *N. neodobsoni* from Greenland and *N. neoantarctica*, *N. neochilensis* and *N. paradobsoni* from Ile de la Possession, Crozet Archipelago.

Material and Methods

Isolation, cultivation and microscopic observation

Amoeba strains were isolated from material obtained by Arctic/Antarctic expeditions in 2010 through 2015 (see Table 1 for details). Samples were collected in ice-free freshwater or terrestrial habitats during the summer seasons and kept at low temperatures (< 20 °C) until transferred to the laboratory wherein they were subsequently stored at 4 °C. Transport times varied between 4 and 18 days. Two different isolation methods were applied. Samples were placed either onto NN agar medium and moistened regularly with sterile Page's saline solution until the appearance of a homogeneous population of trophozoites (Dyková and Kostka 2013) or in a 50 ml culture flask containing 7 ml of sterile Page's saline solution and thereafter maintained in an incubator at 10 °C, the temperature acceptable to polar strains and ensuring a satisfactory culture growth. Subculturing was performed by transfer of a parent culture suspension onto fresh NN agar medium at variable time intervals depending on the phase of growth. Trophozoites, cysts, and eventually flagellates were observed and documented using an Olympus BX60 light microscope equipped with Nomarski differential interference contrast (DIC) and a DP71 digital camera. The morphology of the trophozoites was examined and documented in hanging-drop preparations at room temperature (RT) immediately after removal of the preparations from the 10 °C incubator. Additionally, all strains (except TT13, TT19) were observed at RT after a 24 h incubation at 20 °C. Measurements of the trophozoites and cysts were taken using ImageJ software (Schneider et al. 2012). Material for transmission electron microscopy was prepared as described in Dyková and Kostka (2013) with the only exception that cells were fixed with 3% glutaraldehyde diluted with Page's saline solution. Neither the flagellation test (Page 1988) nor the sorocarp induction test (Brown et al. 2012) was performed. However, presence of flagellate forms was checked in the course of the routine trophozoite observation.

DNA extraction, amplification, sequencing, and gene sequence analyses

The trophozoites or cysts were washed off the agar plates with Page's saline solution and concentrated by centrifugation. DNA was extracted from fresh cells using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) or from cells stored in TNES buffer (Asahida et al. 1996) by the standard phenol-chlorofom protocol (Sambrook and Russell 2001). The PCRs were performed as described in Dyková et al. (2008) unless otherwise specified. A fragment of 18S rDNA was amplified using the Naeg1 and Naeg4 primers (Table 2) at 30 cycles (95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min 30 s) following incubation at 95 °C for 5 min. A potential presence of a twintron in 18S rDNA was inferred from the size of the Naeg1-Naeg4 fragments. In the case of F11 strain, 18S rDNA was amplified using 18e (Hillis and Dixon 1991) and Erib10 primers (Barta et al. 1997). The NuF and NuR primers (Pélandakis et al. 2000) were used in ITS1-5.8S-ITS2 region amplification, which consisted of 30 cycles (95 °C for 1 min, 46 °C for 2 min, and 72 °C for 1 min) following incubation at 95 °C for 5 min. Each DNA sample was amplified at least two times independently to verify sequence

Table 1. List of strains with year of collection, locality of origin and type of habitat.

Strain	Year of coll.	Origin			
		Locality	Coordinates		Habitat
AB02	2010	Petuniabukta, Svalbard, Arctic	N 78°43′ 49″	E 16°26′41″	wet hummock meadow
AB03N	2010	Petuniabukta, Svalbard, Arctic	N 78°43′49″	E 16°26′41″	wet hummock meadow
AB05	2010	Petuniabukta, Svalbard, Arctic	N 78°43′49″	E 16°26′41″	wet hummock meadow
AB07	2010	Petuniabukta, Svalbard, Arctic	N 78°43′49″	E 16°26′41″	wet hummock meadow
AB11	2010	Petuniabukta, Svalbard, Arctic	N 78°43′49″	E 16°26′41″	wet hummock meadow
BA03	2011	Brucebyen, Svalbard, Arctic	N 78°35′11″	E 16°37′48″	stream
EA4AN	2014	Gåsøyane, Svalbard, Arctic	N 78°27′8″	E 16°12′33″	freshwater lake
GRZ3	2013	Zackenberg, Greenland, Arctic	N 74°24′36″	W 20°14′23″	dry creek bed
F11	2013	Vega Island, Antarctica	S 63°49′21″	W 57°20′2″	freshwater lake
F13	2013	Vega Island, Antarctica	S 63°49′30″	W 57°19′48″	freshwater lake
F20	2013	Vega Island, Antarctica	S 63°49′17″	W 57°19′30″	freshwater lake
F21M	2013	Vega Island, Antarctica	S 63°49′17″	W 57°19′30″	freshwater lake
F21V	2013	Vega Island, Antarctica	S 63°49′18″	W 57°19′30″	freshwater lake
F25B	2013	Vega Island, Antarctica	S 63°49′7″	W 57°19′52″	freshwater lake
F26	2013	Vega Island, Antarctica	S 63°49′16″	W 57°19′2″	freshwater lake
F32	2013	Vega Island, Antarctica	S 63°49′2″	W 57°19′56″	freshwater lake
TT13	2015	James Ross Island, Antarctica	S 63°48′3″	W 57°49′39″	moss bed
TT19	2015	James Ross Island, Antarctica	S 63°47′17″	W 57°47′54″	algal mat from dried up lake

differences among strains. The PCR products were purified with the High Pure PCR product purification kit (Roche Diagnostics GmbH, Mannheim, Germany), and their nucleotide sequences were determined using a combination of amplification and internal primers (Table 2) at Macrogen Europe Inc. (Amsterdam, the Netherlands). The sequences were aligned and analysed within the Geneious R7.1.4 software (Geneious, Auckland, New Zealand) using relevant plug-ins. Three datasets, i.e., (I) 18S rDNA of 76 sequences, (II) the entire ITS region of 86 sequences, and (III) the entire ITS region of 12 sequences, were aligned and adjusted manually by MAFFT v7.017 (Katoh et al. 2002) using the G-INS-i algorithm. The final alignments of the I, II, and III datasets contained 1512, 298, and 310 bps, respectively. The best-fit model of evolution was determined using the AIC selection from Modeltest v3.7 (Posada and Crandall 1998) for each alignment. Phylogenetic analyses of alignments I and II were conducted using three different methods: (i) Bayesian analysis (BA) carried out in MrBayes v3.2.2 (Huelsenbeck and Ronquist 2001); (ii) analysis of Maximum Likelihood (ML) carried out in RAxML v7.2.8 (Stamatakis 2006); and (iii) analysis employing the Fitch-Margoliash method with LogDet distances (LD) performed with PAUP* v4.0a144 (Swofford 2002). In BA, four simultaneous Markov-Monte Carlo chains (temperature 0.2) were run for 2,000,000 generations and sampled every 2000 generations under the covarion GTR + I + G substitution model (alignment I) or GTR + G (alignment II). The first 500,000 generations were discarded as the burnin. The –lnL were plotted against the generation to check that burnin was sufficient. ML analyses were run using the GTR GAMMA I model (alignment I) or GTR GAMMA model (alignment II) and rapid bootstrapping with search for the best-scoring ML tree algorithm. In LD analyses, heuristic searches were conducted. Alignment III was executed in PAUP* using ML analysis and the K81uf + I substitution model. The number of bootstrap replicates was 1000 in all cases. Phylogenetic

Table 2. Sequences of primers used in the study.

Primer	Sequence (5′–3′)	Reference
460F	CAGCAGGCGCGCAAATTA	Dyková et al. (2008)
1200F	GATCAGATACCGTCGTAGTC	Dyková et al. (2008)
1350R	CCGTCAATTCCTTTAAGTTTC	Dyková et al. (2008)
NuF	GAACCTGCGTAGGGATCATTT	Pélandakis et al. (2000)
NuR	TTTCTTTTCCTCCCCTTATTA	Pélandakis et al. (2000)
1700R	GGCATCACAGACCTGTTAT	Dyková et al. (2008)
Naeg1	TAGCCACTGGAAAGTTTACAAGG	This study
Naeg2	CCTTCGGGGTGGTAGTAGTAT	This study
Naeg3	GGGTTATCTACACCCAAATCATG	This study
Naeg4	GCCTCACGGATGAACCGT	This study

trees were visualised in iTOL (Letunic and Bork 2007). In order to generate pairwise distances between 11 heterogeneous *Allovahlkampfia* sequences, three other alignments were produced: (a) 5.8S rDNA sequences without any further trimming or modification, (b) 18S rDNA sequences trimmed at their 5′ end (according to the F11 sequence) and (c) manually modified alignment b (deleting introns of the NL64 and PV66 strains in the 459–1840, 1887–1894, 1929–1936, and 1955–1961 positions). MAFFT with the G-INS-I algorithm was used to determine the final alignments a, b, and c of 163, 3335, and 1930 bps lengths, respectively.

Results

Isolation and cultivation

The 18 amoeba strains of Arctic and Antarctic origin that are presented in this study were isolated from a total of 179 samples of wet soil and freshwater with sediments. Seven strains were isolated from three localities on the Svalbard archipelago, 1 from Greenland and 10 from Vega and James Ross Island in the Antarctic. Sampling localities, their coordinates and habitat details are summarized in Table 1. Since many primary isolates and initial subcultures were lost due to overload with bacteria and contaminating fungi (subculturing in liquid media with antibiotics and antimycotic solutions did not solve this problem), the agar plate method of subculturing was slightly modified: selected populations of trophozoites from the parental cultures were suspended in Page's saline solution and 4–5 drops of this suspension were spread on the surface of each new agar plate. This procedure substantially improved the growth of the cultures, shortened subculturing intervals and improved the harvest of cells.

Morphology

Trophozoites of all strains had a monopodial "limax" appearance. They exhibited the typical "vahlkampfiid" locomotion pattern, i.e., distinct eruptive motion. The mean lengths and breadths with standard deviation values of the locomotive forms are summarized in Table 3. The strains F11 (Fig. 1A), F21V (Fig. 1B) and F21M exceeded 30 μm in mean length (Table 3). The mean lengths of the other strains ranged between 18.7 ± 2.6 μm (AB11 strain, Fig. 1D) and 27.7 ± 4.4 μm (TT19 strain, Fig. 1F). The actively moving trophozoites of F25B strain, however, were predominantly irregularly flabellate or even branched in outline (Fig. 1C). All strains formed cysts. Strain F11 produced relatively small cysts (11.5 ± 1.3 μm in diameter, Fig. 3A, B), considering its large trophozoites (Table 3), whereas F21V (17.4 ± 1.3 μm, Fig. 1B) and F21M (18.5 ± 2.6 μm) strains produced the largest cysts (Fig. 3C) of all the strains. Small cysts, between 9.1 ± 0.9 μm and 12 ± 0.9 μm, were also produced by other strains (Fig. 3D–J). Mature cysts of F11 strain possess a thick wall closely attached to endocyst (Fig. 3L). Spontaneous transformation into flagellated stages with two equal flagella was observed in the strains F25B, F32 and TT13 (Table 3).

Molecular diversity among isolated strains and their phylogenetic position

The essential sequence information about the strains under study is given in Table 3. Blast analysis of the sequences confirmed the affiliation of all strains to Heterolobosea and divided them into three groups: one with a unique representative (F11), the second with two representatives (F21M, F21V) and the third, most numerous one containing 15 strains (AB02, AB03N, AB05, AB07, AB11, BA03, EA4AN, GRZ3, F13, F20, F25B, F26, F32, TT13, and TT19). The blast search suggested that these three groups of strains could be representatives of three heterolobosean genera.

Strain F11: complete ITS1-5.8S-ITS2 region and nearly complete 18S rDNA (1,999 bps) sequences were obtained. The most similar sequences revealed by the blast search in the GenBank database (megablast algorithm) belonged to *Allovahlkampfia*. The highest blast score for the entire ITS region sequence was observed with the KF547911 sequence accessing the *Allovahlkampfia* sp. Tib50 strain isolated from Mila West Slope, Tibet. The 18S rDNA sequence shared 96% identity (and the highest blast score) with sequence EU696948 accessing the *Allovahlkampfia spelaea* SK1 strain isolated from a stromatolitic stalagmite in the Pečina v Borštu karst cave, Slovenia. The pairwise distances between 5.8S rDNA and 18S rDNA (obtained from the trimmed/manually modified alignments) sequences are presented in Table 4. This comparison also showed that both SK1 (*A. spelaea*) and Tib50 strains were the most similar to F11 strain (5.8S rDNA: 99.4% bps identity between SK1–F11; 18S rDNA: 95.7% bps identities between SK1–F11 and Tib50–F11). Among the *Allovahlkampfia* 18S rDNA sequences, F11 reached the lowest values of similarity with the Nl64 and PV66 strains (as well as 18S sequences of other *Allovahlkampfia* strains). Both of these strains possess an intron and therefore sequence dissimilarities decreased significantly after removal of these parts (Table 4). The presence of the 5′-TACACTT-3′ motif in helix 8 of the 18S rDNA was confirmed in the strain F11.

Strains F21V and F21M: they were identical (or nearly identical as F21M strain differed only in the 232nd position of the sequence) with sequence AJ698855 accessing the *Vahlkampfia signyensis* 1105 strain which originated from Changing Col, Signy Island, the Antarctic.

The remaining 15 strains: the closest blast hits to members of the third group assigned them to *Naegleria.* No twintron was determined in their 18S rDNA. Most strains (all except EA4AN) had the highest blast score with 'polar' *Naegleria* spp., i.e., *N. arctica* (AM157659), *N. antarctica* (AJ566628), *N. neoantarctica* (AM157662), *N. polaris* (AM157657), and *N. neopolaris* (AM157658). The pairwise distances between

Table 3. Morphometric data of strains and details of sequences obtained.

Strain	Trophozoites[a]				Cysts[a]	Flagellates[d]	Length (bps)			Acc. No.[d]	
	Length (μm)	Breadth (μm)	L/B ratio[b]	H/L ratio[c]	Diameter (μm)		ITS1	5.8S	ITS2	18S rDNA	ITS region
AB02	26.6 ± 3.6	8.7 ± 1.2	3.0 ± 0.5	0.19 ± 0.03	10.7 ± 0.5*	not observed	36	175	297	ND	LC106132
AB02e	23.0 ± 3.3	9.2 ± 1.4	2.6 ± 0.7	0.21 ± 0.04	NA	not observed	NA	NA	NA	ND	NA
AB03N	24.1 ± 2.6	8.8 ± 1.6	2.8 ± 0.6	0.2 ± 0.03	NM	not observed	36	175	297	ND	LC106133
AB05	27.0 ± 3.8	9.3 ± 1.4	3.0 ± 0.6	0.16 ± 0.03	12 ± 0.9*	not observed	36	175	493	ND	LC106134
AB05[e]	24.6 ± 3.0	8.6 ± 1.2	2.9 ± 0.5	0.46 ± 0.07	NA	not observed	NA	NA	NA	ND	NA
AB07	21.0 ± 3.3	8.1 ± 1.4	2.7 ± 0.6	0.18 ± 0.04	9.1 ± 0.9	not observed	36	175	297	ND	LC106135
AB11	18.7 ± 2.6	7.5 ± 1.8	2.6 ± 0.7	0.23 ± 0.04	9.7 ± 0.7	not observed	36	175	499	ND	LC106136
BA03	21.0 ± 2.0	9.2 ± 1.4	2.3 ± 0.4	0.2 ± 0.04	NM	not observed	37	175	297	ND	LC106137
EA4AN	25.1 ± 3.3	8.0 ± 1.5	3.2 ± 0.6	0.2 ± 0.05	NM	not observed	33	175	192	ND	LC106138
GRZ3	22.0 ± 3.7	8.7 ± 1.5	2.6 ± 0.7	0.17 ± 0.05	9.5 ± 0.8	not observed	36	175	297	LC106128	LC106146
F11	35.8 ± 7.6	11.4 ± 2.3	3.2 ± 0.7	0.16 ± 0.08	11.5 ± 1.3	not observed	110	161	105	LC106122	LC106131
F13	25.2 ± 3.2	9.0 ± 1.4	2.9 ± 0.5	0.18 ± 0.05	10.4 ± 0.7	not observed	36	175	297	LC106123	LC106139
F20	25.9 ± 5.3	9.9 ± 2.3	2.8 ± 0.9	0.19 ± 0.04	10.8 ± 1	not observed	36	175	297	LC106124	LC106140
F21M	30.0 ± 5.5*	17.7 ± 3.2*	1.8 ± 0.5	0.2 ± 0.07	18.5 ± 2.6*	not observed	26	155	120	ND	LC106141
F21V	38.6 ± 6.3	12.5 ± 2.2	3.2 ± 0.9	0.15 ± 0.05	17.4 ± 1.3*	not observed	26	155	120	ND	LC106142
F25B	24.2 ± 4.9	11.9 ± 3.6	2.2 ± 0.7	0.19 ± 0.09	10.1 ± 0.9	present	36	175	465	LC106125	LC106143
F26	19.0 ± 3.2	8.8 ± 2.3	2.3 ± 0.5	0.19 ± 0.05	9.7 ± 0.5	not observed	36	175	297	LC106126	LC106144
F32	23.1 ± 3.6	7.7 ± 1.1	3.1 ± 0.6	0.22 ± 0.06	10.5 ± 0.7	present	36	175	297	LC106127	LC106145
TT13	19.5 ± 2.7	7.8 ± 1.1	2.6 ± 0.5	0.19 ± 0.05	10.1 ± 1.1*	present	36	175	297	LC106129	LC106147
TT19	27.7 ± 4.4	8.1 ± 1.3	3.5 ± 0.8	0.14 ± 0.04	11.9 ± 1.2	not observed	36	175	297	LC106130	LC106148

[a] Measurements are based on n = 30 specimens, asterisk labels < 30 specimens; NA = not applicable, NM = not measured.
[b] Length/Breadth ratio.
[c] Hyaloplasm length/Whole cell length.
[d] NA = not applicable; ND = not done.
[e] Measured at 20 °C.

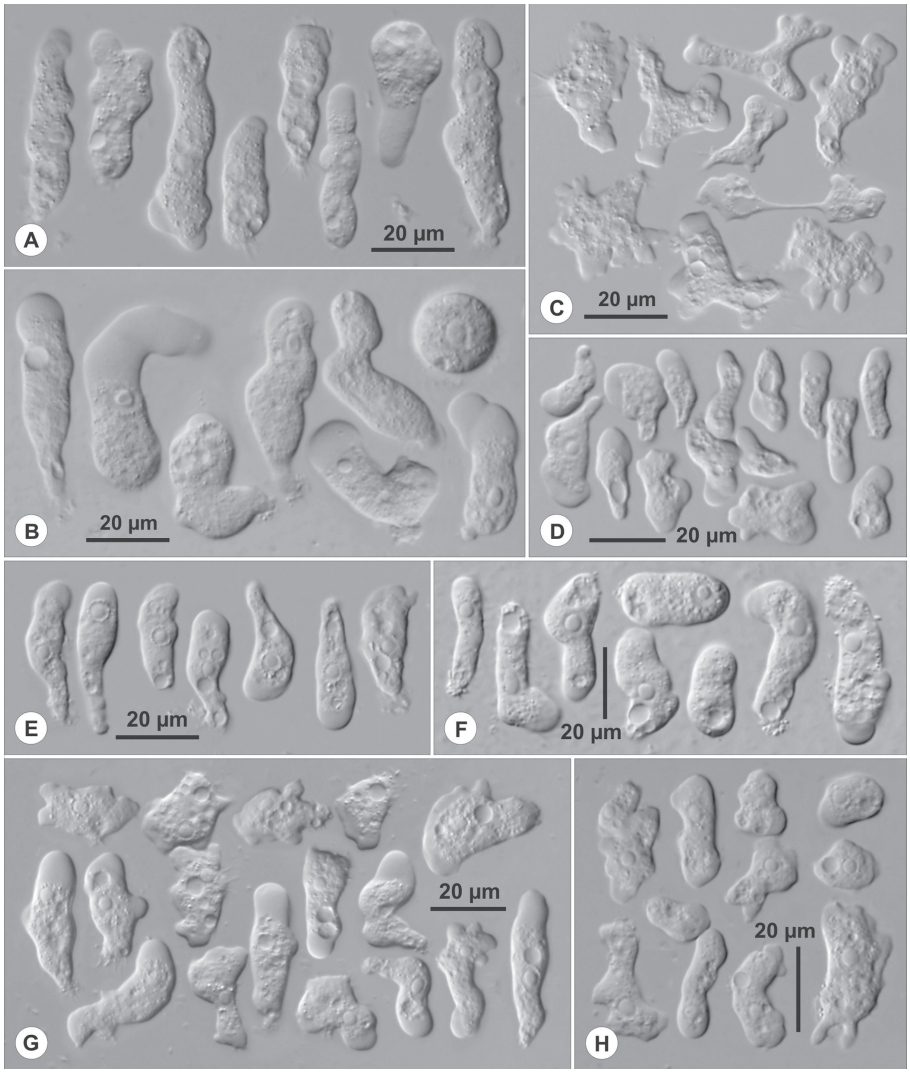

Fig. 1. Heterolobosean strains studied, as seen in hanging-drop preparations. Nomarski differential interference contrast. **A.** *Allovahlkampfia* sp. F11. **B.** *Vahlkampfia signyensis* F21V. **C.** *Naegleria* sp. F25B. **D.** *Naegleria* sp. AB11. **E.** *Naegleria* sp. TT13. **F.** *Naegleria* sp. TT19. **G.** *Naegleria* sp. GRZ3. **H.** *Naegleria* sp. AB07.

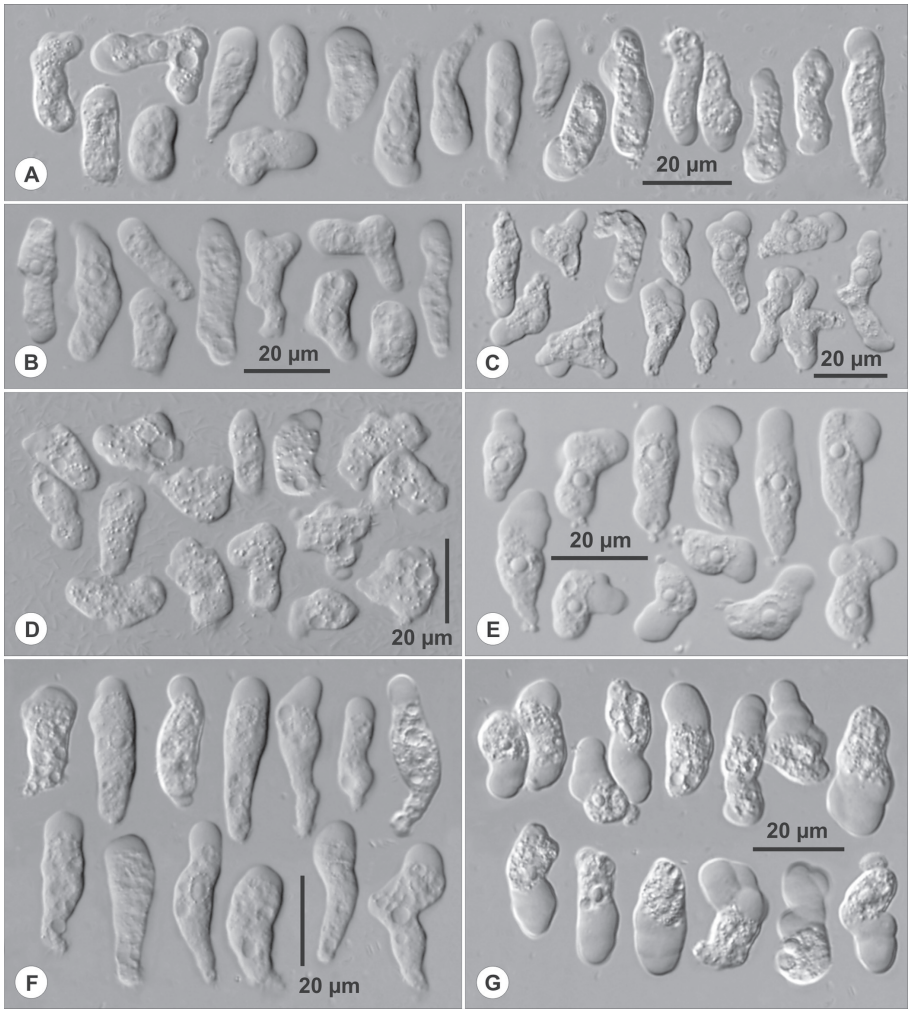

Fig. 2. Trophozoites of *Naegleria* strains, as seen in hanging-drop preparations. Nomarski differential interference contrast. **A.** *Naegleria* sp. AB03N. **B.** *Naegleria* sp. AB02. **C.** *Naegleria* sp. EA4AN. **D.** *Naegleria* sp. BA03. **E.** *Naegleria* sp. F13. **F.** *Naegleria* sp. AB05 incubated at 10 °C. **G.** *Naegleria* sp. AB05 as observed after a 2 h incubation at room temperature.

the ITS1-5.8S-ITS2 region sequences of these strains are shown in Table 5. The only exception was EA4AN strain with 100% bps identity to the *Naegleria* sp. SUM3 V/I strain isolated from a skin lesion on a wels catfish (*Silurus glanis*). The 'polar' cluster of the novel *Naegleria* strains divides into four groups when focusing on the set of the entire ITS sequences: the first and second groups are identical with previously described species, the third group differs in a single base,

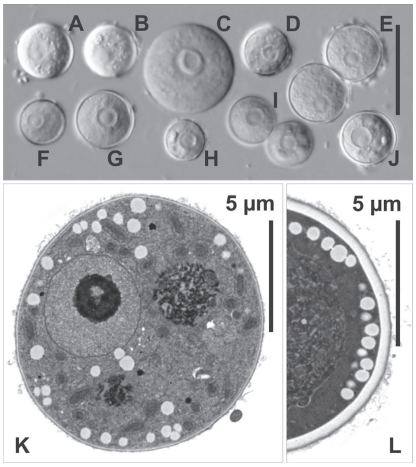

Fig. 3. Cysts of strains included in the study as seen in Nomarski DIC (A–J) and in transmission electron microscope (K, L). **A., B.** *Allovahlkampfia* sp. F11. **C.** *Vahlkampfia signyensis* F21V. **D., G.** *Naegleria* sp. AB07. **E.** *Naegleria* sp. GRZ3. **F.** *Naegleria* sp. AB11. **H.** *Naegleria* sp. TT13. **I.** *Naegleria* sp. AB02. **J.** *Naegleria* sp. TT19. Scale bar for A-J: 20 µm. **K.** Cyst of *Allovahlkampfia* sp. F11 in early stage of formation. **L.** Part of mature *Allovahlkampfia* cyst F11with thick wall closely attached to endocyst.

whereas the strains of the fourth group have a higher degree of sequence dissimilarity both to the described species and among themselves. The first group (AB07, GRZ3, TT13 and TT19 strains) shares the same sequence with *N. neopolaris* (AM157658); the same applies to the strains of the second group (AB02, AB03 N, F20, F26 and F32) and *N. polaris* (AM157657). A single difference separated the sequences of *N. polaris* (AM157657) and the BA03 and F13 strains. For details on the fourth group (F25B, AB11 and AB05) see Table 5.

Phylogenetic analyses based on the 18S rDNA sequences placed the F11 strain among other *Allovahlkampfia*; however, this lineage was weakly supported. In addition, the relationships within this group are also poorly resolved (Fig. 4). The maximum likelihood tree based on the entire ITS region of the *Allovahlkampfia* sequences yielded the same result with respect to the position of F11 strain (Fig. 5). There are, however, two well-supported groups of strains (4165 + PS1073 J, Tib191 + PV66 + Sar37) that did not cluster in the previous analysis (Fig. 4). In the 18S rDNA analysis, two strains (SO/1P) possess the same name, but they are of different origin (the sequence number is used to distinguish them).

The position of the *Naegleria* strains (i.e., F13, F20, F25B, F26, F32, GRZ3, TT13 and TT19) was clearly supported (1.00 Bayesian probability and 100% ML bootstrap) in the 18S rDNA analysis. In a far more detailed analysis focused on the entire ITS *Naegleria* sequences, in which it was possible to analyse our sequences together with representatives of other recognized *Naegleria* clusters, almost all our strains branched within 'polar' cluster 7 (Fig. 6). Only EA4AN strain branched within 'dobsoni' cluster 8, which contains a thermophilic strain (*N. dobsoni* NG257), potentially amphizoic strains (J14Z1, RR11Z/I, SUM3 V/I), and strains of polar (*N. neodobsoni* GR11) or subpolar origin (*N. paradobsoni* IP7). *Naegleria spitzbergeniensis* SP8, another strain of polar origin, tends to be basal to the 'dobsoni' cluster in the majority of analyses (e.g., Fig. 6) although with less support (0.72 Bayesian probability and 38% ML bootstrap).

Using a combination of morphological and molecular characters, and results of phylogenetic analyses, the F11 strain was assigned to *Allovahlkampfia*, the two F21M and F21V strains were identified with *Vahlkampfia signyensis*, and the other 15 novel strains were determined as *Naegleria*. Within the latter group, most of the strains belong to 'polar' cluster 7 and only the EA4AN strain is a member of 'dobsoni' cluster 8. Furthermore, the *Naegleria* strains AB02, AB03N, BA03, F13, F20, F26 and F32 were assigned to *N. polaris* and strains AB07, GRZ3, TT13 and TT19 were assigned to *N. neopolaris*. The strains EA4AN, AB05, AB11, and F25B could not be identified with any *Naegleria* species.

Temperature tolerance

Subculturing problems prevented us from making an exhaustive series of experiments to examine the tolerance of individual strains to temperatures above 10 °C. A limited series of simple experiments consisted in transferring cultures of all isolated strains (except TT13 and TT19) from 10 °C to 20 °C and examining the state of cells at RT 24 hours after the transfer. In 14 of these transferred cultures, the trophozoites encysted and lost the ability of excystment in the next passage (whether at 20 or 10 °C). In contrast to these strains, AB02 and AB05 trophozoites tolerated incubation at 20 °C followed by a 2 h stay at RT and retained viability in the next passage at 10 °C. Whereas the AB02 trophozoites at 20 °C did not exhibit any significant change in morphology and locomotion compared to trophozoites incubated at 10 °C, the AB05 trophozoites developed a more pronounced anterior hyaloplasmic part and intensified their eruptive movement at 20 °C (Fig. 2F, G; see also the H/L ratio in Table 3).

Discussion

The dimensions of the trophozoites and cysts of the isolated strains correspond with those known in the respective three diagnosed genera. *Allovahlkampfia* is known to vary strongly in size; our F11 strain is most similar to *A. spelaea* (trophozoites ~20–40 µm, cysts 16–25 µm; Walochnik and Mulec 2009). There was a noteworthy difference in the dimensions

Table 4. Pairwise distances between *Allovahlkampfia* strain sequences. Percentage of identical bases between 5.8S rDNA sequences (area above the diagonal) and within trimmed/manually modified alignment of 18S rDNA sequences (area below the diagonal).

	F11	*A. spelaea*	Tib32	Tib191	Sar9	4165	PS1073J	Tib50	Sar37	Nl64	PV66
F11 LC106131 LC106122		99.4%	98.8%	95.1%	98.8%	98.8%	98.1%	97.5%	93.9%	98.1%	91.4%
A. spelaea EU696949 EU696948	95.7%		99.4%	95.7%	99.4%	99.4%	98.8%	98.1%	94.5%	98.8%	91.4%
Tib32 KF547910 KF547917	94.5%	94.4%		95.1%	100%	100%	99.4%	97.5%	93.9%	99.4%	90.1%
Tib191 KF547912 KF547919	94.5%	96.1%	94.1%		95.1%	95.1%	94.5%	93.9%	98.8%	94.8%	91.4%
Sar9 KF547907 KF547914	95.3%	95.2%	98.2%	94.7%		100%	99.4%	97.5%	93.9%	99.4%	90.1%
4165 JQ271643 JQ271668	95.2%	95.2%	98.2%	94.6%	99.9%		99.4%	97.5%	93.9%	99.4%	90.1%
PS1073J JQ271644 JQ271669	94.5%	94.2%	99.2%	94.0%	98.2%	98.3%		98.1%	93.3%	100%	89.5%
Tib50 KF547911 KF547918	95.7%	96.4%	95.3%	95.3%	95.7%	95.7%	95.0%		92.6%	98.1%	90.1%
Sar37 KF547908 KF547915	95.5%	95.9%	94.2%	98.3%	94.6%	94.6%	94.0%	95.5%		93.3%	92.6%
Nl64 KF547909 KF547916	53.3% (91.5%)	53.1% (91.2%)	56.0% (96.3%)	53.0% (91.0%)	55.2% (94.8%)	55.1% (94.8%)	55.6% (95.6%)	53.7% (92.3%)	53.0% (91.1%)		89.5%
PV66 JQ271645 JQ271670	53.7% (91.8%)	53.7% (91.8%)	53.2% (91.0%)	54.3% (92.8%)	53.4% (91.4%)	53.4% (91.4%)	53.1% (90.8%)	53.7% (91.8%)	54.5% (93.1%)	80.2% (89.8%)	

Table 5. Pairwise distances between ITS1-5.8S-ITS2 region sequences of the 'polar' and 'dobsoni' *Naegleria* clusters. Area above the diagonal = number of non-identical bases, area below the diagonal = percentage of identical bases.

Strain or species/ Sequence No.	AJ566628	AM157662	AB05	AB11	F25B	AM157659	AM157658	AB07	GRZ3	TT13	TT19	AM157657	AB02	AB03N	F20	F26	F32	BA03	F13	AJ566627	AM157661	AM157664	EA4AN	AM157660
N. antarctica AJ566628		6	60	81	48	40	250	250	250	290	250	252	252	252	252	252	252	253	253	345	345	345	339	373
N. neoantarctica AM157662	99.1%		64	79	46	42	252	252	252	252	252	252	252	252	252	252	252	253	2523	345	345	345	339	373
AB05 strain LC106134	91.5%	90.9%		28	62	96	306	306	306	306	306	308	308	308	308	308	308	309	309	402	402	402	396	429
AB11 strain LC106136	88.6%	88.9%	96.1%		36	93	317	317	317	317	317	317	317	317	317	317	317	318	318	405	405	405	400	436
F25B strain LC106143	92.9%	93.2%	91.3%	94.9%		60	284	284	284	284	284	284	284	284	284	284	284	285	285	372	372	372	367	402
N. arctica AM157659	94.0%	93.7%	86.6%	87.0%	91.2%		273	273	273	273	273	273	273	273	273	273	273	274	274	355	355	355	350	387
N. neopolaris AM157658	63.4%	63.2%	58.5%	57.3%	59.9%	60.8%		0	0	0	0	8	8	8	8	8	8	9	9	195	197	194	194	234
AB07 strain LC106135	63.4%	63.2%	58.5%	57.3%	59.9%	60.8%	100.0%		0	0	0	8	8	8	8	8	8	9	9	195	197	194	194	234
GRZ3 strain LC106146	63.4%	63.2%	58.5%	57.3%	59.9%	60.8%	100.0%	100.0%		0	0	8	8	8	8	8	8	9	9	195	197	194	194	234
TT13 strain LC106147	63.4%	63.2%	58.5%	57.3%	59.9%	60.8%	100.0%	100.0%	100.0%		0	8	8	8	8	8	8	9	9	195	197	194	194	234
TT19 strain LC106148	63.4%	63.2%	58.5%	57.3%	59.9%	60.8%	100.0%	100.0%	100.0%	100.0%		8	8	8	8	8	8	9	9	195	197	194	194	234
N. polaris AM157657	63.1%	63.2%	58.2%	57.3%	59.9%	60.8%	98.4%	98.4%	98.4%	98.4%	98.4%		0	0	0	0	0	1	1	194	196	193	193	232
AB02 strain LC106132	63.1%	63.2%	58.2%	57.3%	59.9%	60.8%	98.4%	98.4%	98.4%	98.4%	98.4%	100.0%		0	0	0	0	1	1	194	196	193	193	232
AB03N strain LC106133	63.1%	63.2%	58.2%	57.3%	59.9%	60.8%	98.4%	98.4%	98.4%	98.4%	98.4%	100.0%	100.0%		0	0	0	1	1	194	196	193	193	232
F20 strain LC106140	63.1%	63.2%	58.2%	57.3%	59.9%	60.8%	98.4%	98.4%	98.4%	98.4%	98.4%	100.0%	100.0%	100.0%		0	0	1	1	194	196	193	193	232
F26 strain LC106144	63.1%	63.2%	58.2%	57.3%	59.9%	60.8%	98.4%	98.4%	98.4%	98.4%	98.4%	100.0%	100.0%	100.0%	100.0%		0	1	1	194	196	193	193	232
F32 strain LC106145	63.1%	63.2%	58.2%	57.3%	59.9%	60.8%	98.4%	98.4%	98.4%	98.4%	98.4%	100.0%	100.0%	100.0%	100.0%	100.0%		1	1	194	196	193	193	232
BA03 strain LC106137	63.0%	63.1%	58.1%	57.2%	59.8%	60.7%	98.2%	98.2%	98.2%	98.2%	98.2%	99.8%	99.8%	99.8%	99.8%	99.8%	99.8%		1	195	197	194	194	233
F13 strain LC106139	61.8%	61.8%	56.8%	55.9%	58.5%	59.5%	98.2%	98.2%	98.2%	98.2%	98.2%	99.8%	99.8%	99.8%	99.8%	99.8%	99.8%	99.8%		188	190	187	187	226
N. dobsoni AJ566627	47.2%	47.3%	43.2%	43.2%	45.2%	46.8%	61.8%	61.8%	61.8%	61.8%	61.8%	62.0%	62.0%	62.0%	62.0%	62.0%	62.0%	61.8%	61.6%		7	13	47	135
N. neodobsoni AM157661	47.2%	47.2%	43.1%	43.1%	45.1%	46.7%	61.3%	61.3%	61.3%	61.3%	61.3%	61.5%	61.5%	61.5%	61.5%	61.5%	61.5%	61.4%	61.1%	98.3%		17	48	136
N. paradobsoni AM157664	47.2%	47.3%	43.2%	43.2%	45.2%	46.8%	62.0%	62.0%	62.0%	62.0%	62.0%	62.2%	62.2%	62.2%	62.2%	62.2%	62.2%	62.0%	61.8%	96.9%	95.9%		45	135
EA4AN strain LC106138	46.0%	48.1%	43.9%	43.7%	45.8%	47.4%	61.8%	61.8%	61.8%	61.8%	61.8%	62.0%	62.0%	62.0%	62.0%	62.0%	62.0%	61.9%	61.6%	88.7%	88.4%	89.1%		118
N. spitzbergensis AM157660	42.7%	42.8%	39.1%	38.7%	40.6%	41.9%	54.0%	54.0%	54.0%	54.0%	54.0%	54.4%	54.4%	54.4%	54.4%	54.4%	54.4%	54.3%	53.7%	67.6%	57.3%	67.5%	70.7%	

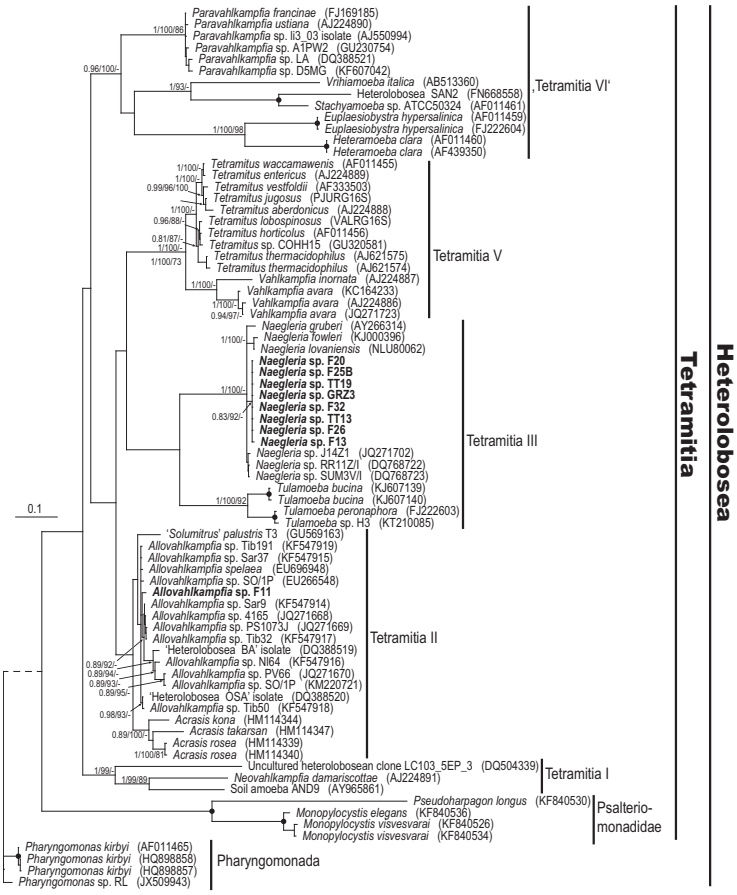

Fig. 4. Bayesian phylogenetic tree of a representative set of Tetramitia based on 18S rDNA sequences. The novel sequences are in bold. Branch support values for three different reconstruction methods are shown (BA posterior probabilities/ML bootstraps/LD bootstraps). Only values higher than 0.8 or 80 are shown and black dots indicate 1/100/100 support values. *Pharyngomonas* spp. served as the outgroup.

between the two novel strains of *Vahlkampfia signyensis* (F21M and F21V); for this we have no other explanation than, perhaps, different culture conditions or limited number of measured cells. Similar to findings of De Jonckheere (2004, 2006), we could not distinguish our new *Naegleria* strains based solely on morphological features; notwithstanding, we consider it appropriate to present standard documentation of actively moving trophozoites as these data have so far been missing for all nominal species of the 'polar' and 'dobsoni' clusters.

An amendment to the diagnosis of the genus *Allovahlkampfia*, as proposed by Geisen et al. (2015a), defines the highest possible genetic distance between strains (>95% similarity of SSU and 5.8S rDNA sequences). However, this arbitrary genetic yardstick does not apply even to some strains studied by Geisen et al. (2015a); similarly, some strains added into the dataset by us also exceeded the 95% threshold (Table 4). The strains Nl64 and PV66 possess the highest degree of genetic dissimilarity both to other *Allovahlkampfia* as well as between themselves. This is caused by the

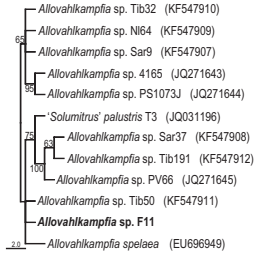

Fig. 5. Phylogenetic relationships among *Allovahlkampfia* strains obtained from entire ITS region sequences using Maximum Likelihood analysis and K81uf + I substitution model. Bootstrap supports are given at the nodes.

presence of a long group I intron in both strains. The pairwise distances presented in Geisen et al. (2015a) are based on a manually modified alignment. We do not consider this appropriate for genus definition because, in principle, manual modification cannot be unbiased. In consequence, we propose to omit the genetic yardstick from the diagnosis of *Allovahlkampfia*. The amended diagnosis basically follows Geisen et al. (2015a) and should thus read as follows. Molecular patterns: with diverse molecular sequence patterns such as motif 5′-TACACTT-3′ in helix 8 only shared by members of this clade. Closest phylogenetic relationship based on the SSU rDNA with the soil amoeba AND12 (∼10% bp difference) and the genus *Acrasis* (>15% bp difference). All species show sequence differences in the V4 region of the SSU rDNA.

Our results confirm the high sequence heterogeneity of *Allovahlkampfia* as previously stressed by Geisen et al. (2015a). In fact, the present addition of the new strain sequences makes the relationships within the *Allovahlkampfia* clade even more complicated. The phylogenetic inference at the species level in *Allovahlkampfia* can be improved by further DNA sampling (from cultures and environment) or introduction of a new molecular marker. Our phylogenetic analysis of 18S rDNA sequences placed '*Solumitrus*' *palustris* inside the *Allovahlkampfia* lineage and thus corresponds well with the results of Brown et al. (2012), Harding et al. (2013), and Geisen et al. (2015a). '*Solumitrus*' was established by Anderson et al. (2011) as a close relative to *Allovahlkampfia spelaea*. However, Brown et al. (2012) and Geisen et al. (2015a) suggested that '*Solumitrus*' *palustris* could be eventually transferred into *Allovahlkampfia* if a re-sequencing would be done and confirm the present phylogenetic placement.

The 'polar' and 'dobsoni' *Naegleria* clusters reach high values of support compared with other clusters established by De Jonckheere (2004, 2006). Whereas the 'dobsoni' cluster contains also four strains from temperate regions, of which at least one (*N. dobsoni* NG257) multiplies at 34 °C (Robinson et al. 1992), the 19 'polar' cluster strains not only are prevalent and widespread in the hostile habitats of the Arctic and Antarctic, but their known distribution is limited exclusively to these regions (the DDBJ/NCBI/EMBL databases contain no environmental sequence disrupting their polar geographic definition) (see Fig. 6). A distribution limited to the Arctic or the Antarctic has been reported for numerous species and higher taxa of 'polar' microorganisms (Gutt et al. 2010). Among these, some are regarded as 'bipolar', i.e., limited in their distribution to both polar regions. Often, however, the purportedly bipolar taxa may not be identical (or not closely enough related) and their geographic distribution may not be reliably known due to insufficient taxon sampling. In fact, the relationships between the 'polar' taxa members can be polyphyletic since 'non-polar' data disrupt monophyly. The strains of the 'polar' *Naegleria* cluster, or at least *Naegleria polaris* and *N. neopolaris*, however, appear to be true polar organisms, given the ample availability of sequence data. There is only one other bipolar taxon of free-living amoebae, the marine genus *Vermistella* (Tyml et al. 2016), which belongs to Amoebozoa. In both cases, i.e. 'polar' *Naegleria* cluster and *Vermistella*, the bipolar distribution can be caused by dispersion. Whereas *Vermistella* spp. do not form cysts (Moran et al. 2007) and therefore have a limited dispersal potential, *Naegleria* spp. are cyst-forming and their ability to disperse via air has been proven (Kingston and Warhurst 1969). Although De Jonckheere's (2006) and our results strongly suggest that the distribution of these heteroloboseans is bipolar, a conclusive proof, however, cannot be given. *Naegleria* spp. appear to be studied intensively in respect to their distribution. Indeed, most studies have been focused on pathogenic *N. fowleri* or at least on thermophilic *Naegleria* spp. In our view, thorough and non-biased targeted sampling should be conducted between both polar regions to clarify status of the putative bipolar *Naegleria* spp.

It is interesting, but not necessarily significant, that EA4AN strain was isolated from a freshwater lake on the Islands of Gåsøyane, Svalbard. These islands occupy a small area where Isfjorden splits into Billefjorden and Sassenfjorden. The freshwater lakes on the islands are therefore enclosed by seawater. The area is a bird reserve that harbours a large colony of the pink-footed goose (*Anser brachyrhynchus*) among other bird species. The Svalbard population of pink-footed geese overwinters in Belgium, the Netherlands, and Denmark and migrates to Svalbard via the Norwegian mainland (Madsen and Fox 1997). It is conceivable that pink-footed geese are carriers of *Naegleria* cysts and introduce those from either of their overwintering or migration localities each year. We mention this situation, which is special both in terms of geography and habitat, in order to illustrate that precise location (ideally including coordinates and habitat description) may be an important part of species records even if they are microbes. Unfortunately, some reports on amoebae from Arctic and Sub-Antarctic regions (De Jonckheere 2006) lack such details.

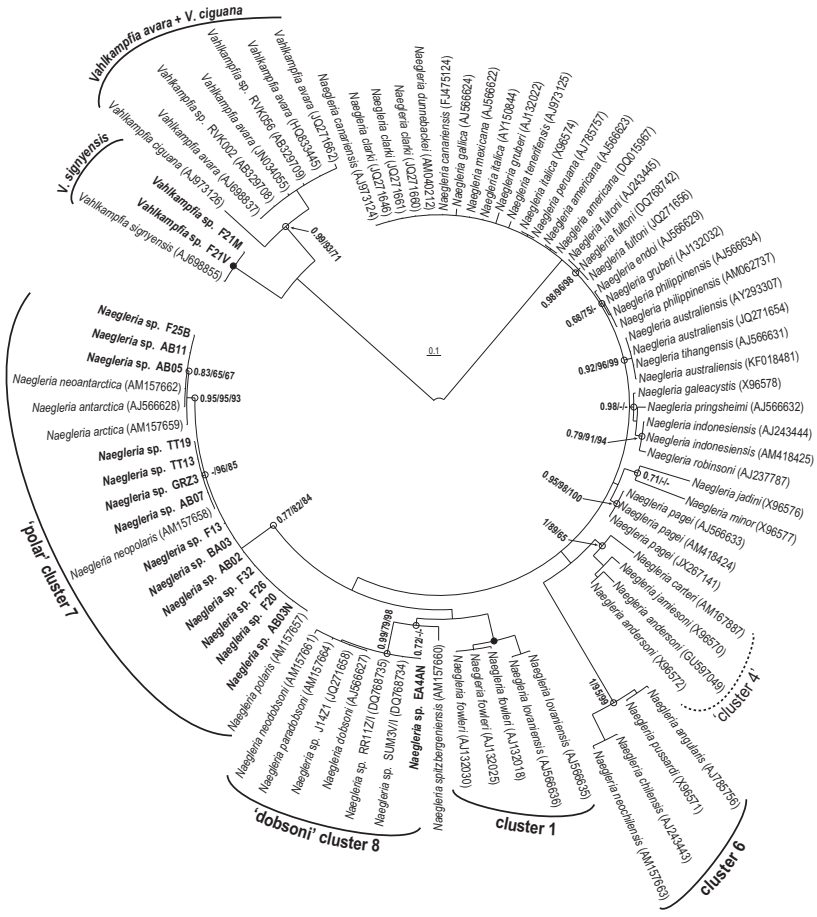

Fig. 6. Bayesian phylogenetic tree of *Naegleria* strains based on ITS1-5.8S-ITS2 sequences. The novel sequences are in bold. Branch support values for three different reconstruction methods are shown at the outline circles (BA posterior probabilities/ML bootstraps/LD bootstraps). Only values higher than 0.8 or 80 are shown, black dots indicate 1/100/100 support values. *Vahlkampfia* spp. served as the outgroup.

In comparison to the number of samples used for isolations, the number of isolated strains is somewhat low, which may give the impression that heterolobosean amoebae are rare in polar regions. We are convinced that the opposite is true, as evidenced by the previous findings of heterolobosean in various parts of the polar and subpolar regions (e.g., De Jonckheere 2006; Dixon 1939; Smith 1978). Indeed, the proportion of our primary isolates was pretty high (>80%); unfortunately, the majority of our primary isolates could not be successfully subcultured to strains due to a heavy bacterial overload and negative response of the amoebae to antibiotics. Apparently, the natural balance of amoebae and bacteria in the samples had been impaired due to delayed laboratory processing, and neither of the

standard culturing methods (agar plate or liquid media) sufficiently imitated the complex natural conditions in which the amoebae could thrive and outgrow the bacterial load.

Polar heterolobosean amoebae thrive in habitats that are typically at subzero temperatures for a substantial part of the year. They must have many adaptations to be able to survive freeze-thaw cycles and take advantage of the very short summer season. For example, vahlkampfiids have been isolated from permafrost in Siberia (Shatilovich et al. 2009) and from a glacier in Greenland (Marquardt et al. 1966), places in which they had been lying dormant for at least several centuries. It is also feasible that some polar localities or regions are colonized due to episodic or repeated dispersion (Frenot et al. 2005). Such colonizations may well be possible in our EA4AN strain (see above); in the *Naegleria* 'polar' cluster, as well as in *Vahlkampfia signyensis*, however, this is not suspected (at least, not to a significant extent). Understanding of heterolobosean amoebae diversity in the polar regions is still fragmentary, similarly as in other regions. Along with culture-dependent methods, a substantial improvement of such knowledge can be expected especially from culture independent large scale sequencing of environmental DNA samples and comparison of data obtained from similar (i.e., polar and alpine) habitats. Nevertheless, these methods have limitations of their own. Geisen et al. (2015b) provided experimental evidence that high-throughput sequencing introduces significant biases and stressed that data obtained need careful interpretation. The presence of introns as well as the strongly diverging and generally long 18S rDNA qualify *Allovahlkampfia* among underestimated or even undetectable taxa in PCR-based high-throughput methods (Geisen et al. 2015a, 2015b). *Naegleria* was found in a metatranscriptomic study, although heteroloboseans comprised only about 1% of all protist transcripts (Geisen et al. 2015c). However, limited resolution provided by the 18S rDNA restricts an accurate classification of *Naegleria* spp. Taken together, targeted approaches using group/genus specific primers in the high-throughput methods may be a promising way how to decipher the heterolobosean diversity.

Acknowledgements

This work was supported by funding from the Ministry of Education, Youth and Sports (LM2015078), European Social Fund Project (CZ.1.07/2.2.00/28.0190), Czech Science Foundation (505/12/G112), and PICTO-2010-0096 Project. We are very grateful to the Czech Research Arctic Infrastructure (University of South Bohemia), J. G. Mendel Antarctic station (Masaryk University), and Argentine Antarctic Institute which facilitated this work in many ways. We also extend our thanks to Otakar Strunecký for providing samples collected during his stay at the Zackenberg Research Station, Greenland. We are grateful to Keith Edwards who corrected the English text.

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Section VII

Marine amoebae with cytoplasmic and perinuclear symbionts

SCIENTIFIC REPORTS

OPEN

Marine amoebae with cytoplasmic and perinuclear symbionts deeply branching in the *Gammaproteobacteria*

Received: 16 May 2015
Accepted: 24 July 2015
Published: 25 August 2015

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Amoebae play an important ecological role as predators in microbial communities. They also serve as niche for bacterial replication, harbor endosymbiotic bacteria and have contributed to the evolution of major human pathogens. Despite their high diversity, marine amoebae and their association with bacteria are poorly understood. Here we describe the isolation and characterization of two novel marine amoebae together with their bacterial endosymbionts, tentatively named '*Candidatus* Occultobacter vannellae' and '*Candidatus* Nucleophilum amoebae'. While one amoeba strain is related to *Vannella*, a genus common in marine habitats, the other represents a novel lineage in the Amoebozoa. The endosymbionts showed only low similarity to known bacteria (85–88% 16S rRNA sequence similarity) but together with other uncultured marine bacteria form a sister clade to the *Coxiellaceae*. Using fluorescence *in situ* hybridization and transmission electron microscopy, identity and intracellular location of both symbionts were confirmed; one was replicating in host-derived vacuoles, whereas the other was located in the perinuclear space of its amoeba host. This study sheds for the first time light on a so far neglected group of protists and their bacterial symbionts. The newly isolated strains represent easily maintainable model systems and pave the way for further studies on marine associations between amoebae and bacterial symbionts.

Unicellular eukaryotes, in particular free-living amoebae, are major players in the environment. Free-living amoebae are ubiquitous in soil, fresh- and seawater, but can also be found in anthropogenic environments, such as cooling towers, water pipes and waste-water treatment plants[1,2]. Taxonomically, free-living amoebae are scattered across the eukaryotic tree of life, with the supergroup Amoebozoa containing a substantial part of known free-living amoebae, such as naked lobose amoebae (gymnamoebae)[3,4]. In total, there are more than 200 described species of gymnamoebae classified into over 50 genera[5]. A substantial proportion of this diversity is found in marine environments, and some genera represent exclusively marine lineages. Yet our current knowledge of marine amoebae is still scarce.

Free-living amoebae shape microbial communities; they control environmental food webs by preying on bacteria, algae, fungi and other protists and contribute to elemental cycles in diverse ecosystems[1]. Free-living amoebae typically take up their food by phagocytosis. However, some bacteria have developed strategies to survive digestive processes and eventually use amoebae as niche for intracellular

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replication[6–9]. Free-living amoebae are thus considered to have served as evolutionary training ground for intracellular microbes. Bacteria such as *Legionella pneumophila*, *Francisella tularensis*, or *Mycobacterium* species transiently exploit these protists as a vehicle to reach out for higher eukaryotic hosts. Others engage in long-term, stable associations with free-living amoebae[7,8], which can be beneficial, neutral or parasitic for their hosts. These obligate intracellular symbionts include a diverse assemblage of phylogenetically different bacterial groups[10–12] and their analysis has provided unique insights into the evolution of the intracellular life style[13,14]. However, virtually nothing is known about bacterial symbionts in marine amoebae.

Here we report on the isolation and characterization of two novel marine amoeba strains harboring obligate intracellular bacterial symbionts. Both bacteria represent deeply branching novel lineages in the *Gammaproteobacteria* affiliated with the *Coxiellaceae*. While one of the symbionts replicates in the amoeba cytoplasm, the other exploits a highly unusual intracellular niche, its host's perinuclear space.

Methods

Amoeba isolation and cultivation. Lago di Paola is a meso-eutrophic lake located on the Tyrrhenian coast of Central Italy (Latium). Two narrow artificial channels at the northwestern and southeastern ends of the lake allow for a limited water exchange with the sea, sustaining a high degree of salinity throughout the year (33.7 during sampling). A surface water sample was collected on October 1, 2013, from station SAB2[15]. The number of protist-sized particles per milliliter lake water was determined with a Neubauer counting chamber. Between one and ten protist-sized particles were placed in wells on a 96-well plate (Corning Costar, Sigma-Aldrich, Germany) containing 200 µl artificial seawater (ASW, DSMZ 607) and *E. coli tolC*- as well as ampicillin (200 ng/ml)[11]. The amoeba strain A1, which was propagating on these plates was screened for the presence of bacterial endosymbionts with fluorescence *in situ* hybridization (FISH), and maintained in cell culture flasks (Nunclon delta-surface, Thermo Scientifc, Germany) containing ASW and *E. coli tolC*- as well as ampicillin (200 ng/ml).

Grains of wet sand collected on a sea shore (Montego Bay, Jamaica) were placed onto a MY75S agar plate and moistened daily with ASW (75%) as described previously[16]. Two weeks later, a morphologically uniform group of cells was transferred onto a new MY75S plate, and the newly established amoeba strain JAMX8 was sub-cultured either on plates or in liquid medium containing ASW and *E. coli tolC*- as well as ampicillin (200 ng/ml).

Trophozoites of both strains were observed in hanging drop preparations and documented using an Olympus BX51 microscope equipped with a Nomarski differential interference contrast (DIC) and an Olympus DP70 camera (Olympus Optical Co. Ltd, Japan). Trophozoites and cellular structures were analyzed using ImageJ software[17].

Transmission electron microscopy. For transmission electron microscopy (TEM), ASW in culture flasks containing strain A1 was replaced with 3% glutaraldehyde in 0.1 M Na-cacodylate buffer. Trophozoites of strain JAMX8 were fixed *in situ* on MY75S plates with the same fixative. Pelleted trophozoites were rinsed in 0.1 M Na-cacodylate buffer, post-fixed in 1% osmium tetroxide, dehydrated in graded acetone series, and embedded in Spurr's resin. Ultrathin sections were stained with 2% uranyl acetate in 50% methanol and Reynold's lead citrate and examined using a JEOL JEM 1010 electron microscope (Jeol Ltd, Japan) operating at 80 kV.

Fluorescence *in situ* hybridization. Amoeba cells were harvested by centrifugation (3000 × g, 8 min), washed with ASW and left to adhere on slides for 30 min prior to fixation with 4% formaldehyde (15 min at room temperature). The samples were hybridized for two hours at 46 °C at a formamide concentration of 25% using standard hybridization and washing buffers[18] and a combination of the following probes: symbiont specific probes JAMX8_197 (5′-GAAAGGCCAAAACCCCCC-3′) or A1_1033 (5′-GCACCTGTCTCTGCATGT-3′), together with EUK-516 (5′-ACCAGACTTGCCCTCC-3′ [19]) targeting most eukaryotes and the EUB338 I-III probe mix (5′-GCTGCCTCCCGTAGGAGT-3′, 5′-GCAGCCACCCGTAGGTGT-3′, 5′-GCTGCCACCCGTAGGTGT-3′ [20]) targeting most bacteria. FISH probes were designed based on a multiple 16S rRNA sequence alignment in the software ARB[21] using the integrated probe-design tool. Furthermore, thermodynamic parameters and binding specificity were evaluated with the web-based tools mathFISH and probeCheck[22,23]. All probes were purchased from ThermoFisher Scientific (Germany). Cells were subsequently stained with DAPI (0.5 µg/ml in double distilled water, 3 min), washed once and embedded in Citifluor (Agar-Scientific, UK). Slides were examined using a confocal laser scanning microscope (SP8, Leica, Germany).

DNA extraction, PCR, cloning and sequencing. DNA was extracted from infected amoeba cultures using the DNeasy Blood and Tissue Kit (Qiagen, Austria). Amoebal 18S rRNA genes were amplified by PCR using primers 18e (5′-CTGGTTGATCCTGCCAGT-3′) and RibB (5′-TGATCCTTCTGCAGGTTCACCTA-3′) at an annealing temperature of 52 °C[24,25]. Bacterial 16S rRNA genes were amplified using primers 616 V (5′-AGAGTTTGATYMTGGCTCAG-3′) and 1492R (5′-GGYTACCTTGTTACGACTT-3′) at an annealing temperature of 52 °C[26,27]. PCR reactions typically contained 100 ng template DNA, 50 pmol of each primer, 1 unit of Taq DNA polymerase (TopBio, Czech Republic for 18S rDNA; Fermentas, Germany for 16S rDNA), 10x Taq buffer with KCl and 2 µM MgCl2 and 0.2 µM of each deoxynucleotide

in a total volume of 50 μl. PCR products were purified using the PCR Purification Kit (Qiagen, Germany) and cloned using the TOPO TA Cloning Kit (Invitrogen, Germany) following the manufacturer's instructions. Nucleotide sequences were determined at Microsynth (Vienna, Austria) and Macrogen Europe (Amsterdam, Netherlands). Newly obtained rRNA gene sequences were deposited at Genbank/EMBL/DDBJ (accession numbers LC025958, LC025959, LC025974, LC025975).

Phylogenetic analysis. To infer the phylogenetic position of the isolated amoeba strains in the Amoebozoa a representative dataset of 18S rRNA sequences from a total of 57 taxa was compiled. The length of the final trimmed alignment was 1226 nt; alternative alignments obtained by altering taxon sampling and/or trimming stringency were also analyzed to check the stability of deeper nodes. A more detailed analysis of the position of strain A1 in the Vannellidae was performed, comprising in total 19 taxa, both nominal species and unnamed sequences assigned to morphologically characterized strains. The alignment was processed as described above and trimmed to a final length of 1440 nt. Both datasets were analyzed using with RAxML 8.0.20[28], with the GTR gamma model of evolution and rapid bootstrapping (1000 replicates). Bayesian interference analysis was computed for both datasets in MrBayes 3.1.2[29] with default options, GTR gamma model and 10^6 generations; burnin 25%.

For phylogenetic analysis of bacterial 16S rRNA sequences the sequence editor integrated in the software ARB was used to build alignments based on the current Silva ARB 16S rRNA database[21,30], which was updated with sequences from GenBank/EMBL/DDBJ obtained by sequence homology searches using BLASTn available at the NCBI web site (National Centre for Biotechnology Information[31,32]). The alignment was trimmed to the length of the shortest sequence, manually curated and exported from ARB using a 50% conservation filter. The resulting alignment comprised 61 sequences and 1417 positions. For Bayesian analysis, PhyloBayes[33] was used with two independent chains under GTR and the CAT + GTR model. Both analyses ran until convergence was reached (maxdiff < 0.1) and as burnin 25% of the sampled trees were removed. Posterior predictive tests were performed in PhyloBayes with the ppred program (sampling size 1000 trees).

Results

Two novel stenohaline amoebae containing bacterial symbionts. Two novel strains of marine amoebae, initially referred to as A1 and JAMX8, were isolated from samples taken from a coastal lake in Italy and a sea shore in Jamaica, respectively. Both amoeba strains were successfully cultivated only in artificial seawater. They were truly stenohaline, shown by their incapability to grow under varying salt concentrations. In hanging drop preparations, strain A1 exhibited a flattened, oval to fan-shaped locomotive form (Fig. 1A) with an average length of 17.5 μm (S.D. 2.5, n = 50), width of 15.7 μm (S.D. 2.4, n = 50) and a length/width ratio of 0.8–1.6 (in average 1.1). The anterior hyaloplasm typically occupied about half the cell length. At the ultrastructure level, the cytoplasm contained a nucleus with a peripheral nucleolus or nucleoli, oval mitochondria and food vacuoles (Fig. 1Bi). The cell surface was covered with fine, hair-like filamented glycostyles (length of 71 ± 9 nm) (Fig. 1Bii). The mitochondria possessed branching tubular cristae (Fig. 1Biii). The partial 18S rRNA gene sequence (1889 nt) of strain A1 was most similar to *Vannella plurinucleolus* and other *Vannella* species (98% sequence similarity). In our phylogenetic analyses the placement of strain A1 in the family Vannellidae was highly supported (Figs 2 and S1) and further confirmed by a more detailed analysis focusing on the Vannellidae only, in which *V. plurinucleus* appeared as closest relative (Fig. 2 inset, S1).

The second isolate, strain JAMX8, showed flattened trophozoites with variable shape with an average length of 19.9 μm (S.D. 3.9, n = 26), width of 16.3 μm (S.D. 3.5, n = 26) and length/width ratio 0.88–1.84 (in average 1.25) (Fig. 3A). A frontal irregular hyaline zone occupied about one third of the cell length and was clearly separated from the granuloplasm containing a large quantity of spherical granules. The hyaloplasm possessed typically one to three longitudinal ridges. The locomotive cells often produced short dactylopodia (usually not more than 5 μm in length) that could freely move horizontally or vertically. No cysts were observed during subculturing. Floating forms consisted of a spherical central body with an average size of 4.8 μm in diameter (S.D. 0.7, n = 20) and thin radiating pseudopodia not longer than 10 μm (6.3 μm in average). A single, vesicular nucleus was located near the border of the granuloplasm (Fig. 3A,Bi,v). The cell surface was covered with a thin and amorphous cell coating (Fig. 3Bii). The cytoplasm contained numerous phagosomes (Fig. 3Bi), rounded or ovoid mitochondria (Fig. 3Bi,iii) with tubular cristae (Fig. 3Biii) and a Golgi complex organized as dictyosome (Fig. 3Biv). Comparison of the partial 18S rRNA sequence (2081 nt) of strain JAMX8 with known sequences revealed the absence of highly similar sequences in the NCBI nr/nt database. Taxa with moderate sequence similarity (< 88%) were scattered among various amoebozoan lineages. In our phylogenetic analyses the JAMX8 strain represented a deeply branching novel lineage in the Amoebozoa with no clear affiliation to described taxa (Figs 2, S1).

Electron microscopy and staining with the DNA dye DAPI readily revealed the presence of bacterial endosymbionts in both amoeba strains (Figs 1 and 3).

Bacterial endosymbionts in the amoeba cytoplasm and perinuclear space. In addition to ingested bacteria in food vacuoles (Fig. 1Bi), amoeba strain A1 harbored morphologically different rod-shaped bacteria with a diameter of about 0.44 μm and a maximum length of 1.2 μm (Fig. 1Bi,iv–vi).

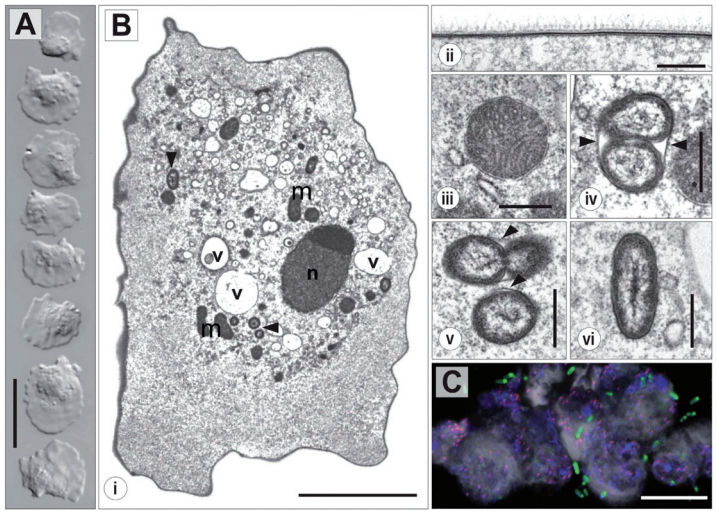

Figure 1. ***Vannella*** **sp. A1 and its bacterial endosymbiont '*Candidatus* Occultobacter vannellae'.** (**A**) Trophozoites as seen in hanging drop preparations (scale bar = 20 μm). (**B**) Fine structure of *Vannella* sp. A1 and its bacterial symbiont. (i) Section of an amoeba trophozoite: cell organelles located within granuloplasm; nucleus (n) with laterally located nucleolus, mitochondria (m), vacuoles (v), bacterial endosymbionts (arrowheads) (scale bar = 5 μm). (ii) Cell surface of trophozoite with amorphous glycocalyx (scale bar = 200 nm). (iii) Mitochondria with tubular cristae (scale bar = 500 nm). (iv–vi) Bacterial endosymbionts in detail: (iv, v) host-derived vacuolar membranes (arrowheads) enclosing endosymbionts undergoing cell division, (vi) longitudinal section through an endosymbiont (scale bar = 500 nm). (**C**) Fluorescence *in situ* hybridization image showing the intracytoplasmic location of '*Candidatus* Occultobacter vannellae' (*Occultobacter*-specific probe A1_1033, pink) in its *Vannella* sp. A1 host (probe EUK516, grey) with DAPI stained nuclei (blue) and food bacteria (general bacterial probe EUB338-mix, green); scale bar indicates 10 μm.

These bacteria were predominantly located enclosed in vacuoles (arrowheads in Fig. 1Biv,v), in which dividing cells were observed (Fig. 1Bv). The bacterial endosymbionts appeared to be few in numbers and scattered throughout the cytoplasm. However, nearly 100% of all amoeba trophozoites were infected.

Ultrastructural analysis of amoeba strain JAMX8 revealed bacterial symbionts at a conspicuous location within the cells (Fig. 3Bi,v,vi); rod-shaped bacteria of about 0.41 μm in diameter and a maximum length of 1.7 μm were found enclosed in the perinuclear space, between the inner and the outer nuclear membrane. Bacteria were never observed within the nucleoplasm. Nearly 100% of amoeba cells were infected. Both amoeba strains showed no apparent signs of symbiont-induced stress or lysis; the symbiotic associations could be stably maintained non-axenically in the lab.

Novel gammaproteobacteria related to the *Coxiellaceae*. Sequencing of the 16S rRNA gene revealed that the endosymbiont of amoeba strain A1 showed highest 16S rRNA sequence similarity to *Legionella longbeachae* (85%) in the NCBI RefSeq database, which contains only sequence information of well described organisms[34]. The bacterial symbiont was tentatively named "*Candidatus* Occultobacter vannellae A1" (referring to the hidden location of these bacteria inside their *Vannella* sp. host and their small cell size; hereafter: *Occultobacter*). The endosymbiont of amoeba strain JAMX8 was most similar to *Coxiella burnettii* (88% 16S rRNA sequence similarity), and is provisionally referred to as "*Candidatus* Nucleophilum amoebae JAMX8" (referring to the association of these bacteria with the amoeba nucleus; hereafter: *Nucleophilum*). The rRNA sequences of both endosymbionts had a similarity of 85% with each other.

For phylogenetic analysis we first calculated trees with the CAT + GTR and the GTR models in PhyloBayes[33]. We then used posterior predictive tests to compare the fit of the models to the data (observed diversity: 2.866), indicating that CAT + GTR (posterior predictive diversity: 2.859 +/− 0.027,

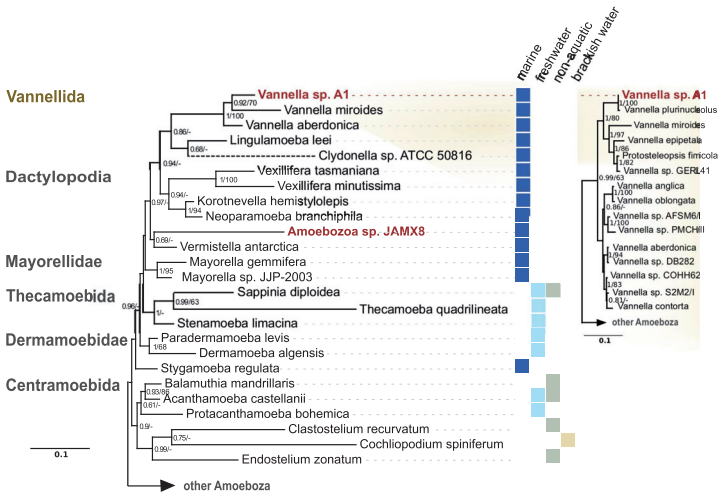

Figure 2. Phylogenetic relationships of *Vannella* sp. A1 and amoeba isolate JAMX8 within the Amoebozoa. Phylogenetic 18S rRNA-based trees of the Amoebozoa (left panel) and Vannellidae (right panel) constructed using the Bayesian inference method. Bayesian posterior probabilities (>0.6) and RaxML bootstrap support values (>60%) are indicated at the nodes; the dashed line indicates a branch shortened by 50% to enhance clarity. Colored squares indicate the typical habitat of the respective amoeba species (left panel). A detailed version of the trees including accession numbers is available as supplementary Figure S1.

p-value: 0.57) was superior to the GTR (posterior predictive diversity: 3.138 +/− 0.027, p-value: 0). We therefore used the CAT + GTR model to assess the phylogenetic position of the two endosymbionts, demonstrating that both represent deeply branching lineages in the *Gammaproteobacteria* (Figs 4, S2). In our analysis they grouped with several marine and freshwater clones, together forming a sister clade to the *Coxiellaceae* (Bayesian posterior probability = 0.78). *Occultobacter* and *Nucleophilum* were also moderately related to a clade comprising the two unclassified amoeba-associated bacteria CC99 and HT99 (Bayesian posterior probability = 0.98)[35]. We searched published 16S rRNA amplicon and metagenomic sequence datasets using an approach described recently[63], but did not find significant numbers of similar sequences to *Occultobacter* or *Nucleophilum* at a 97% similarity threshold.

In order to demonstrate the intracellular location of the bacterial symbionts FISH experiments were performed by combining symbiont-specific probes with a universal bacterial probe mix[20]. The positive hybridization reaction with both probes respectively confirmed the location of *Occultobacter* in the cytoplasm of its *Vannella* sp. A1 host (Fig. 1C) and the association of *Nucleophilum* with the nucleus of its JAMX8 amoeba host (Fig. 3C).

Discussion

Here we report on the recovery of two novel stenohaline amoeba from marine samples. Based on light microscopy, amoeba strain A1 was readily identified as a member of the ubiquitous family Vannellidae whose members are also frequently found in marine environments[36]. Nuclear structure (laterally located nucleolus/nucleoli) and trophozoite size allow an assignment of this strain to *Vannella plurinucleolus* (Fig. 1). However, the shape of its cell surface is in conflict with the diagnostic features of *V. plurinucleolus*. Yet, in our phylogenetic analyses strain A1 clustered together with *V. plurinucleolus* strain 50745 (Fig. 2). As the taxonomy of *V. plurinucleolus* strain 50745 is under debate[37], we decided to leave strain A1 undetermined at the species level. Morphological and molecular characterization of additional *Vannella* strains will be required to resolve species identification.

By light microscopy, trophozoites of strain JAMX8 showed a combination of morphological features typical of the genera *Mayorella* and *Korotnevella*[38]. However, neither essential diagnostic features, like a surface cuticle or surface microscales, nor any other distinct characteristics were found (Fig. 3). We were thus not able to assign strain JAMX8 to any described gymnamoeba species or genus based on morphological criteria. Furthermore, the placement of JAMX8 within the Amoebozoa could not be

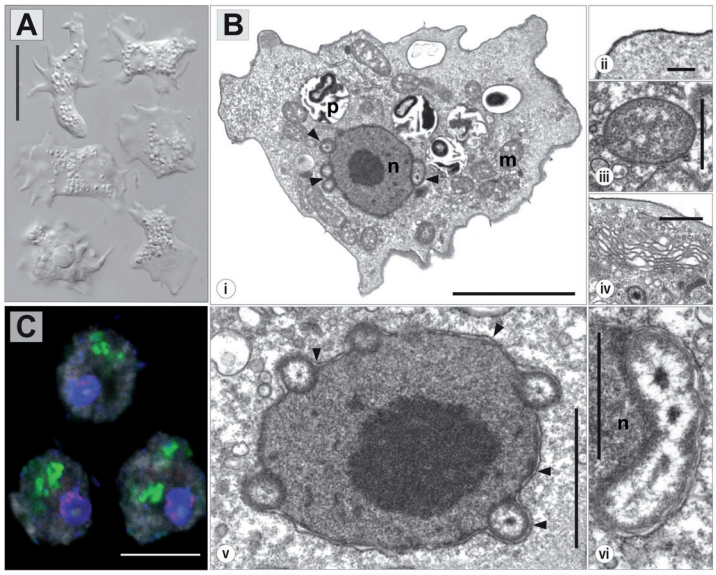

Figure 3. Amoeba isolate JAMX8 and its bacterial endosymbiont '*Candidatus* Nucleophilum amoebae'. (**A**) Trophozoites as seen in hanging drop preparations (scale bar = 20 μm). (**B**) Fine structure of JAMX8 and its bacterial endosymbiont inhabiting the perinuclear space. (i) Section of an amoeba trophozoite: vesicular nucleus (n), phagosomes (p), mitochondria (m), bacterial endosymbionts associated with the nuclear envelope (arrowheads) (scale bar = 5 μm). (ii) Amorphous and tenuous cell coat (scale bar = 200 nm). (iii) Mitochondria with tubular cristae (scale bar = 1 μm). (iv) The Golgi complex organized as dictyosome (scale bar = 1 μm). (v,vi) Bacterial endosymbionts located within the perinuclear space, between inner and outer nuclear membrane. (v) Nucleus in detail with numerous endosymbiotic bacteria in transverse section (scale bar = 2 μm). Arrowheads indicate outer nuclear membrane. (vi) Longitudinal section through a rod-shaped bacterial endosymbiont, nucleus (n) (scale bar = 1 μm). (**C**) Fluorescence *in situ* hybridization image showing the co-localization of '*Candidatus* Nucleophilum amoebae' (*Nucleophilum*-specific probe JAMX8_197, pink) with its host nucleus (DAPI, blue); food bacteria (general bacterial probe EUB338-mix, green) enclosed in the amoeba cytoplasm (probe EUK516, grey); scale bar indicates 10 μm.

unambiguously determined in our phylogenetic analyses (Fig. 2). The deeper nodes in our Amoebozoa tree are generally rather unstable, which is consistent with previous studies[39,40], and the tree topology was dependent on taxon sampling and alignment trimming stringency. However, strain JAMX8 groups with low statistical support with *Vermistella antarctica* (Bayesian posterior probability 0.69, maximum likelihood bootstrap value 16%), and this sister taxa relationship was recovered repeatedly in different analyses. Taken together, the exact relationship of strain JAMX8 to known amoebae remains elusive. However, morphological and phylogenetic data suggest that JAMX8 is a representative of a new taxon within the Amoebozoa.

To our knowledge this is the first molecular identification and characterization of bacterial symbionts of marine amoebae. In the last two decades numerous reports described the discovery of obligate intracellular amoeba symbionts[8,10]. However, these studies were often biased towards the isolation of *Acanthamoeba*, *Naegleria* or *Vermamoeba* (former *Hartmannella*) strains, usually from anthropogenic or freshwater habitats or clinical samples[7,41,42]. This is not surprising as these amoebae are in the focus of medical and parasitological research, and standard isolation protocols are available[2,16,43,44]. Interestingly, bacterial symbionts previously found in these amoebae were frequently very similar to each other; although isolated from geographically distant places, the endosymbionts belonged to symbiont clades either in the *Alpha-* or *Betaproteobacteria*, the *Bacteroidetes*, or the *Chlamydiae*[10,11,45–48]. In addition, *Gammaproteobacteria*, such as *Coxiella, Francisella, Legionella*, and *Legionella*-like amoebal pathogens, may also be associated with amoebae[7,48–50], but these are mostly facultative associations. These bacteria

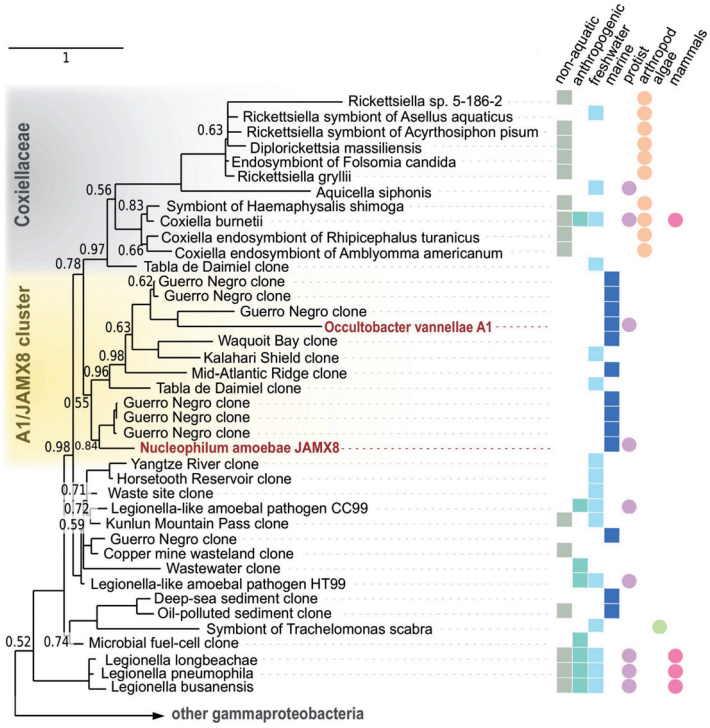

Figure 4. Phylogenetic relationship of '*Candidatus* Occultobacter vannellae' and '*Candidatus* Nucleophilum amoebae' with the *Gammaproteobacteria*. The phylogenetic tree (PhyloBayes, CAT + GTR) is based on the 16S rRNA sequences, Bayesian posterior probabilities are indicated at the nodes (only values < 0.99 are shown). Colored squares indicate the environmental origin of the respective sequence; colored circles indicate host association. A detailed version of this tree including accession numbers is available as supplementary Figure S2.

show a parasitic life style, and many also infect higher eukaryotes[7]. Our study for the first time reports on *Gammaproteobacteria* naturally living in a stable association with their amoeba hosts, i.e. host and symbiont can together be maintained in culture over extended periods of time without apparent signs of host cell lysis and 100% of amoebae being infected.

The two gammaproteobacterial symbionts *Occultobacter* and *Nucleophilum* represent novel phylogenetically deeply branching lineages, with only low 16S rRNA sequence similarity to known bacteria (85% and 88%, respectively; Fig. 4). In addition, no close relatives (>97% 16S rRNA sequence similarity) could be retrieved in any of the numerous 16S rRNA amplicon studies targeting biodiversity of marine environments, suggesting that both endosymbionts are rather rare and/or their hosts have not been captured during sampling. Our phylogenetic analysis showed that the exact position of the two endosymbionts in the *Gammaproteobacteria* is difficult to resolve. *Occultobacter* and *Nucleophilum* group together in a well-supported monophyletic clade with the *Coxiellaceae* and a cluster comprising the *legionella*-like amoebal pathogens HT99 and CC99 (Fig. 4). The topology within this clade is, however, not very robust. This is in agreement with previous observations, highlighting the challenge of resolving the phylogeny of the major *Gammaproteobacteria* groups[51].

The closest relatives of *Occultobacter* and *Nucleophilum* are other symbionts and pathogens of eukaryotes. The *Coxiellaceae* mainly include bacteria infecting arthropods, which occasionally also invade mammalian or protozoan hosts[49,52,53]. The bacteria referred to as HT99 and CC99 were associated with

amoebae found in a hot tub and a cooling tower, respectively[35]. Worth noting, while the *Coxiellaceae*, CC99, HT99 and related taxa mainly originate from freshwater, anthropogenic and non-marine habitats, many of the closest relatives of *Occultobacter* and *Nucleophilum* were detected in marine environments (Fig. 4).

The two symbionts described here colonize fundamentally different intracellular niches. Whereas, similar to many known intracellular bacteria, *Occultobacter* establishes replication inside host-derived vacuoles and is also occasionally found as single cell inside the cytoplasm, *Nucleophilum* is associated with its host cell's nucleus (Figs 1 and 3). The latter is a very unusual life style[54,55], but there are few reports on bacteria located in the nuclear compartment of other amoebae, namely the chlamydial symbiont of *Naegleria* 'Pn'[56,57], the two gammaproteobacteria HT99 and CC99[35], and the alphaproteobacterium *Nucleicultrix amoebiphila*[58]. Bacteria capable to invade the nucleus possibly benefit from a nutrient-rich environment, protection from cytoplasmic defense mechanisms and a direct path to vertical transmission during host cell replication[55]. However, the pattern of how bacteria settle in this compartment shows striking differences; while *Nucleicultrix* is spread out in the nucleoplasm, Pn is associated with the nucleolus, and *Nucleophilum* is located in the perinuclear space[56–58]. Embedded in between the inner and outer nuclear membrane, the bacteria thus do not have direct access to the nucleoplasm. The perinuclear space, which is continuous with the endoplasmic reticulum, serves as a calcium storage[59] and has regulatory impact on processes in the nucleus, such as gene expression[60]. The exact physicochemical conditions of this compartment remains currently unknown[59], however, in contrast to the nucleoplasm or the cytoplasm it likely contains less substrates to support bacterial growth. We therefore expect *Nucleophilum* to have evolved unconventional strategies to target its peculiar perinuclear niche and to satisfy its nutritional requirements. Previously, it has been shown and hypothesized that some intranuclear bacteria confer beneficial effects to their hosts, such as an increased survival under adverse environmental conditions or protection against co-infection by cytoplasmic bacteria[55,61,62]. However, at the moment we have no indication that the symbiosis between *Nucleophilum* and its amoeba host is a mutualistic association. Genome analysis in combination with functional approaches, such as transcriptomics and proteomics, will help to gain insights into the infection process, interaction mechanisms, possible benefits for host and symbiont, and the evolution of this unique lifestyle.

This is the first report on the concomitant isolation and characterization of marine amoebae and their bacterial endosymbionts. The low degree of relationship of the symbionts to known bacteria and the discovery of a symbiont thriving in the host perinuclear space, a niche not reported previously for intracellular microbes, indicates that marine habitats represent a rich pool of hidden symbiotic associations.

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Acknowledgments

This work was supported by a DOC fellowship of the Austrian Academy of Sciences to F.S., by the Czech Science Foundation to T.T. and I.D. (grant 505/12/G112), by the Grant Agency of University of South Bohemia to T.T. (140/2013/P), by a European Research Council grant to M.H. (ERC grant 'EvoChlamy' #281633) and by a grant of the Austrian Science Fund (I1628-B22) to M.H. I.P. and S.F. contributions were supported by the National Flag program RITMARE (SP3-WP2-A2-UO5).

Author Contributions

I.P., S.F., T.T. performed the sampling; F.S., T.T., I.D., M.K. and M.H. conceived the experiments; F.S., T.T., I.P. and M.K. conducted the experiments; F.S., T.T. and M.K. analyzed the results. All authors discussed and commented the results. F.S. and T.T. prepared figures; F.S., T.T. and M.H. wrote the main manuscript text. All authors reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Schulz, F. *et al.* Marine amoebae with cytoplasmic and perinuclear symbionts deeply branching in the *Gammaproteobacteria*. *Sci. Rep.* **5**, 13381; doi: 10.1038/srep13381 (2015).

Marine amoebae with cytoplasmic and perinuclear symbionts deeply branching in the *Gammaproteobacteria*

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Supplementary Material

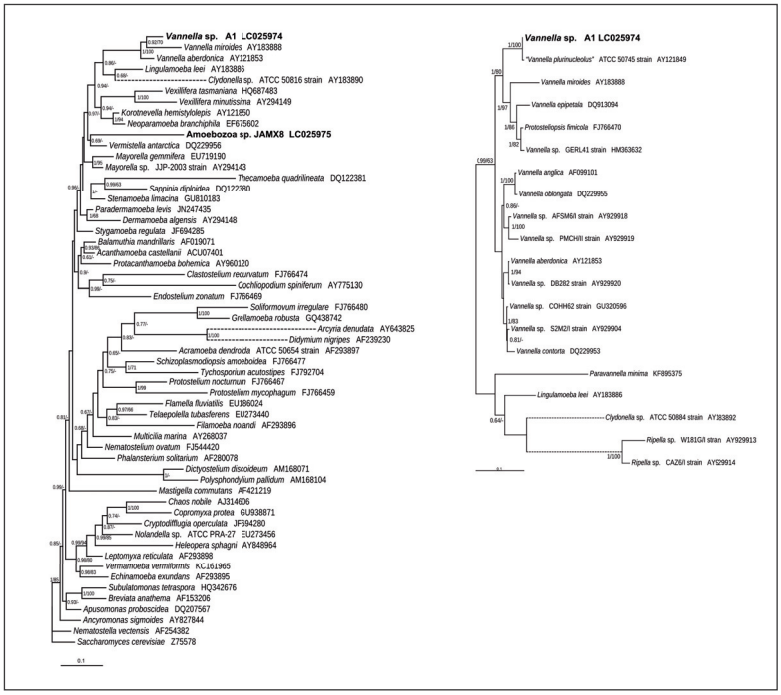

Figure S1. Phylogenetic relationships of *Vannella* sp. A1 and amoeba isolate JAMX8 within the Amoebozoa. Phylogenetic 18S rRNA-based trees of the Amoebozoa (left panel) and Vannellidae (right panel) constructed using the Bayesian inference method. Bayesian posterior probabilities 8 (> 0.6) and RaxML bootstrap support values (> 60%) are indicated at the nodes; the dashed line indicates a branch shortened by 50% to enhance clarity.

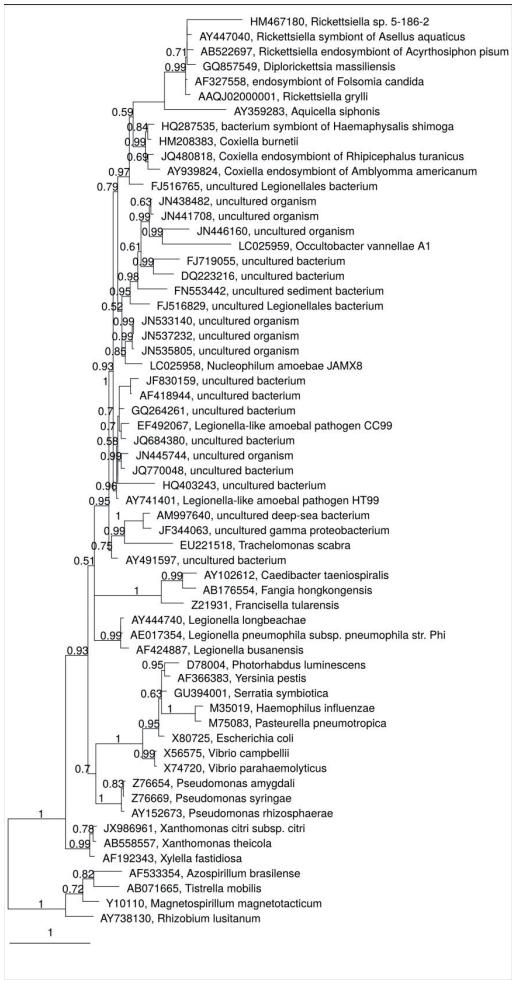

Figure S2. Phylogenetic relationship of '*Candidatus* Occultobacter vannellae' and '*Candidatus* Nucleophilum amoebae' with the *Gammaproteobacteria*. The phylogenetic tree (Phylobayes, CAT+GTR) is based on the 16S rRNA sequences, Bayesian posterior probabilities are indicated at the nodes.

Section VIII

Vexillifera **as a host of** ***Neptunochlamydia*** **and seasonality of chlamydiae**

environmental microbiology

Environmental Microbiology (2016) **18**(8), 2405–2417 doi:10.1111/1462-2920.13111

Chlamydial seasonal dynamics and isolation of '*Candidatus* Neptunochlamydia vexilliferae' from a Tyrrhenian coastal lake

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Summary

The *Chlamydiae* are a phylum of obligate intracellular bacteria comprising important human and animal pathogens, yet their occurrence in the environment, their phylogenetic diversity and their host range has been largely underestimated. We investigated the seasonality of environmental chlamydiae in a Tyrrhenian coastal lake. By catalysed reporter deposition fluorescence *in situ* hybridization, we quantified the small planktonic cells and detected a peak in the abundance of environmental chlamydiae in early autumn with up to 5.9×10^4 cells ml^{-1}. Super-resolution microscopy improved the visualization and quantification of these bacteria and enabled the detection of pleomorphic chlamydial cells in their protist host directly in an environmental sample. To isolate environmental chlamydiae together with their host, we applied a high-throughput limited dilution approach and successfully recovered a *Vexillifera* sp., strain harbouring chlamydiae (93% 16S rRNA sequence identity to *Simkania negevensis*), tentatively named '*Candidatus* Neptunochlamydia vexilliferae'. Transmission electron microscopy in combination with fluorescence *in situ* hybridization was used to prove the intracellular location of these bacteria representing the first strain of marine chlamydiae stably maintained alongside with their host in a laboratory culture. Taken together, this study contributes to a better understanding of the distribution and diversity of environmental chlamydiae in previously neglected marine environments.

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Introduction

The bacterial phylum *Chlamydiae* was discovered about a century ago. Until recently, research was focused on a single chlamydial family, the *Chlamydiaceae,* which includes the aetiological agents of important animal and human diseases such as trachoma, sexually transmitted diseases and pneumonia (WHO, 2001; Kalayoglu and Byrne, 2006; Horn, 2008; WHO, 2008). Knowledge of chlamydial diversity drastically changed in the 1990s with the discovery of the first '*Chlamydia*-like bacteria' (Kahane *et al.*, 1995; Amann *et al.*, 1997; Birtles *et al.*, 1997; Fritsche *et al.*, 2000; Horn, 2008). Today, these bacteria are referred to as environmental chlamydiae, occurring as symbionts of free-living amoebae and insects and as pathogens of crustaceans, bovines, fishes, bats and other vertebrates (Horn, 2008; Taylor-Brown *et al.*, 2015). These bacteria are the closest relatives of pathogenic *Chlamydiaceae* and so far constitute eight additional families in the phylum of *Chlamydiae*: *Parachlamydiaceae*, *Simkaniaceae* (Everett *et al.*, 1999), *Waddliaceae* (Rurangirwa *et al.*, 1999), *Rhabdoclamydiaceae* (Corsaro *et al.*, 2007), *Criblamydiaceae* (Corsaro *et al.*, 2009), *Clavochlamydiaceae* (Karlsen *et al.*, 2008), *Piscichlamydiaceae* (Draghi *et al.*, 2004) and the recently discovered *Parilichlamydiaceae* (Stride *et al.*, 2013). While the pathogenic chlamydiae are a genetically homogeneous group (Read *et al.*, 2003), the genomes of environmental chlamydiae are more diverse (Bertelli *et al.*, 2010; Collingro *et al.*, 2011; Domman *et al.*, 2014; Pilhofer *et al.*, 2014).

All members of the phylum *Chlamydiae* are characterized by a developmental cycle consisting of two morphologically and physiologically different life stages. The infectious life stage called the elementary body (EB) upon uptake by the host, differentiates into a larger, pleomorphic, metabolically active reticulate body (RB). As soon as the RB has divided several times by binary fission, it goes through a secondary differentiation process back to the infectious EB, which leaves the host by exocytosis or lysis to start a new round of infection (Subtil and Dautry-Varsat, 2004; Horn, 2008). Elementary bodies previously considered to be metabolically inert are now recognized as having some limited metabolic capacities (Haider *et al.*, 2010; Omsland *et al.*, 2012; 2014; Sixt *et al.*, 2013). Reticulate bodies are morphologically similar among all members of *Chlamydiae*, while EBs significantly vary in size and shape (Siegl and Horn, 2012; Rusconi *et al.*, 2013).

Aquatic systems have been shown to be a potential source of environmental chlamydiae (Corsaro and Venditti, 2009). They were recently found in high abundance in the water column of a Tyrrhenian coastal marine lake (Pizzetti *et al.*, 2012), and meta-analysis of amplicon sequence data indicated that a high diversity of yet unrecognized chlamydiae exists in marine environments (Lagkouvardos *et al.*, 2014b). The role of free-living amoebae as reservoir for the novel chlamydiae may be important since amoebae can successfully colonize man-made water systems such as cooling towers, humidifiers, hospital water networks or drinking water distribution systems. It deserves particular attention since there is evidence for a potential pathogenicity of some environmental chlamydiae (Corsaro and Greub, 2006), such as *Simkania negevensis*, *Waddlia chondrophila* and *Parachlamydia acanthamoebae*, present in amoebae and mammalian cells, associated with respiratory disease and/or miscarriage in humans (Dilbeck *et al.*, 1990; Kahane *et al.*, 2008; Greub, 2009; Baud and Greub, 2011). However, the impact of environmental chlamydiae on public health is currently under debate (Corsaro and Greub, 2006; Lamoth *et al.*, 2011).

Our aim was to study, for the first time, seasonal dynamics and isolate new chlamydiae together with their hosts from a coastal marine lake. Between May and November 2013, we localized and quantified *Chlamydiae* in the Tyrrhenian Lago di Paola, Italy, by catalysed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) and super-resolution microscopy. Using a high-throughput limited dilution approach, we were also able to isolate a chlamydia together with their natural host (*Vexillifera* sp.) and tentatively named it '*Candidatus* Neptunochlamydia vexilliferae'. This is the first strain of marine chlamydiae stably maintained in a laboratory culture together with its host.

Results

Seasonal variability of physico-chemical parameters

Physical parameters such as temperature, salinity and pH did not show significant differences among the three sampling sites (Table S1). Average values for temperature varied between 10.4°C in November and 30.7°C in July. The average salinity values ranged between 28.4 (registered both in May and November) and 33.7 (in September). The pH showed average values around 8 between May and November.

The concentration of dissolved organic carbon (DOC) slightly varied between the three stations (Table S1). The highest concentration of DOC was detected in September at SAB1, while at SAB2 and SAB3, the highest concentrations of DOC were observed in August (Table S1). Oxygen concentrations at SAB1 were in the range of 7.2 mg l^{-1} (June–July) to 21.2 mg l^{-1} (September) while there was less variation at the other stations with concentrations of between 9.6 mg l^{-1} (November) and 14.0 mg l^{-1} (July) at SAB2 and between 9.4 mg l^{-1} (November) and 15.0 mg l^{-1} (October) at SAB3 (Table S1). Chlorophyll *a* (chl *a*) strongly varied between May and November at SAB1 ranging from a minimum of 3.7 μg l^{-1} in November and a maximum of 267.0 μg l^{-1} in September, the latter representing the highest value registered in the lake. It fluctuated between 6.9 μg l^{-1} (November) and 71.0 μg l^{-1} (October) at SAB2, and at SAB3 it ranged between 9.4 μg l^{-1} (May) and 56.1 μg l^{-1} (September) (Table S1). Concentrations of NO_2^- and NO_3^- showed similarly increasing trends between May and November at the three stations (Table S1), while PO_4^{3-} concentration displayed its highest values in July, September and November (Table S1).

Picoplankton abundance

Picoplankton abundance, as determined by 4′,6-diamidino-2-phenylindole (DAPI) counting, showed different trends at the three stations (Fig. 1). Picoplankton counts at SAB1 increased from 5.0×10^6 cells ml^{-1} in May to 3.1×10^7 cells ml^{-1} in July, then decreased to 6.1×10^6 cells ml^{-1} in September, slightly increasing again in October (8.1×10^6 cells ml^{-1}) and reaching the lowest value in November (3.2×10^6 cells ml^{-1}) (Fig. 1). *Cyanobacteria* peaked in June, when they accounted for 25% of the total picoplankton.

Picoplankton at SAB2 developed similarly, also increasing in abundance between May (5.7×10^6 cells ml^{-1}) and July (2.6×10^7 cells ml^{-1}), then decreasing in August (1.3×10^7 cells ml^{-1}) and September (1.5×10^7 cells ml^{-1}) and reaching their lowest value in November (3.4×10^6 cells ml^{-1}) (Fig. 1). *Cyanobacteria* in July interestingly accounted for 48% of the total picoplankton,

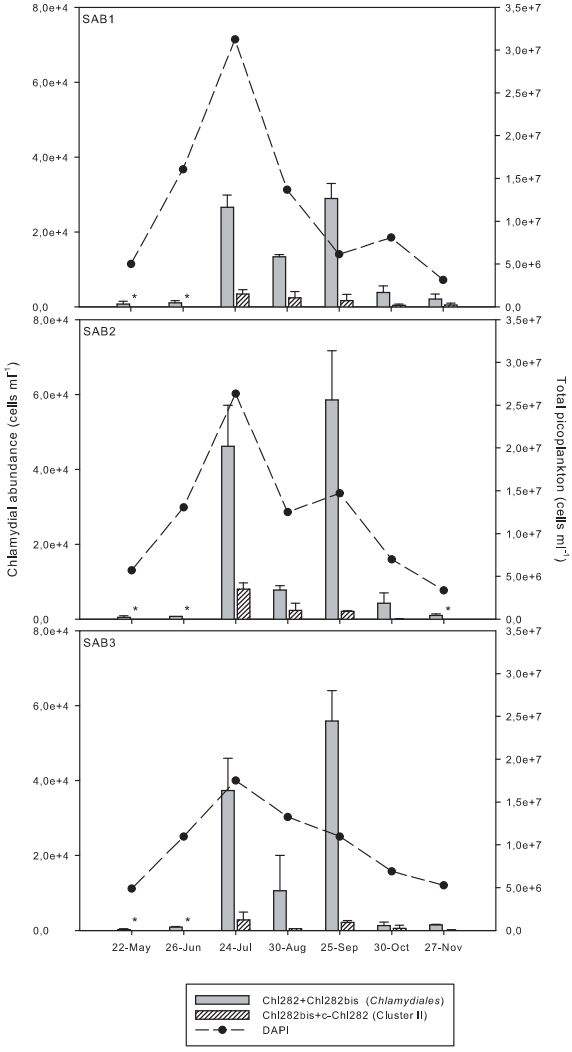

Fig. 1. Total picoplankton and chlamydial abundances at the three sampling sites SAB1, SAB2 and SAB3 between May and November 2013. Means ± standard deviation of two replicates are shown for *Chlamydiae*. *not detected.

representing their highest value registered in the lake throughout the year.

All picoplankton at SAB3 showed increasing abundance between May (4.9×10^6 cells ml^{-1}) and July (1.8×10^7 cells ml^{-1}), decreased from July and reached their lowest value in November (5.3×10^6 cells ml^{-1}) (Fig. 1). *Cyanobacteria* again peaked in July, accounting for 27% of the total picoplankton.

Seasonality of chlamydiae

Using CARD-FISH, we identified and quantified abundant picoplankton cells with mainly coccoid morphology. The combination of probes Chl282 and Chl282bis was used to target all known members of the phylum *Chlamydiae,* while we used Chl282bis with unlabelled Chl282 as a competitor to quantify only cluster II, a new chlamydial family previously discovered and described in the water column of Lago di Paola (Pizzetti *et al*., 2012).

At the three stations, the highest values of chlamydiae and cluster II were registered in summer and early autumn (Fig. 1). For cluster II, at each sampling date and station (with the exception of SAB2 in July) less than one cell per counting grid on average was counted, meaning that the lower limit for exact quantification was exceeded.

At SAB1, chlamydiae showed values of $2.7 \pm 0.3 \times 10^4$ cells ml^{-1}, $1.3 \pm 0.1 \times 10^4$ cells ml^{-1} and $2.9 \pm 0.4 \times 10^4$ cells ml^{-1} in July, August and September respectively. The lowest values were registered in October ($3.9 \pm 1.7 \times 10^3$ cells ml–1) and November ($2.1 \pm 1.4 \times 10^3$ cells ml^{-1}). In May and June, chlamydiae were also detected although numbers were again too low for exact counting.

Cluster II was absent in May and June, had its highest abundance in July ($3.5 \pm 1.2 \times 10^3$ cells ml^{-1}) and was then still present in the lake until November (Fig. 1).

At SAB2, chlamydial cell numbers reached a maximum at the end of September with up to $5.9 \pm 1.3 \times 10^4$ individual small cells per millilitre. A high abundance was also observed in July ($4.6 \pm 1.1 \times 10^4$ cells ml^{-1}). In August and October, lower values were recorded, with $7.8 \pm 1.2 \times 10^3$ cells ml^{-1} and $4.3 \pm 2.8 \times 10^3$ cells ml^{-1} respectively. In May, June and November chlamydiae were present but due to low abundance not quantifiable. Cluster II could be detected only from July to October, with the highest value observed in July ($8.1 \pm 1.6 \times 10^3$ cells ml^{-1}) (Fig. 1).

At SAB3, the highest values of chlamydiae were detected in July, August and September, respectively, $3.7 \pm 0.9 \times 10^4$ cells ml^{-1}, $1.1 \pm 0.9 \times 10^4$ cells ml–1 and $5.6 \pm 0.8 \times 10^4$ cells ml^{-1}. At the other sampling time points, chlamydiae were present but the lower limit for quantification was exceeded. Cluster II was present from July to November with the highest abundance of $2.8 \pm 2.1 \times 10^3$ cells ml^{-1} observed in July but it was absent in May and June (Fig. 1).

Environmental parameters and chlamydial abundance

To elucidate the main relationships between chlamydial and picoplankton abundance with environmental factors, we conducted a principal component analysis (PCA) (Gauch, 1982) in order to reduce the amount of studied variables to a few components explaining most of the observed variability. The two first principal components explained 65.5% of the variability in the original data (Fig. 2). The first principal component, which explained 45.2% of the variability, clearly discriminates between summer and autumn sampling points, describing an environment in summer characterized by higher temperature, pH, salinity, DOC, chl *a* and dissolved oxygen (DO) and lower NO_3^-, NO_2^- and PO_4^{3-}. Picoplankton together with chlamydial abundance were strongly associated with summer conditions. In autumn, the environment was characterized by higher NO_3^-, NO_2^- and PO_4^{3-} and lower temperature, pH, salinity, DOC, chl *a* and DO (Fig. 2). In addition, to test for seasonal differences, we used analysis of similarities (ANOSIM), which indicated a strong dissimilarity between spring, summer and autumn ($R = 0.88$; $P = 0.0001$). Pairwise ANOSIMs between all pairs of groups were provided as a post-hoc test, and comparison results were significant (at $P < 0.05$).

Super-resolution microscopy

Environmental chlamydiae were identified in Lago di Paola in different developmental stages, both inside and outside the host. For an in-depth study of the relation between chlamydiae in Lago di Paola and their hosts, a confocal laser scanning microscope (CLSM) was used in selected samples combined with a super-resolution system based on structured illumination microscopy. With this approach, the 3D arrangements of environmental chlamydiae within host cells were investigated. The improved resolution allowed a better visualization and, for the first time, permitted the quantification of chlamydiae inside a host cell directly in an environmental sample (Fig. 3; Fig. S1). In fact, we were able to quantify from about 10 to 40 chlamydial cells within unicellular hosts in selected samples particularly from station SAB2.

Recovery of Vexillifera sp. K9 amoeba infected with chlamydial symbionts

Using a cultivation approach based on limiting dilution, we obtained several amoeba cultures. A screening with FISH and DAPI showed the presence of coccoid bacteria in isolate K9. The locomotive forms of this amoeba strain corresponded to genus diagnosis of *Vexillifera*, flattened amoeba of acanthopodial morphotype which were triangular in outline and express slender and non-furcating subpseudopodia (Fig. 4A). The cell size of observed locomotive forms was 10.1–21 μm (mean = 14.8 μm, SD = 2.9 μm) in length and 4.4–13 μm (mean = 8.4 μm, SD = 1.9 μm) in width with a length/width ratio 3.9–9.5 (mean 5.9) and a hyaline zone occupying approximately four tenths of the total cell length. At the ultrastructural level, vesicular nuclei (Fig. 4B), mitochondria with tubular cristae (Fig. 4B, D) and a cell surface coated with amorphous glycocalyx (Fig. 4C) were observed. In addition,

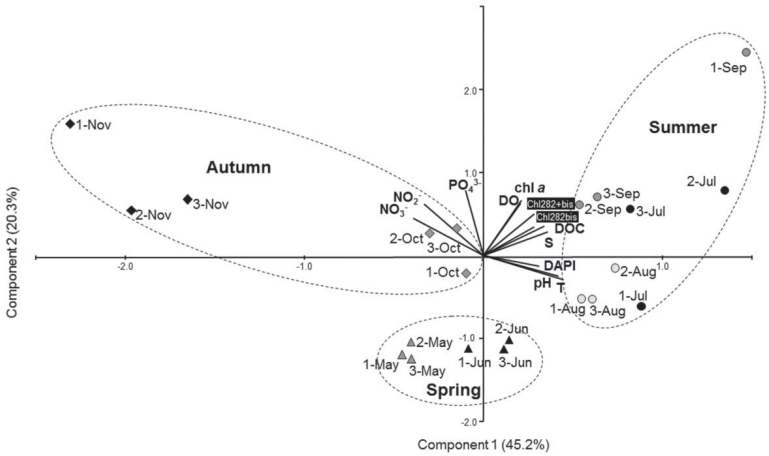

Fig. 2. Two-dimensional plot of the PCA performed including physico-chemical parameters and chlamydial absolute abundances and picoplankton between May and November 2013. Symbols refer to the months. T: Temperature; S: Salinity; chl *a*: Chlorophyll *a*; DOC: Dissolved organic carbon; DO: Dissolved oxygen; DAPI: DAPI-stained cells; Chl282 + bis: *Chlamydiae*; Chl282bis: cluster II. Dotted circles separate spring, summer and autumn. Black lines represent all variables used. The length of each line indicates the degree of correlation with the ordination axis. The percent variation explained by each axis is indicated in parentheses.

peculiar inclusions were present in many trophozoites. These wedge-shaped filamentous structures (Fig. 4B, E) were located variously and without any stable position within the amoeba cytoplasm (Fig. 4B). The internal structure appeared to be either filamentous or containing electron-densed and rhomboid bodies (Fig. 4B). Bacterial cells with a morphology resembling known *Chlamydiae* were observed in the cytoplasm of *Vexillifera* sp. K9. Both elementary and reticulate bodies were present in single-cell inclusions (Fig. 4F) and rarely in larger inclusions (Fig. 4G). The maximal diameter of observed elementary bodies and reticulate bodies was 0.61 μm and 0.93 μm respectively. Nearly 100% of amoebae harboured bacterial symbionts. The isolate could be maintained stably in laboratory culture although lysis of infected amoebae was occasionally observed. *Vexillifera* sp. K9 was only moderately related to known amoeba strains, with its 18S rRNA gene sequence being most similar to *Vexillifera multispinosa* (80% sequence identity). Our phylogenetic analysis placed the isolate basal within a clade of freshwater vexilliferids comprising *V. fluvialis*, *V. multispinosa* and *V. bacillipedes* (Fig. S2).

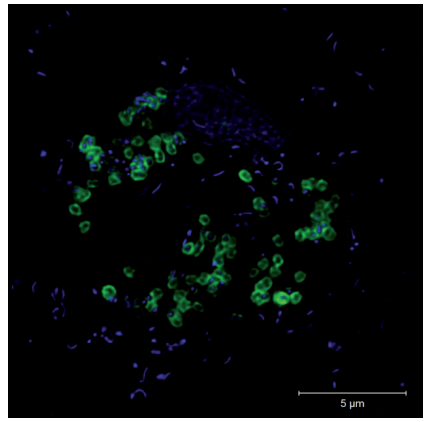

Fig. 3. Identification of host-associated chlamydiae directly in water samples by super-resolution microscopy. Blue, DAPI signals of DNA-containing cells; green, fluorescein signals conferred by CARD-FISH to *Chlamydiae* cells. Bar: 5 μm.

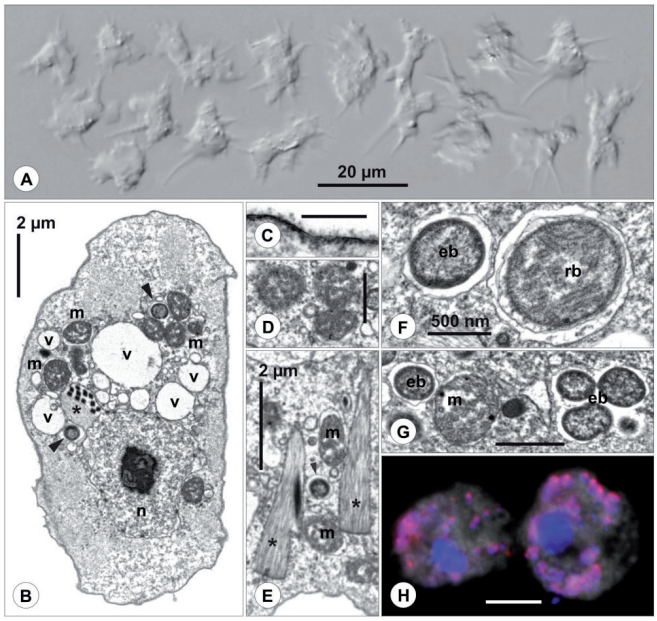

Fig. 4. Microscopic characterization of Vexillifera sp. K9 and its chlamydial endosymbiont '*Candidatus* Neptunochlamydia vexilliferae'.
A. Locomotive forms of *Vexillifera* sp. K9 as seen in hanging drop preparations (Nomarski DIC).
B. Overview of the trophozoite ultrastructure and the presence of '*Candidatus* Neptunochlamydia vexilliferae' as single-cell inclusions in the amoeba cytoplasm: vesicular nucleus (n); mitochondria (m); vacuoles (v); chlamydial reticulate bodies (arrowheads); filamentous structure (*).
C. Cell surface with amorphous glycocalyx (scale bar = 200 nm).
D. Mitochondria with tubular cristae (scale bar = 1 µm).
E. Wedge-shaped filamentous structures enclosed by a membrane (*).
F. Single-cell inclusions of an EB and an RB of '*Candidatus* Neptunochlamydia vexilliferae'.
G. Rare case of an inclusion with multiple chlamydia (EB); mitochondrion (m); scale bar indicate 1 µm.
H. Fluorescence *in situ* hybridization image showing the intracytoplasmic location of '*Candidatus* Neptunochlamydia vexilliferae' (purple; using a symbiont-specific probe) in its *Vexillifera* sp. K9 host (grey) with DAPI stained nuclei (blue).

'Candidatus Neptunochlamydia vexilliferae'

The 16S rRNA gene of the endosymbiont of isolate K9 showed the highest identity to *S. negevensis* (93% sequence identity) in National Center for Biotechnology Information RefSeq, a database which contains mainly sequences of well described organisms (Pruitt *et al.*, 2012). Phylogenetic analysis revealed that it groups in a clade in the *Simkaniaceae,* together with other chlamydiae either originating from marine or freshwater habitats and being associated with animals, such as insects, fish or the marine worm *Xenoturbella* (Fig. 5). Based on its 16S rRNA gene sequence, we designed an oligonucleotide probe for the specific detection of the chlamydial symbiont and successfully used FISH to demonstrate the symbionts within their native *Vexillifera* sp. host cells (Fig. 4H). Due to its origin, a coastal marine lake near Rome, we provisionally referred to the new species as '*Candidatus* Neptunochlamydia vexilliferae K9' (hereafter: *N. vexilliferae*), a member of the proposed novel genus '*Candidatus* Neptunochlamydia', referring to *Neptūnus*, the ancient Roman god of the sea.

Discussion

Monitoring seasonal dynamics of chlamydiae in Lago di Paola was facilitated by significant methodological improvements in the process of quantification of chlamydial abundance. As reported in a previous study (Gomez-Pereira *et al.*, 2010), rare populations in aquatic

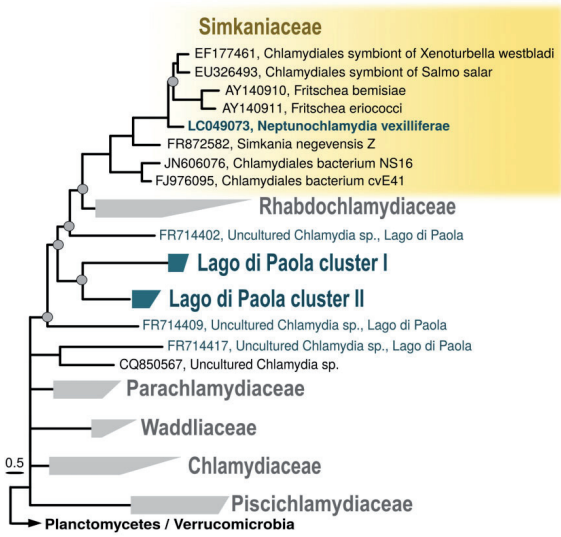

Fig. 5. Phylogenetic relationship of '*Candidatus* Neptunochlamydia vexilliferae' in the *Chlamydiae*. Shown is a 16S rRNA-based phylogenetic tree (MrBayes, GTR) of representative members of the *Chlamydiae*, Bayesian posterior probabilities are indicated at the nodes (full support, no circle; probabilities 0.80–0.99, gray circle), nodes with probabilities of below 0.5 are not resolved. Scale bar indicates substitutions per site.

samples should be enumerated from filters containing high volumes of water samples to overcome the limitation in quantifying them. Here for the first time, chlamydiae were quantified on high-volume filters, allowing for much more accurate counts. Furthermore, using super-resolution microscopy, we were able, for the first time, to quantify chlamydiae directly inside their host in an environmental sample. This method has the potential to improve significantly quantification of chlamydiae in future studies, as it allows researchers to take into account host-associated bacteria.

Monitoring of picoplankton in Lago di Paola over a time period of 7 months has shown that shifts in abundance are mainly linked to temperature changes. Interestingly, at station SAB1 picoplanktonic cells rapidly decreased from July to September and then slightly increased in October, whereas at station SAB2 decreasing values were registered from July to August then slightly increased in September (Fig. 1). This shift in abundance values observed at SAB1 and SAB2 is apparently related to the peak of dissolved organic carbon registered in September at SAB1 and in August at SAB2 (Table S1). Furthermore, it seems that the dissolved organic carbon detected at SAB1 and SAB2 has different origins. At SAB1 the peak of DOC in September is linked to the peak of chlorophyll *a* and oxygen, suggesting an autochthonous origin of the organic carbon. However, at SAB2 the peak of DOC in August is not directly linked to chlorophyll *a* and oxygen, suggesting an allochthonous origin.

Members of the phylum *Chlamydiae* in Lago di Paola showed similar trends in the three stations although with different values that were higher at SAB2 and SAB3 compared with SAB1 (Fig. 1). The highest values were registered in summer (July), but also in early autumn (late September), when instead the picoplankton abundance was lower, mainly due to environmental conditions. *Chlamydiae* belonging to cluster II were detected but they cannot be exactly quantified since they were below the detection limit. Still, cluster II was more present in summer and early autumn. This result is consistent with a previous study in Lago di Paola in which *Chlamydiae* were only quantified in two repeated early autumn settings showing similar abundances to the ones observed in 2013 (Pizzetti *et al.*, 2012).

Summer to early autumn is the time of the highest anthropogenic pressure coinciding with a strong increase of the number of residents in the catchment area of Lago di Paola. Most probably, the highest human impact fosters a higher abundance of protists already well known to be correlated with the level of organic matter present in the water which constitute an ecological niche where amoebae can feed on bacteria (Loret and Greub, 2010). Overall, we suppose that the higher anthropogenic pollution in summer and early autumn triggered higher

abundances of amoebae which favoured the multiplication and dissemination of *Chlamydiae* in Lago di Paola. Particularly, the biphasic abundance pattern of *Chlamydiae* in July and September suggests that the two peeks represent independent events either different host blooms and/or different chlamydial groups present in high abundance at the different time points. However, further studies will be necessary to elucidate this finding.

A general limitation of the isolation of amoebae from the environment is that certain strains cope better with laboratory conditions and thus outcompete other amoebae present in the sample. The approach applied here strongly reduced competition between different protist strains due to the low cell number used as an inoculum ($n = 1$ to 10) and thus increases the chance to retrieve novel amoeba isolates (Schulz *et al*., 2015). The amoeba isolate K9 showed an only 80% 18S rRNA sequence identity to other *Vexillifera* strains found in freshwater and marine habitats. In general, the 18S rRNA sequences of *Vexillifera* spp., are markedly divergent in comparison to the other related Amoebozoa lineages (Dyková *et al*., 2011) and isolate K9 represents such a long-branching taxon (Fig. S2). The distinct filamentous inclusions observed in isolate K9 are similar to the 'trumpet-shaped tapered bundles', which have been described for the gracilipodid amoeba *Telaepolella tubasferens* and characterized as an assembly of microtubules (Lahr *et al*., 2011; Fig. 4E). Interestingly, even more highly similar structures were found in *Vexillifera armata,* showing some similarity to the trichocysts of dinoflagellates and of the ciliate *Drepanomonas dentata* (Page, 1979). To our knowledge, isolate K9 is the third strain of amoebae possessing this peculiar type of the cytoplasmic filamentous structure. Based on phylogenetic and morphological analyses isolate K9 can be assigned to the amoebal genus Vexillifera as '*Vexillifera* sp. K9' (Fig. 4 and Fig. S2).

It was recently shown that Lago di Paola harbours a rich chlamydial diversity, comprising at least two large family-level clusters of phylogenetically deeply branching and so-far unknown chlamydiae (Pizzetti *et al*., 2012). Here, we were able to obtain a novel amoeba strain infected with chlamydiae from this particular marine lake. Surprisingly, these symbionts do not belong to one of the largest Lago di Paola sequence clusters identified earlier but instead represent a so-far unknown chlamydial species (Fig. 5). *Neptunochlamydia vexilliferae* showed features characteristic for members of the phylum *Chlamydiae*. It replicated in host-derived cytoplasmic vacuoles and alternated between the two typical developmental stages, elementary and reticulate bodies (Omsland *et al*., 2014; Fig. 4). Our phylogenetic analysis shows that *N. vexilliferae* is a member of the family *Simkaniaceae* (Fig. 5). However, in contrast to *S. negevensis*, which was found as contaminant of a human cell culture (Kahane *et al*., 1998) with an unknown original host, the chlamydial symbiont described here was found directly in its native protist host. It should be noted that *N. vexilliferae* was not detected in a recent polymerase chain reaction (PCR)-based study on samples from Lago di Paola (Pizzetti *et al*., 2012) targeting chlamydial diversity in the lake. With respect to the limitations of the previously conducted study, our more recent finding is not surprising as the diversity of environmental chlamydiae has been estimated to be much higher than originally assumed, particularly in marine habitats (Lagkouvardos *et al*., 2014b). Consistent with their long evolutionary history as intracellular microbes (Horn *et al*., 2004; Horn, 2008; Subtil *et al*., 2014), all of the thus-far studied chlamydial strains originated from either multicellular hosts or from amoebae, with many of the latter isolated from environments impacting human health concerns such as water distribution systems, cooling towers and waste water treatment plants (Collingro *et al*., 2005; Thomas *et al*., 2008; Corsaro *et al*., 2010). It is notable that this study adds the first species of marine chlamydiae successfully retrieved together with its host. The fact that it can be stably maintained in laboratory paves the way for further experiments on marine chlamydiae, potentially giving insights into adaptation of chlamydiae to saline systems, their host-range and possible impacts on human and animal health.

Taken together, this is the first study that quantified chlamydial abundance and monitored seasonal dynamics of chlamydiae outside and inside their hosts. Our analyses of three different stations at Lago di Paola revealed a striking seasonality likely associated with host abundance. Further studies on chlamydiae in this lake should focus on the summer and early autumn. Ultimately, our results deserve attention also from a sanitary point of view due to the putative pathogenic potential of some environmental chlamydiae.

Experimental procedures

Study site and sampling

Lago di Paola is a shallow, meso-eutrophic lake located on the Tyrrhenian coast of central Italy (*Latium*). Two narrow artificial channels at the northwestern and southeastern ends of the lake allow for a limited water exchange with the sea, which is enough to sustain a high degree of salinity throughout the year. Surface water samples were collected every month between May and November 2013 [22nd May, 26th June (spring/early summer, identified as spring); 24th July, 30th August, 25th September (summer/early autumn, identified as summer); 30th October, 27th November (autumn)] in three stations along Lago di Paola's main axis previously described and named SAB1, SAB2 and SAB3. For a detailed description of the study site and the location of sampling points see (Pizzetti and colleagues (2011a).

Environmental parameters such as temperature, salinity, DO and pH were determined with probes (HQ40d, Hach,

USA). Dissolved organic carbon was determined by oxidative combustion infrared analysis using a Shimadzu Total Organic Carbon (TOC)-VCS. Dissolved inorganic nutrients (nitrite, nitrate, phosphate) were analysed as reported in APAT and IRSA-CNR (2003) and chl *a* according to Lazzara and colleagues (2010) and APAT and IRSA-CNR (2003).

Samples for CARD-FISH were fixed for 1 h at room temperature and up to 24 h at 4°C with formaldehyde solution (37% w/v, Sigma Aldrich; final concentration 1%). Afterwards, aliquots of 5–10 ml (low volume filters) and 15–50 ml (high-volume filters) were filtered at very low vacuum levels (4 inHg) onto 0.2 μm pore-size polycarbonate filters (type GTTP; diameter, 47 mm; Millipore, Eschborn, Germany) in order to avoid damage to the protists and stored at −20°C until further processing.

The sample for the isolation of the native chlamydial host was collected on 1 October 2013. Three litres of lake water were filtered on 5 μm pore-size polycarbonate filters (TMTP type; diameter, 47 mm; Millipore, Eschborn, Germany) to collect a good amount of protists, and 500 ml of the resultant filtrate was then filtered onto 0.2 μm pore-size polycarbonate filters (type GTTP; diameter, 47 mm; Millipore, Eschborn, Germany) to collect the bacterial fraction. The two filters were then re-suspended in 50 ml autoclaved lake water and kept at 4°C until further processing.

DAPI-staining and CARD-FISH

Untreated filter sections (low-volume filters) were stained with DAPI at a final concentration of 1 μg ml^{-1} to quantify the total picoplankton. A minimum of 20 microscopic fields were counted comprising at least 800 DAPI-stained cells. Photosynthetic picoplankton (*Cyanobacteria*) was discriminated for the reddish autofluorescence (excitation wavelength 550 nm; CY3). In order to confirm their presence, emission peaks were evaluated by the use of the lambda scan function of CLSM (FV1000 Olympus, Tokyo, Japan). *Cyanobacteria* showed the two expected emission peaks, one at 680 nm (chlorophyll *a*) and one between 550 and 650 nm (phycobiliproteins).

Catalysed reporter deposition fluorescence *in situ* hybridization was performed according to previous protocols (Pernthaler *et al.*, 2004; Fazi *et al.*, 2005; 2007) and slightly modified by Pizzetti and colleagues (2011a,b). Horseradish peroxidase-labeled oligonucleotide probes (Biomers, Germany) were used to quantify the known members of the phylum *Chlamydiae* (Chl282 and Chl282bis; Pizzetti *et al.*, 2012) and a new chlamydial family recently discovered in Lago di Paola called cluster II (Chl282bis with unlabelled Chl282 as competitor; Pizzetti *et al.*, 2012) which has also been recognized by Lagkouvardos and colleagues (2014b) as a new 'Predicted Chlamydial Family'. The probe NON338 (Wallner *et al.*, 1993) was used as a negative control. No false-positive signals were detected in the samples. Chlamydial counts were done on high-volume filters (15–50 ml) to allow for reliable quantifications of populations as small as 0.1% (Gomez-Pereira *et al.*, 2010). The probe-conferred fluorescence was counted in 20 microscopic fields and the lower limit for quantification was set to 1 cell per counting grid on average (Gomez-Pereira *et al.*, 2010).

Isolation and cultivation of marine amoebae

Protist-sized particles present in coastal lake water (Lago di Paola, Italy) were counted with a Neubauer counting chamber. Between 1 and 10, protist-sized particles were placed in wells on a 96-well plate (Corning Costar, Sigma-Aldrich, Germany) containing 200 μl artificial sea water (ASW, DSMZ 607) with *Escherichia coli tolC* serving as food (Lagkouvardos *et al.*, 2014a). Amoebae propagating on these plates were screened for the presence of bacterial endosymbionts with FISH, upscaled and maintained in cell culture flasks (Nunclon delta-surface, Thermoscientifc, Germany) containing ASW and *E. coli tolC*. Trophozoites were observed and documented in hanging drop preparations using a BX53 Olympus microscope equipped with Nomarski differential interference contrast (DIC) and a DP72 camera (Olympus Optical, Japan).

FISH

Amoeba cells were harvested by centrifugation (3000 × g, 8 min), washed with ASW and left to adhere at slides for 30 min prior to fixation with 4% formaldehyde (15 min at room temperature). The samples were hybridized for 2 h at 46°C in a formamide concentration of 25% using standard hybridization and washing buffers (Daims *et al.*, 2005) and a combination of the *N. vexilliferae* specific probe (K9_443, 5′-TGCTCTCTCTTGTTCCCT-3′) together with EUK-516 (5′-ACCAGACTTGCCCTCC-3′, Amann and Binder, 1990) targeting most eukaryotes and the EUB338 I–III (5′-GCTGCCTCCCGTAGGAGT-3′, 5′-GCAGCCACCCGTAGGTGT-3′, 5′-GCTGCCACCCGTAGGTGT-3′; Daims *et al.*, 1999) probe mix targeting most bacteria. All probes were purchased from ThermoFisher Scientific (Germany). Cells were subsequently stained with DAPI [0.5 μg ml^{-1} in Page's amoebic saline (PAS; 0.12 g l^{-1} NaCl, 0.004 g l^{-1} $MgSO_4 \times 7H_2O$, 0.004 g l^{-1} $CaCl_2 \times 2H_2O$, 0.142 g l^{-1} Na_2HPO_4, 0.136 g l^{-1} KH_2PO_4), 3 min], washed once with PAS and embedded in Citifluor (Agar-Scientific, UK). Slides were examined using a confocal laser scanning microscope (SP8, Leica, Germany).

Microscopy

Super-resolution structured illumination fluorescence microscopy with a resolution of 100–130 nm (Schermelleh *et al.*, 2010) was done with a Zeiss LSM 780 CLSM combined with the super-resolution system ELYRA PS.1. No specific sample preparation was required to study the intracellular localization and abundance of chlamydial cells in their protist hosts in filter-colllected formalin-fixed water samples.

For transmission electron microscopy, ASW medium in a culture flask was replaced with cacodylate buffered 3% gluraldehyde to fix adhered trophozoites *in situ*. The fixed trophozoites were washed off after 30 min, pelleted and postfixed with 1% osmium tetroxide for 1 h. The pellet was dehydrated in an acetone series and embedded in Spurr resin. Ultrathin sections were routinely stained with uranyl acetate and lead citrate and observed in a JEOL JEM 1010 electron microscope operating at 80 kV. Measurements of cellular structures were made using the software ImageJ (Schneider *et al.*, 2012).

DNA extraction, PCR, cloning and sequencing

Deoxyribonucleic acid was extracted from infected amoeba cultures using the DNeasy Blood and Tissue Kit (Qiagen, Austria). The partial 18S rRNA gene was amplified by PCR using primers 460F (5′-CAGCAGGCGCGCAAATTA-3′, Dyková *et al.*, 2008) and Erib10 (5′-CTTCCGCAGGT TCACCTACGG-3′, Barta *et al.*, 1997). Bacterial 16S rRNA genes were amplified using primers PLA46 (5′-GACTTGCATGCCTAATCC-3′; Neef *et al.*, 1998) and Univ-1392a (5′-ACGGGCGGTGTGTRC-3′; Lane *et al.*, 1985) at an annealing temperature of 52°C. Polymerase chain reactions contained 100 ng template DNA, 50 pmol of each primer, 1 unit of Taq DNA polymerase (TopBio, Czech Republic for 18S rDNA; Fermentas, Germany for 16S rDNA), 10x Taq buffer with KCl and 2 μM MgCl2 and 0.2 μM of each deoxynucleotide in a total volume of 50 μl. Bacterial 16S rRNA PCR products were purified using the PCR Purification Kit (Qiagen, Germany) and were cloned using the TOPO TA Cloning Kit (Invitrogen, Germany) following the manufacturers' instructions. Amoebal 18S rRNA PCR product was purified using the same PCR Purification Kit and sequenced directly. Nucleotide sequences were determined at Microsynth (Vienna, Austria) and Macrogen Europe (Amsterdam, the Netherlands). Newly obtained partial rRNA gene sequences were deposited at Genbank/EMBL/DDBJ (accession numbers: LC049073, LC049074).

Phylogenetic analysis and probe design

For bacterial phylogeny, the alignment editor integrated in the software ARB was used to build alignments based on the 115 Silva ARB 16S rRNA database (Ludwig *et al.*, 2004; Quast *et al.*, 2013). The alignment was trimmed to the length of the shortest sequence, manually curated and exported from ARB using a 50% conservation filter. The resulting alignments comprised 84 sequences and 1258 positions. For Bayesian analysis, the parallel version of MrBayes 3.2.6 (Ronquist and Huelsenbeck, 2003; Altekar *et al.*, 2004) was used in two runs with 4 Markov Chain Monte Carlo chains under the General Time Reversible (GTR) invgamma model and in total 5×10^5 generations. As burnin, 25% of the sampled trees were discarded. The endosymbiont-specific FISH probe 'K9_443' was designed in ARB (Ludwig *et al.*, 2004) and evaluated *in silico* with MATHFISH (Yilmaz *et al.*, 2011) and PROBECHECK (Loy *et al.*, 2008). To assess the phylogenetic position of the novel amoeba strain, a data set containing 18S rRNA sequences of 27 dactylopodid amoebae (including all available sequences of *Vexillifera* spp.) and five vannellid amoebae serving as the outgroup was selected. The sequences were aligned with the MAFFT 7 software (Katoh and Standley, 2013) and checked for ambiguously aligned positions. The final length of the alignment was 1491 positions and GTR + G + I model was selected using JMODELTEST 2 (Darriba *et al.*, 2012) as the best fitting. The data set was analysed with RAxML 8.0.20 (Stamatakis, 2014) with rapid bootstrapping including 1000 replicates. Bayesian inference analysis was run in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) with the GTR invgamma model of evolution and 2×10^6 generations. A burnin of 25% of the sampled trees was chosen.

Statistical analysis

For an ordination of sampling sites, a PCA was performed with the software PAST (PALAEONTOLOGICAL STATISTICS, ver. 2.01) (http://folk.uio.no/ohammer/past/; Hammer *et al.*, 2001). Component loadings visualized in a biplot were calculated using all physico-chemical parameters, picoplankton and absolute abundances of *Chlamydiae*. All variables were normalized using division by their standard deviations, and loading values were computed by the data correlation matrix.

The one-way ANOSIM, a non-parametric test of significant difference between two or more groups, based on any distance measure (Clarke, 1993), was also performed with the software PAST. All data were log-transformed. Euclidean was used as the distance measure. The one-tailed significance was computed by permutation of group membership, with 9999 replicates. Pairwise ANOSIMs between all pairs of groups were provided as a post-hoc test.

Acknowledgements

This work was funded by the National Flag programme RITMARE (SP3-WP2-AZ2-UO5). We thank Andreas Ellrott, Max Planck Institute for Marine Microbiology for the help with super-resolution microscopy, Simona Crognale for the help in water sampling and Francesca Di Pippo for providing *Cyanobacteria* emission peaks (both Water Research Institute, IRSA-CNR, Rome). We also acknowledge Andrea Butturini from the University of Barcelona for the DOC analysis. This work was supported by a DOC fellowship of the Austrian Academy of Sciences to FS, by Czech Science Foundation to TT (grant 505/12/G112), by a European Research Council grant to MH (ERC grant 'EvoChlamy' #281633) and by a grant of the Austrian Science Fund (I1628-B22) to MH.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1. Three-dimensional video obtained from super-resolution microscopy (Zeiss LSM 780 CLSM with the super-resolution system ELYRS PS.1). Blue, DAPI signals of DNA-containing cells; green, fluorescein signals conferred by CARD-FISH to *Chlamydiae* cells.
Fig. S2. Phylogenetic placement of *Vexillifera* sp. K9 within Dactylopodida, Amoebozoa. 18S rRNA-based maximum likelihood tree comprising 27 dactylopodids and 5 vannellids as outgroup. Bayesian posterior probabilities and RAxML bootstrap support values are indicated at the nodes. Scale bar indicates substitutions per site.
Table S1. Physico-chemical parameters in SAB1, SAB2, SAB3 between May and November 2013.

Supporting information

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Fig. S1. 3D video obtained from super-resolution microscopy (Zeiss LSM 780 CLSM with the super-resolution system ELYRS PS.1). Blue, DAPI signals of DNA-containing cells; green, fluorescein signals conferred by CARD-FISH to *Chlamydiae* cells.

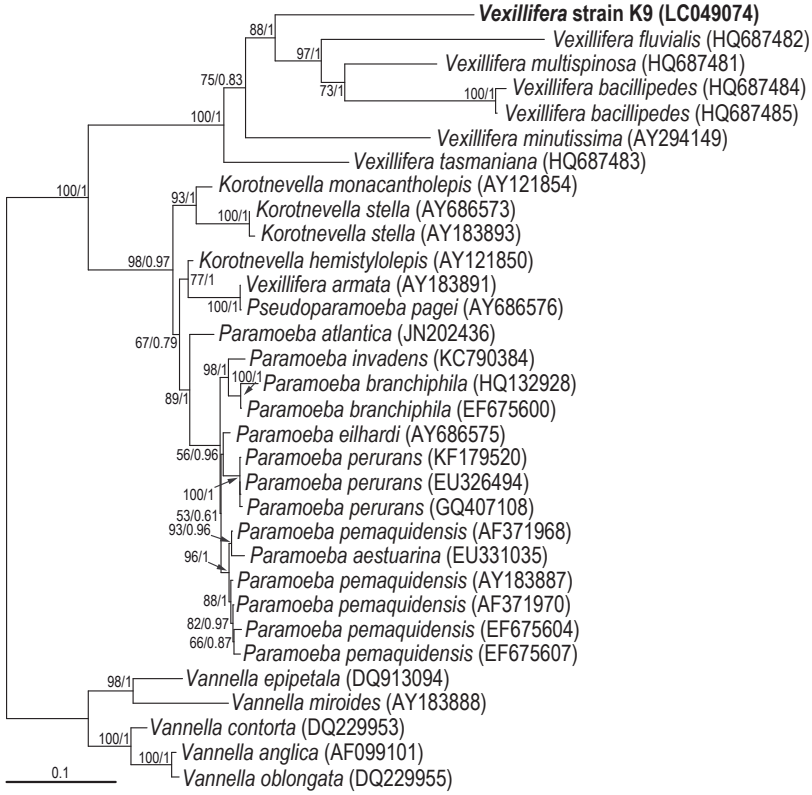

Fig. S2. Phylogenetic placement of *Vexillifera* sp. K9 within Dactylopodida, Amoebozoa. 18S rRNA-based Maximum likelihood tree comprising 27 dactylopodids and 5 vannellids as outgroup. Bayesian posterior probabilities and RaxML bootstrap support values are indicated at the nodes. Scale bar indicates substitutions per site.

Table S1. Physico-chemical parameters in SAB1, SAB2, SAB3 between May and November 2013.

Stations	Dates	Temp (°C)	Sal	pH	DO (mg l^{-1})	Chl *a* (µg l^{-1})	DOC (mg l^{-1})	PO_4^{3-} (µg l^{-1})	NO_2^- (µg l^{-1})	NO_3^- (µg l^{-1})
SAB1	22/05	22.4	28.2	8.3	10.7	13.6	2.9	22.1	17.8	173.9
	26/06	26.2	30.6	8.2	7.2	11.9	4.0	92.7	32.2	192.8
	24/07	30.9	32.3	8.3	7.2	21.5	4.6	112.6	23.3	68.3
	30/08	28.4	32.6	8.4	9.3	23.7	4.5	84.9	24.4	75.6
	25/09	26.5	34.0	8.6	21.2	267.0	7.2	173.4	73.3	151.7
	30/10	21.8	32.7	8.4	10.6	64.1	3.3	70.7	78.9	471.1
	27/11	10.1	27.8	7.4	9.3	3.7	3.1	438.7	181.1	5910.6
SAB2	22/05	22.4	27.7	8.5	11.7	19.6	3.3	18.0	36.7	705.0
	26/06	26.8	30.5	8.5	11.5	11.7	3.8	33.3	12.2	371.1
	24/07	30.6	30.5	8.6	14.0	42.2	4.6	216.6	58.9	74.4
	30/08	27.6	31.4	8.5	12.6	32.6	6.4	76.4	20.0	263.3
	25/09	25.6	32.9	8.3	11.9	56.1	3.8	139.3	72.2	894.4
	30/10	21.5	31.6	8.4	12.8	71.0	3.7	77.1	138.9	1627.8
	27/11	10.0	28.1	7.6	9.6	6.9	2.9	149.9	194.4	3805.6
SAB3	22/05	22.4	29.2	8.4	9.9	9.4	2.9	11.7	16.7	233.3
	26/06	26.8	31.0	8.5	10.2	13.1	3.6	62.8	8.9	<LOD
	24/07	30.6	33.0	8.5	11.1	17.7	4.8	351.7	52.2	39.4
	30/08	27.4	33.2	8.5	10.8	20.0	5.5	66.8	16.7	16.7
	25/09	24.2	34.2	8.4	12.3	56.1	3.5	229.2	41.1	67.2
	30/10	22.1	31.6	8.4	15.0	46.2	4.2	73.6	157.8	650.6
	27/11	11.2	29.3	7.6	9.4	17.6	2.9	229.2	226.7	1681.7

Section IX

Perspectives of future research

Perspectives of future research

Introduction of new molecular methods, improvement of widely used techniques and availability of efficient sequencing facilities have opened new perspectives in all aspects of FLA studies. Based on the ever expanding amount of (meta)genomic and (meta)trancriptomic data, a substantial progress can be expected especially in:

- capturing species diversity of FLA including "bacteria-sized" species;
- unveiling new FLA lineages within the tree of eukaryotes;
- life cycle research on sexuality of FLA;
- exploration, characterization and interpretation of FLA diversity within the context of natural environment, based on sampling efforts across diverse geographical regions, ecosystems and time-scales;
- collection of data shedding new light upon FLA evolution and mechanisms involved in diversification of FLA;
- pathogenomics, i.e., research on genetic diversity of pathogens that affect human and animal health, and studies of genetic background of host-amoeba interactions involved in disease status;
- understanding the role of FLA in communities of heterotrophic protists;
- search for possibilities of barcode-based species identification in FLA;
- development of new (routine) biomonitoring approaches;
- studies of obligate/transient symbiotic associations of FLA with prokaryotes

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Diversity, phylogeny and phylogeography of free living amoebae.
Ph.D. Thesis Series, 2016, No. 13

The cover page is composed of lithographs by A. Hoen & Co. published in Schaeffer's Taxonomy of Amebas (1926).

Printed in the Czech Republic by Typodesign
Edition of 20 copies

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