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Diverzita a fylogeneze archaméb

Diversity and phylogeny of Archamoebae

Disertační práce

Školitel: doc. RNDr. Ivan Čepička, Ph.D.

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Prohlášení:

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V Praze, 22.1.2016

Eliška Zadrobílková

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Obsah

Abstrakt

Zástupci skupiny Archamoebae jsou volně žijící nebo endobiotičtí améboidní bičíkovci nebo měňavky. Protože se vyskytují v anoxickém nebo mikrooxickém prostředí, jejich mitochondrie jsou značně redukované. Zpočátku se dokonce předpokládalo, že mitochondrie postrádají úplně, a proto byly považovány za jedny z nejpůvodnějších eukaryotických organismů vůbec. Tato hypotéza byla později vyvrácena a dnes víme, že archaméby náleží do říše Amoebozoa a spolu s aerobními hlenkami (Macromycetozoa) a sběrným taxonem Variosea vytváří skupinu Conosa.

Charakteristickým znakem bičíkatých archaméb je poměrně jednoduchý mikrotubulární cytoskelet, který se skládá z jednoho bazálního tělíska, ze kterého vychází bičík, postranního kořene a mikrotubulárního koše. U bezbičíkatých zástupců byl tento cytoskelet zcela redukován.

V minulosti bylo vytvořeno asi 350 jmen archaméb na druhové úrovni. Popisy druhů jsou převážně založeny na morfologických znacích, které jsou ale často nedostačující, a proto je identita druhů nejistá a je pravděpodobné, že řada druhů bude v budoucnu synonymizována. Problémem je také nedostatek sekvenčních dat.

V našem projektu se nám podařilo výrazně rozšířit dataset DNA sekvencí převážně volně žijících archaméb. Na základě kombinace molekulárních a morfologických dat jsme popsali 13 nových druhů archaméb. Potvrdili jsme, že rod *Rhizomastix* patří mezi archaméby a že vykazuje nový typ cytoskeletárního uspořádání u této skupiny. Jako první jsme provedli multigenovou analýzu této skupiny. Z našich fylogenetických analýz vyplývá, že se archaméby rozpadají na čtyři hlavní linie: Entamoebidae, Pelomyxidae, Rhizomastixidae a Mastigamoebidae. Ukázali jsme, že rod *Mastigella* je parafyletický, protože *Pelomyxa* představuje jeho vnitřní větev, a že převážně parazitický rod *Entamoeba* je sesterský zbytku archaméb. Z našich výsledků také vyplývá, že společný předek archaméb byl pravděpodobně volně žijící a parazitismus se v této skupině objevil nejméně třikrát nezávisle na sobě.

Abstract

Members of the group Archamoebae are free-living or endobiotic amoeboid flagellates and amoebae. They live in anoxic or microoxic habitats, and their mitochondria have been reduced. They were originally thought to lack mitochondria and represent one of the earliest eukaryotes. However, this hypothesis has been refuted, and now it is evident that the Archamoebae belongs to the lineage Conosa within the supergroup Amoebozoa, together with aerobic slime molds (Macromycetozoa) and variosean amoebae and flagellates.

Relatively simple microtubular cytoskeleton is a characteristic feature of Archamoebae. It consists of a single basal body from which a flagellum arises, lateral root, and microtubular cone. Cytoskeleton of aflagellated genera has been completely reduced.

About 350 species names of Archamoebae have been created so far. However, most descriptions were based on inadequate morphological features. The identity of numerous species is uncertain, and many of them are likely synonymous. Another problem is a small amount of available molecular data.

During our project, we have substantially improved the dataset of DNA sequences of archamoebae. On the basis of molecular and morphological data, we described 13 new species. We showed that genus *Rhizomastix* belongs to Archamoebae and displays a new type of the cytoskeletal arrangement within the group. We carried out the first multigene analysis of Archamoebae with reasonable taxon sampling. On the basis of our phylogenetic analysis, we conclude that Archamoebae splits into four major lineages: Entamoebidae, Pelomyxidae, Rhizomastixidae and Mastigamoebidae, the first one being sister to the rest. We showed that *Pelomyxa* forms an internal branch of paraphyletic *Mastigella*. We suppose that the last common ancestor of Archamoebae was free-living, and the parasitism has evolved at least three times independently within the group.

1. Úvod a cíle práce

Archaméby (Archamoebae) jsou malou skupinou anaerobních nebo mikroaerofilních améb a améboidních bičíkovců, která náleží do eukaryotické říše Amoebozoa. Archaméby žijí endobioticky v trávicím traktu bezobratlých i obratlovců nebo jsou volně žijící a obývají organicky bohaté vodní sedimenty. Buňky archaméb postrádají “stacked“ Golgiho aparát, plastidy a zprvu se předpokládalo, že nemají ani mitochondrie a peroxisomy. Proto byly archaméby považovány za archezoa, tj. za potomky prastarých eukaryotických organismů, které se vyvinuly ještě před vznikem těchto organel (Cavalier-Smith 1983). Později byly u archaméb objeveny anaerobní deriváty mitochondrie, které ale tuto hypotézu vyvrátily (Gill et al. 2007; Tovar et al. 1999). Navíc se začíná uvažovat, že se u archaméb zachovaly také peroxisomy (Žárský 2012). Archaméby jsou známy především díky lidskému patogenu *Entamoeba histolytica*, kterým se dlouhodobě zabývá většina studií o archamébách. Naproti tomu volně žijícím zástupcům nebyla v minulosti věnována příliš velká pozornost, i přesto, že by jejich studium mohlo přispět k pochopení evoluce parazitismu v této skupině.

Původně se archaméby dělily podle způsobu života na dvě skupiny: na volně žijící bičíkaté pelobionty, kam se řadily pelomyxy a mastigaméby, a na endobiotické bezbičíkaté entaméby. S příchodem molekulárně-fylogenetických metod bylo ale zjištěno, že vnitřní vztahy ve skupině budou patrně složitější, než se ze začátku předpokládalo (Cavalier-Smith et al. 2004). Přestože nám první získané sekvence DNA pomohly zhruba nastínit základní členění archaméb, podrobnější fylogeneze skupiny zůstávala nadále skryta. Důvodem, proč se situace příliš nezlepšovala, bylo, že ve středu zájmu zůstaly opět hlavně parazitické entaméby (Silberman et al. 1999). Sekvenční data z volně žijících zástupců nadále chyběla, a proto byl fylogenetický strom značně nevyvážený a neměl příliš velkou informativní hodnotu. Později sice výrazně vzrostlo procento známých DNA sekvencí také dalších archaméb (Edgcomb et al. 2002), doposud ale stále existují rody, ze kterých DNA data chybí nebo jsou jen ojedinělá. Mezi takové případy patří rody *Endamoeba*, *Tricholimax* a *Mastigina*, které byly dlouhou dobu, nebo stále jsou, řazeny mezi archaméby pouze na základě morfologické podobnosti, ale jinak zůstávají prakticky neprobádané. Konkrétně na rod *Rhizomastix* byla zaměřena výraznější pozornost až poměrně nedávno (Cepicka 2011; Ptáčková et al. 2013; Zadrobílková et al. 2015b). Dalším příkladem je rod *Mastigella*, k němuž, jak se později ukázalo, byla přiřazena sekvence DNA patřící jinému organismu (Ptáčková et al. 2013). Záhadou dlouhou dobu zůstával také druh *Mastigamoeba invertens*, který ve starších fylogenetických analýzách často spadal mezi jiné eukaryotické linie a budil dojem, že archaméby nejsou monofyletické (Bolivar et al. 2001; Edgcomb et al. 2002). Později se ale ukázalo, že se vůbec nejedná o archamébu a organismus byl přejmenován na *Breviata anathem*a (Walker et al. 2006).

Pokud pomineme skutečnost, že z většiny výše zmíněných málo prostudovaných rodů jsou k dispozici pouze omezená sekvenční data, s přibývajícím počtem fylogenetických analýz se začíná vynořovat další problém. Pro rekonstrukci fylogeneze zůstává obecně nejpoužívanějším markrem sekvence genu pro molekulu RNA malé ribosomální podjednotky (SSU rDNA). U archaméb se také používá sekvence genu pro aktin (Fahrni et al. 2003; Zadrobílková et al. 2015a, b). Je však evidentní, že fylogenetické stromy založené pouze na těchto dvou genech nejsou schopné vyřešit příbuzenské vztahy mezi hlavními liniemi archaméb a roste potřeba získat alespoň z některých zástupců potřebná data, která by umožnila provést vícegenovou analýzu.

Kromě nedostatku sekvenčních dat, pomocí kterých by mohl být vytvořen kvalitní fylogenetický strom, který by co nejpřesněji mapoval evoluci archaméb, zde existuje ještě další problém. V průběhu dvacátého století bylo formálně popsáno velké množství druhů, které však byly obvykle charakterizovány na základě málo přesvědčivých znaků, jako je tvar buňky nebo počet a pozice kontraktilních vakuol. Vzhledem k tomu, že jsou popisy navíc často neúplné, existence těchto druhů zůstává otázkou (Bernard et al. 2000). Hlavním problémem při definování druhů byla neschopnost autorů dlouhodobě kultivovat jednotlivé izoláty a zachytit jejich morfologickou variabilitu. Je tak velmi pravděpodobné, že některé popsané druhy ve skutečnosti představují pouze vývojová stádia nebo morfologické varianty druhu jiného.

Při prvotním plánování projektu se zdály být naše cíle poměrně jasné. Během jejich plnění jsme především chtěli eliminovat výše zmíněné nedostatky a rozšířit dostupná data o skupině Archamoebae. V průběhu času jsme ale zjistili, že ne všechny cíle jsou snadno dosažitelné.

Cíle dizertační práce:

1. Získat a dlouhodobě kultivovat nové izoláty především volně žijících, ale i endobiotických zástupců archaméb.
2. Získané izoláty porovnat s již popsanými druhy, případně popsat druhy nové.
3. Pomocí molekulárních markerů co nejpřesněji zrekonstruovat fylogenetické vztahy v rámci archaméb a pokusit se zmapovat evoluci parazitismu uvnitř této skupiny.
4. Nalézt platné morfologické znaky hlavních linií archaméb.

2. Stavba buňky archaméb

Archaméby jsou améby nebo amébovití bičíkovci, kteří se pohybují jak pomocí pseudopodií, tak díky bičíku. Pseudopodie jsou eruptivní a jejich tvar je poměrně variabilní. Nejčastěji se ale vytváří prstovité lobopodie a někdy také krátké a tenké filopodie. K pohybu buňky a jejímu přichycení k povrchu výrazně přispívá uroid, který se formuje na zadním konci buňky a může mít cibulovitý nebo klkovitý tvar, vytvářet krátké tenké výběžky nebo mít tvar dlouhého vlákna. U některých druhů je snadno odlišitelná vnější hyalinní vrstva ektoplasmy od vnitřní zrnité endoplasmy, která obsahuje důležité organely a vakuoly (viz Brugerolle a Patterson 2000).

2. 1 Bičíky a mikrotubulární cytoskelet

Počet bičíků se může u jednotlivých skupin archaméb lišit, nicméně obecně lze říci, že zde převládají rody, pro které jsou typické buňky s jedním bičíkem (*Mastigamoeba*, *Mastigella*, *Mastigina*, *Rhizomastix*, *Tricholimax*). Jedná se převážně o volně žijící zástupce. Naproti tomu u parazitických rodů *Entamoeba*, *Endamoeba*, *Endolimax* a *Iodamoeba* došlo pravděpodobně důsledkem jejich způsobu života k sekundární ztrátě jak bičíku, tak přidruženého vnitřního cytoskeletu (Ptáčková et al. 2013). Opačným směrem se ubíraly některé druhy rodu *Pelomyxa*, u kterých se naopak bičíky zmnožily a jejich buňky jich mohou obsahovat desítky až stovky (Chistyakova a Frolov 2011; Frolov et al. 2005, 2006, 2011). Přestože bylo řečeno, že pro rody *Mastigella* a *Rhizomastix* je typická přítomnost jediného bičíku, buňky typového druhu rodu *Mastigella*, *M. polymastix*, mohou obsahovat až čtyři bičíky (Frenzel 1897) a u druhu *R. biflagellata* převládají dvoubičíkaté buňky (Cepicka 2011). Naproti tomu rod *Pelomyxa* zahrnuje druh *P. corona*, který je nejspíš bezbičíkatý (Frolov et al. 2004). Navíc ve skupině Archamoebae existuje velká variabilita forem jednotlivých druhů v průběhu životního cyklu a není výjimkou, pokud se např. jinak typicky jednobičíkatý druh vyskytuje ve formě améby nebo jeho buňka obsahuje dva bičíky (Chávez et al. 1986; Ptáčková et al. 2013; Simpson et al. 1997).

Pokud pomineme počet bičíků, kanonické uspořádání bičíkatého aparátu archaméb zahrnuje jediné bazální tělísko, ze kterého vychází ven z buňky bičík (jedná se tedy o monokinetidu) a směrem dovnitř buňky postranní kořen a mikrotubulární koš neboli konus (Brugerolle 1982; Simpson et al. 1997; Walker et al. 2001). Cytoskelet mnohobičíkatých zástupců rodu *Pelomyxa* se skládá z více samostatných monokinetid (Frolov et al. 2005; Chistyakova et al. 2014; Seravin a Goodkov 1987). Vzájemná pozice mikrotubulárního koše a jádra je důležitým diagnostickým znakem. U rodů *Mastigamoeba* a *Tricholimax* je konus asociovaný s jadernou membránou (Brugerolle 1982; Chistyakova et al. 2012; Frenzel 1897; Simpson et al. 1997; Walker et al. 2001), zatímco v případě rodu *Mastigella* spojení mezi mikrotubulárním košem a jádrem chybí a obě struktury jsou od sebe v buňce poměrně vzdáleny (Goldschmidt 1907; Walker et al. 2001).

Pro rod *Mastigamoeba* platí, že zde existují dva typy uspořádání mastigontu, která korelují s fylogenezí. Pro skupinu Mastigamoebidae A je typické, že mikrotubuly koše vycházejí ze stran bazálního tělíska, přechodová zóna bičíku je dlouhá a může obsahovat denzní sloupek. Naproti tomu zástupcům skupiny Mastigamoebidae B odstupuje mikrotubulární koš podélně z blízkosti báze bazálního tělíska, přechodová zóna bičíku je krátká a neobsahuje žádné další elementy (Pánek et al., *in press*).

Uspořádání bičíkatého aparátu rodu *Pelomyxa* je variabilní, ale jeho základní typy lze také rozdělit do dvou hlavních skupin. Pro první je typické dlouhé bazální tělísko (např. *P. gruberi, P. flava*) (Frolov et al. 2006, 2011) a druhá skupina je charakteristická krátkým bazálním tělískem a náhodným vnitřním uspořádáním bičíku (např. *P. binucleata*, *P. palustris*, *P. stagnalis*) (Chistyakova a Frolov 2011; Frolov et al. 2005, 2007). Dále zde existuje nejméně jedna přechodná forma reprezentovaná druhem *P. paradoxa* (Chistyakova et al. 2014). Ať už buňky obsahují desítky nebo stovky bičíků, z každého bazálního tělíska bičíku vychází vlastní mikrotubulární koš.

Zdaleka ne všechny rody archaméb jsou dopodrobna prostudovány natolik, abychom znali přesné vnitřní uspořádání jejich buňky. Na příklad rod *Rhizomastix*, jehož první studie ultrastruktury byla publikována teprve v roce 2013 (Ptáčková et al. 2013), byl do této skupiny zprvu zařazen pouze na základě podobnosti morfologie buněk a jádra (Cepicka 2011). Dlouhou dobu tedy nebylo možné blíže specifikovat rhizostyl, který je pro tento rod typický. Na základě pozorování ve světelném mikroskopu bylo pouze možné tvrdit, že se jedná o jakousi fibrilu, která vychází z bazálního tělíska bičíku a obvykle vede okolo jádra až k zadnímu konci buňky (Alexeieff 1911). Díky zmíněné ultrastrukturní studii se prokázalo, že je tato fibrila složená z mikrotubulů a pravděpodobně je pozůstatkem mikrotubulárního koše, jak ho známe např. u rodu *Mastigamoeba* (Ptáčková et al. 2013). Zajímavé je, že i v rámci rodu *Rhizomastix* nalezneme určitou variabilitu v uspořádání mastigontu. Jedná se především o rozdílné umístění rhizostylu v buňce, variabilní počet mikrotubulů, kterými je rhizostyl tvořen nebo přítomnost druhého postranního kořene u druhu *R. elongata* (Ptáčková et al. 2013; Zadrobílková et al. 2015b). Tyto rozdíly by mohly souviset s odlišným způsobem života jednotlivých druhů – bylo pozorováno, že bičík endobiotických druhů je delší a méně pohyblivý než bičík volně žijících druhů (Zadrobílková et al. 2015b). Zatím nejsou k dispozici data, která by detailně popisovala mastigont rodu *Mastigina*.

Pokud jsou buňky archaméb opatřeny bičíkem, jeho vnitřní struktura a také pohyblivost může být různá. Bičíky rodů *Mastigamoeba* a *Mastigella* mají typické eukaryotické uspořádání axonemy „9x2+2“, avšak chybí zde vnější dyneinová raménka (Walker et al. 2001). Pravděpodobně důsledkem zmíněné absence není pohyb bičíku příliš rychlý a především v případě rodu *Mastigella* je často spíše mdlý a nevýrazný (van Bruggen et al. 1985; Walker et al. 2001; Zadrobílková et al. 2015a). Stejně tak rody *Tricholimax* a *Pelomyxa* mají bičíky jen málo pohyblivé nebo zcela nepohyblivé, postrádající vnější dyneinová raménka, což by mohlo ukazovat na případnou příbuznost těchto tří rodů (Zadrobílková et al. 2015a). Axonema rodů *Tricholimax* a *Pelomyxa* se ale navíc vyznačuje variabilním počtem a uspořádáním centrálních a periferních mikrotubulů (Brugerolle 1982; Chistyakova a Frolov 2011; Frolov et al. 2005, 2006, 2007, 2011; Griffin 1988). Bičík archaméb se pohybuje od špičky k bázi, stejně jako u většiny ostatních protist. Důsledkem toho je anterokontní pohyb, což znamená, že buňky archaméb mají tažný bičík směřující dopředu před buňku.

2. 2 Anaerobní deriváty mitochondrie

Už ze samotného názvu skupiny vyplývá, že byly archaméby („praměňavky“, v češtině se však používá spíš jméno panoženky) zprvu považovány za velmi staré organismy. Původně se předpokládalo, že jejich buňky neobsahují mitochondrie, peroxisomy a plně vyvinutý Golgiho systém, a proto byly dokonce považovány za nejstarší eukaryota vůbec, která se vyvinula ještě před vznikem těchto organel. Společně se skupinami Parabasalia, Metamonada a Microsporidia byly řazeny do říše Archezoa (Cavalier-Smith 1983). Tato hypotéza se však ukázala jako neplatná, když byly postupně u bývalých archezoí, včetně archaméb, nalézány různé typy mitochondriálních derivátů. Na základě lokalizace typických mitochondriálních proteinů, jako je chaperonin cpn60 a protein teplotního šoku Hsp60, pomocí ultrastrukturních fotografií a studiem metabolismu byl charakterizován mitosom rodu *Entamoeba histolytica* (Chan et al 2005; Clark a Roger 1995; Ghosh et al. 2000; León-Avila a Tovar 2004; Mai et al. 1999; Mi-ichi et al. 2011; Tovar et al 1999). Jedná se o poměrně malou organelu odvozenou od mitochondrie, která přišla o vlastní genom a jejíž energetický metabolismus je redukovaný a nepodílí se na produkci ATP (Müller et al. 2012). Váčky obalené dvojitou membránou, které by mohly mít mitochondriální původ, byly pozorovány také u mnoha dalších archaméb, a to konkrétně u druhů *Endolimax piscium*, *Mastigamoeba punctachora*, *Mastigamoeba schizophrenia*, *Mastigamoeba simplex*, *Mastigella commutans*, *Mastigella ineffigiata*, *Mastigella rubiformis*, *Pelomyxa palustris*, *Rhizomastix elongata* a *Rhizomastix libera* (Constenla et al. 2013; Ptáčková et al. 2013; Seravin and Goodkov 1987; Simpson et al 1997; Walker et al. 2001; Zadrobílková et al. 2015a, b). Volně žijícím modelovým druhem archaméb, na kterém jsou dlouhodobě studovány také biochemické dráhy uvnitř mitochondriálního derivátu, je *Mastigamoeba balamuthi*. Zjistilo se, že metabolismus jeho derivátů mitochondrie je komplexnější než u *Entamoeba histolytica* a že zde jsou lokalizovány některé proteiny, které jsou charakteristické pro hydrogenosom (Gill et al. 2007; Nývltová et al. 2013). Jedná se především o pyruvát:ferredoxin oxidoreduktázu (PFO) a Fe-hydrogenázu (Gill et al. 2007). PFO dekarboxyluje pyruvát, který je koncovým produktem glykolýzy, za vzniku CO_2 a acetyl-CoA, a zároveň redukuje ferredoxin, který přenáší elektrony na Fe-hydrogenázu. Hydrogenáza předává elektrony vodíkovým kationtům za vniku plynného vodíku (Müller et al. 2012).

Klíčovou roli v metabolismu eukaryotických organismů, ale také prokaryot, hrají proteiny obsahující železo-sirné (Fe-S) klastry. Ty se účastní mnoha enzymatických reakcí v buňce a nalezneme je v mitochondrii, cytoplasmě, jádře a v plastidech (viz Tsaousis et al. 2012). Na skládání těchto klastrů se u eukaryotických organismů mohou podílet tři různé systémy bakteriálního původu: mitochondriální železo-sirný systém (ISC) pocházející od α-proteobakterie (Tachezy a Dolezal 2007), plastidový systém mobilizace síry (SUF) pocházející od sinice (Ye et al. 2006) a systém fixace dusíku (NIF), který má ε-proteobakteriální původ (Ali et al. 2004; van der Giezen et al. 2004). Za ancestrální stav je považována přítomnost systému ISC, který je exprimován v mitochondrii převážné většiny eukaryotických organismů (Tsaousis et al. 2012). Jinak je tomu ale u archaméb, kde byl nalezen systém NIF (Ali et al. 2004; Dolezal et al. 2010; Gill et al. 2007; Mi-ichi et al. 2009; Nývltová et al. 2013; van der Giezen et al. 2004). U druhu *Mastigamoeba balamuthi* bylo prokázáno, že je tento systém lokalizovaný jak v mitochondrii, tak v cytoplasmě (Nývltová et al. 2013; 2015). U druhu *Entamoeba histolytica* se ale výsledky poněkud rozcházejí. Existuje studie, která u tohoto druhu dokazuje duální lokalizaci NIF systému (Maralikova et al. 2010), zatímco jiní autoři zjistili jeho přítomnost především v cytoplasmě a distribuce v mitosomu nebyla jednoznačně prokázána (Dolezal et al. 2010; Mi-ichi et al. 2009; Nývltová et al. 2013, 2015). Na základě dostupných dat lze předpokládat, že NIF systém byl přítomný již u posledního společného předka archaméb (Pánek et al., *in press*). Ten byl pravděpodobně nejprve získán laterálním genovým transferem (LGT) od ε-proteobakterie a poté došlo k duplikaci genů kódujících komponenty systému, takže byla jedna kopie přítomná v cytoplasmě a druhá v hydrogenosomu (Nývltová et al. 2013, 2015). Během přestavby hydrogenosomu na mitosom následně došlo u *E. histolytica* ke ztrátě hydrogenosomální kopie (Nývltová et al. 2015). Alternativní možností je, že k duplikaci NIF systému došlo pouze v linii vedoucí k *M. balamuthi* (Nývltová et al. 2015).

Další zajímavostí, která byla dosud nalezena pouze u derivátů mitochondrie některých archaméb a jinak u žádných dalších mitochondrií, je přítomnost dráhy aktivace sulfátu (Mi-ichi et al. 2009; Nývltová et al. 2015). U *E. histolytica* bylo prokázáno, že některé enzymy, které se účastní aktivace sulfátu, hrají významnou roli při tvorbě sulfolipidů a buněčné proliferaci (Mi-ichi et al. 2011). Tato dráha, podobně jako NIF systém, byla pravděpodobně přítomna již u posledního společného předka archaméb (Pánek et al., *in press*), který ji získal laterálním genovým transferem od proteobakterií (Mi-ichi et al. 2009).

2. 3 Jádro a jeho struktura

Počet jader v buňce archaméb je poměrně variabilní, ale obecně lze říct, že obvykle obsahují jader málo, nejčastěji pouze jedno. Tento stav je typický pro bičíkaté trofozoity rodů *Mastigamoeba*, *Mastigella*, *Mastigina*, *Rhizomastix* a *Tricholimax*. Samozřejmě, že ne všichni zástupci výše jmenovaných rodů jsou jednojaderní. Mezi výjimky patří *Mastigamoeba schizophrenia*, která je typická svými dvěma navzájem se dotýkajícími jádry (Simpson et al. 1997). Stejně tak trofozoiti rodu *Mastigella erinacea* jsou nejčastěji dvoujaderní (Zadrobílková et al. 2015a). *Mastigamoeba balamuthi* sice vytváří jednojaderné trofozoity, ale v kultuře ji nalezneme spíše ve formě vícejaderných bezbičíkatých plasmodií (Chávez et al. 1986). Mnohojaderné stádium se může vyskytovat také v životním cyklu *Mastigamoeba aspera* a předpokládá se, že je typické pro mnoho pelobiontů (Bernard et al. 2002; Chystyakova et al. 2012).

U některých druhů rodu *Pelomyxa* došlo k tak masivnímu zvýšení počtu jader, že jich jediná buňka může čítat až stovky (Whatley a Chapman-Andresen 1990). Výjimkou jsou druhy jako *P. binucleata*, *P. flava*, *P. gruberi*, *P. paradoxa* a *P. schiedti*, které mají nejčastěji dvě nebo jen jedno jádro, přestože v životním cyklu vytváří i vícejaderná stádia (Chistyakova et al. 2014; Frolov et al. 2005, 2011, 2006; Zadrobílková et al. 2015a).

Trofozoiti entaméb jsou obvykle jednojaderní, ale pro diagnostiku, a to především druhů vyskytujících se u člověka, jsou významné hlavně jejich cysty (Fotedar et al. 2007). Cysty jsou čtyřjaderné (*E. histolytica*, *E. hartmanni*), osmijaderné (*E. coli*) nebo mají jádro pouze jedno (*E. polecki*) (Burrows 1959; Wenyon 1926). *E. gingivalis*, kterou můžeme nalézt v ústní dutině, cysty nevytváří vůbec (Wenyon 1926). Čtyřjaderné cysty má také druh *Endolimax nana* (Wenyon 1926). Pro rod *Rhizomastix* jsou charakteristické cysty se dvěma jádry, která se navzájem nedotýkají (Alexeieff 1911; Cepicka 2011; Zadrobílková et al. 2015b), na rozdíl od také dvoujaderných cyst druhu *Mastigamoeba schizophrenia* (Simpson et al. 1997). Cysty ostatních archaméb byly obvykle pozorované jen zřídka nebo vůbec (Bernard et al. 2000; Frolov et al. 2007; Ptáčková et al. 2013; Stensvold et al 2012; Zadrobílková et al. 2015a; Zaman et al. 1998).

Také uspořádání chromatinu uvnitř jádra není u všech archaméb stejné. Pro většinu zástupců je obvyklá přítomnost centrálního jadérka různé velikosti (Brugerolle 1991; Constenla et al. 2014; Goldschmidt 1907; Walker et al. 2001), které je u rodů *Rhizomastix* a *Entamoeba* navíc obklopeno periferními chromatinovými granulemi (Alexeieff 1911; Martínez-Palomo 1993; Ptáčková et al. 2013; Zadrobíková et al. 2015b). Zcela netypickou morfologii jádra nalezneme u rodu *Endamoeba*, které je oválné a charakteristické centrálním alveolárním útvarem ohraničeným silnou vrstvou velkých chromatinových granulí (Wenyon 1926). Největší variabilitu ve struktuře jader nalezneme u rodu *Pelomyxa*. Velmi často je uspořádání chromatinu v jádře chaotické bez jasného vzoru (Chistyakova a Frolov 2011; Chistyakova et al. 2014; Frolov et al. 2005, 2006, 2011; Griffin 1988). Někdy jsou chromatinové granule umístěny periferně (Frolov et al. 2007) nebo mohou jádra obsahovat pouze malý počet drobných jadérek (Frolov et al. 2004). U některých zástupců rodu *Pelomyxa* byla navíc uvnitř jader nalezena blíže nespecifikovaná tělíska různého tvaru (Chistyakova a Frolov 2011; Chistyakova et al. 2014). Pro druh *Mastigamoeba punctachora* je typická přítomnost extranukleární granule (Bernard et al. 2000; Ptáčková et al. 2013; Walker et al. 2001).

Na fotografiích struktury jader druhu *Pelomyxa schiedti* bylo pozorováno drobné, pod světelným mikroskopem velmi obtížně rozeznatelné jadérko spolu s periferním chromatinem. Stejná struktura jádra je přítomna také u druhu *Mastigella rubiformis* (Zadrobílková et al. 2015a), což by mohlo naznačovat, že variabilita v uspořádání chromatinu v jádře může být charakteristická pro celou čeleď Pelomyxidae. U dalších doposud popasných zástupců rodu *Mastigella* totiž nalezneme pro archaméby nejčastější uspořádání jádra s jedním velkým centrálním jadérkem (Walker at al. 2001; Zadrobílková et al. 2015a).

3. Systém a taxonomie archaméb

V průběhu dvacátého století bylo popsáno velké množství druhů archaméb (viz Supplementary Table S1 v Ptáčková et al. 2013). Původní popisy ale obvykle neobsahovaly detailní informace založené na dlouhodobém pozorování, a proto byly často nekompletní. To znamenalo, že je velmi nesnadné spolehlivě určit nové nálezy pelobiontů a zařadit je do již existujících druhů. Takto vznikala stále nová druhová jména a celá systematika archaméb je poměrně zmatená. Předpokládá se, že velké množství druhů bude v budoucnosti synonymizováno (Bernard et al. 2000). Navíc velká míra polymorfismu a pleomorfismu, která je přítomna u jednotlivých druhů, pravděpodobně způsobila, že každá odchylka tvaru nebo velikosti buňky byla zaznamenána jako nový druh (viz Simpson et al. 1997). Na přelomu 20. a 21. století se potvrdilo, že variabilita bičíkatých archaméb je opravdu vysoká (Bernard et al. 2000; Walker et al. 2001) a že k lepšímu pochopení systému skupiny Archamoebae bude potřeba použít také molekulární markery (Edgcomb et al. 2002). Dále se ukázalo, že pro morfologické srovnání jednotlivých druhů mezi sebou je nejvhodnější se detailně zaměřit především na tzv. „gliding“ neboli klouzavé formy buněk. Tyto formy mají v rámci stejného druhu obvykle poměrně stabilní velikost a tvar buněk a lze na nich nejlépe pozorovat významné diagnostické znaky (Ptáčková et al. 2013).

Mezi archaméby se v současné době zahrnují rody *Mastigamoeba*, *Mastigella*, *Mastigina*, *Tricholimax*, *Pelomyxa*, *Endolimax*, *Iodamoeba*, *Entamoeba* a *Endamoeba* (Adl et al. 2012; Stensvold et al. 2012) a nově také rod *Rhizomastix* (Cepicka 2011; Ptáčková et al. 2013).

3. 1 Rod *Mastigamoeba* Schulze, 1875

Rod *Mastigamoeba* zahrnuje především jednobičíkaté archaméby, pro které je charakteristická přítomnost jediného bazálního tělíska, ze kterého vychází bičík, jeden postranní mikrotubulární kořen a kužel mikrotubulů, který je v kontaktu s jediným jádrem přítomným v buňce. Pohyb může být zajištěn jak panožkami, tak bičíkem, který je při plavání obvykle nasměrován dopředu. Bičíkaté stádium rodu *Mastigamoeba* může za určitých podmínek bičík ztratit a přeměnit se do stádia améby, nebo naopak bičík opět vytvořit (Bernard et al. 2000; Chávez et al. 1986; Walker et al. 2001). Za jakých podmínek k této přeměně dochází, zatím není jasné. Za nepříznivých podmínek můžou buňky rodu *Mastigamoeba* přežívat ve formě jednojaderných cyst, které ale byly zatím pozorovány jen u několika druhů (Bernard et al. 2000; Chávez et al. 1986; Simpson et al. 1997). Tento rod může vytvářet široké spektrum různých forem, z nichž jsou pro morfologický popis nejvýznamější především gliding formy (Ptáčková et al. 2013). Typovým druhem rodu *Mastigamoeba* je *M. aspera* Schulze, 1875, který vykazuje charakteristické znaky rodu *Mastigamoeba*, jako je převaha jednojaderného a jednobuněčného stádia v životním cyklu, tvorba prstovitých panožek nebo typická organizace jádra a bičíkatého aparátu (Chystyakova et al. 2012). Penard (1909) se domníval, že se pravděpodobně jednalo o stejný organismus, který popsal Leidy (1874) jako *Dinamoeba mirabilis* (tedy o rok dříve, než Schulze popsal *Mastigamoeba aspera*), což později podporují také Chystyakova et al. (2012). Podle mezinárodních pravidel zoologické nomenklatury by mělo mít starší jméno prioritu (International Commission for Zoological Nomenclature 1999), a jména *Mastigamoeba* a *Mastigamoeba aspera* by proto měla být mladší synonyma jmen *Dinamoeba* a *Dinamoeba mirabilis*. Leidyho pojmenování se ale příliš nevžilo, a proto se pro označení rodu používá jméno *Mastigamoeba*.

Druh *Mastigamoeba aspera* se vyznačuje také některými vlastnostmi, které u jiných zástupců rodu *Mastigamoeba* obvykle nenajdeme. Kromě přítomnosti glykokalyxu, který je charakteristický spíše pro rod *Pelomyxa* (Chystyakova et al. 2012), se jedná o velikost buněk, kdy jejich délka může dosahovat až 250 µm a šířka 50 – 100 µm, což mnohonásobně převyšuje průměrnou velikost buněk všech ostatních doposud známých zástupců rodu *Mastigamoeba* (Bernard et al. 2000; Chystyakova et al. 2012; Simpson et al. 1997; Walker et al. 2001). Z typového druhu ale zatím chybí jakákoliv sekvenční data.

3. 2 Rod *Mastigella* Frenzel, 1897

Zástupci rodu *Mastigella* jsou nejčastěji jednobičíkatí a jejich mastigont se podobá rodu *Mastigamoeba*. Narozdíl od něj zde však chybí jakékoliv spojení nebo blízká poloha mikrotubulárního koše a jádra buňky (Goldschmidt 1907). Bičík plovoucích buněk obvykle vyrůstá z tenkého výběžku hyaloplasmy v přední části buňky. Trofozoiti některých druhů se mohou přeměnit na améby s jedním nebo více jádry. V životním cyklu rodu *Mastigella* byly také pozorovány cysty (viz Frenzel 1897). Stejně jako je tomu u druhu *Mastigamoeba aspera*, nejsou doposud z typového druhu, *Mastigella polymastix* Frenzel, 1897, k dispozici žádná sekvenční data.

Jelikož mají rody *Mastigamoeba* a *Mastigella* velmi podobnou morfologii, předpokládalo se, že jsou oba tyto rody sesterské. Na základě toho byly tradičně řazeny do společné čeledi Mastigamoebidae (Adl et al. 2012; Cavalier-Smith et al. 2004; Chatton

1925; Goldschmidt 1907; Griffin 1988). Blízkou příbuznost rodů *Mastigella* a *Mastigamoeba* potvrzují také některé fylogenetické analýzy, které zahrnují po dlouhou dobu jedinou známou DNA sekvenci z druhu *Mastigella commutans* (Cavalier-Smith et al. 2004; Edgcomb et al. 2002; Lahr et al. 2011; Nikolaev et al. 2006; Stensvold et al. 2012). Později se ale ukázalo, že tato sekvence patří pravděpodobně druhu *Mastigamoeba punctachora* (Ptáčková et al. 2013). Podle struktury jádra a přítomnosti endosymbiotických prokaryot se někteří autoři domnívali, že *Mastigella* se spíše podobá rodu *Pelomyxa* (Cavalier-Smith 1991; Frolov 2011; van Bruggen et al. 1985; Walker et al. 2001). Tato hypotéza byla potvrzena nedávnou fylogenetickou analýzou založenou na genech pro SSU rDNA a pro aktin stejně tak první multigenovou analýzou archaméb (Pánek et al., *in press*; Zadrobílková et al. 2015a).

3. 3 Rod *Mastigina* Frenzel, 1897

Rod *Mastigina* s typovým druhem *Mastigina chlamys* (Frenzel 1897) nebyl v původním popisu příliš dobře odlišený od ostatních pelobiont. Goldschmith (1907) poté definoval tento rod na základě slimákovité neboli limax formy buněk, kde se jádro nachází na předním konci buňky a téměř se dotýká báze bičíku. Ten je obvykle velmi málo pohyblivý. Charakteristická je také absence postranních panožek a přítomnost fontánovitého proudění cytoplasmy (Goldschmith 1907). Později ale Lemmerman (1914) použil jméno *Mastigina* jako mladší synonymum pro rod *Mastigamoeba* a vytvořil novou kombinaci *Mastigamoeba chlamys*, a to i přesto, že se rod *Mastigamoeba* nevyznačuje fontánovitým prouděním cytoplasmy. Vnesl tak zmatek do charakteristiky jak rodu *Mastigina*, tak *Mastigamoeba*.

Do rodu *Mastigina* byl často řazen také rod *Tricholimax*, proto studie ultrastruktury druhu *Tricholimax hylae* byla prezentována pod jménem *Mastigina hylae* (Brugerolle 1982). Na morfologii *T. hylae* byl založen také souhrnný popis rodu *Mastigina* (Brugerolle a Patterson 2000). Z toho vyplývá, že dodnes nebyla ve skutečnosti publikována žádná ultrastrukturní data rodu *Mastigina* a stejně tak nejsou k dispozici ani data sekvenční. O existenci a fylogenetické pozici tohot rodu v systému archaméb lze tedy pouze spekulovat.

3. 4 Rod *Tricholimax* Frenzel, 1897

Rod *Tricholimax* zahrnuje jediný druh *Tricholimax hylae* Frenzel, 1897, který byl několikrát nalezen ve střevě žáby (např. Becker 1925; Frenzel 1897). Jeho buňky se vyznačují slimákovitým tvarem buňky bez postranních pseudopodií a fontánovitým prouděním cytoplasmy. Na zadním konci buňky se vytváří uroid, pomocí kterého se organismus přichycuje k podkladu (Becker 1925). Bičík je v těsném kontaktu s jádrem a je velmi krátký a nepohyblivý. Z bazálního tělíska vychází struktura, která připomíná rhizostyl některých endobiotických bičíkovců (Becker 1925; Frenzel 1897). Z fotografií z transmisního elektronového mikroskopu je patrné, že mastigont druhu *T. hylae* se skládá z mikrotubulárního koše, který je pomocí mikrofibril spojený s jadernou membránou, a postranního kořene mikrotubulů. Axonema bičíku je složena z neuspořádaného svazku mikrotubulů (Brugerolle 1982). Aberantní uspořádání mikrotubulů axonemy, nepohyblivost bičíku a monopodiální tvar buňky jsou typické pro rod *Pelomyxa*. Ani zde však nejsou k dispozici molekulární data, která by potvrdila možnou blízkou příbuznost rodů *Tricholimax* a *Pelomyxa*.

3. 5 Rod *Pelomyxa* Greeff, 1874

Rod *Pelomyxa* je obvykle charakterizován jako poměrně velká mnohojaderná améba s monopodiálním pohybem, uroidem na zadním konci buňky a jedním nebo více nepohyblivými bičíky (Whatley a Chapman-Andresen 1990). Bičík má různý počet mikrotubulů a i jejich uspořádání je variabilní (Chistyakova a Frolov 2011; Frolov et al. 2005, 2006, 2007, 2011; Griffin 1988). V buňkách jednotlivých druhů jsou velmi často přítomny různé morfologické typy prokaryotických endosymbiontů (Frolov et al. 2005, 2006, 2011; Griffin 1988; Gutiérrez 2012; Whatley a Chapman-Andresen 1990), nicméně jejich přesná funkce není zatím zcela objasněna.

Typovým druhem je *Pelomyxa palustris* Greeff, 1874, jehož identita je ale otázkou, protože se předpokládá, že se ve skutečnosti jedná o druhový komplex (Frolov et al. 2004; Goodkov et al. 2004). Na druhou stranu organismy popsané jako samostatné druhy, u kterých nebyl pozorován kompletní životní cyklus, mohou představovat pouze určitá vývojová stádia *P. palustris* a dalších druhů (Zadrobílková et al. 2015a). Tím že rod *Pelomyxa* zahrnuje jedno- až mnohojaderné jedince s různým počtem bičíků, proměnlivou velikostí a různou morfologií jádra, je definice jednotlivých druhů bez použití molekulárních dat poměrně problematická. Až studie z posledních několika let výrazněji zvýšily počet známých DNA sekvencí z rodu *Pelomyxa* (Ptáčková et al. 2013; Zadrobílková et al. 2015a, Pánek et al., *in press*)

3. 6 Rod *Entamoeba* Casagrandi & Barbagallo, 1895

Rod *Entamoeba* zahrnuje bezbičíkaté améby, u kterých došlo ke kompletní ztrátě mikrotubulárního cytoskeletu. Trofozoiti jsou obvykle jednojaderní a pohybují se pomocí lobopodií (Martínez-Palomo 1993). Převážná většina druhů entaméb jsou endobiotické organismy, kteří se mezi hostiteli přenášejí pomocí cyst. Jediným druhem, který nevytváří cysty, je *Entamoeba gingivalis*, která může u lidí způsobovat periodontitidu, zánětlivé onemocnění dutiny ústní, kdy se trofozoiti přenáší přímo pomocí slin (Bonner et al. 2014). Zatím pouze u tří druhů entaméb bylo zjištěno, že mohou být fakultativně nebo obligátně volně žijící. Nejznámější *Entamoeba moshkovskii* byla opakovaně nalezena jak v odpadních vodách, tak u pacientů s gastrointestinálními problémy (Heredia et al. 2012; Fotedar et al. 2007). Druhý druh, *Entamoeba ecuadoriensis*, byl izolovaný pouze jednou, a to z odpadních vod (Clark a Diamond 1997). Nejnověji objevená *Entamoeba marina* byla nalezena v sedimentu přílivové zóny moře poblíž ústí řeky Shiira v Japonsku (Shiratori a Ishida 2015).

Nejznámějším a z hlediska patogenity pro člověka nejvýznamnějším zástupcem rodu *Entamoeba* je druh *E. histolytica*, který způsobuje střevní potíže a ve vážných případech také jaterní absces. Nejvyšší procento výskytu amébové dysenterie je především v rozvojových zemích tropického pásma a udává se, že je druhou nejčastější parazitární příčinou úmrtí na světě vůbec (Isea et al. 2012). Každoročně lze zaznamenat kolem deseti autochtonních případů onemocnění dokonce i na území ČR (Hůzová a Tolarová 2012). Typovým druhem je *Entamoeba coli* Grassi, 1879, která byla poprvé pozorována Fedorem Löschem jako domnělý původce dysenterie. (viz Issa 2014).

3. 7 Rod *Endamoeba* Leidy, 1879

Málo známý rod *Endamoeba* zahrnuje amébovité mikroorganismy, které žijí endobioticky ve střevech hmyzu. Díky netypické struktuře jádra jej lze poměrně dobře odlišit od rodu *Entamoeba*. V jádře rodu *Endamoeba* lze na první pohled zřetelně rozlišit dvě strukturně odlišné zóny. Je zde přítomna periferní zóna, kde se vyskytují chromatinová granula, a centrální zóna připomínající svým vzhledem vakuolu (Wenyon 1926). Přesto především v první polovině 20. století panovala jistá nejednotnost v užívání rodových jmen *Endamoeba* a *Entamoeba*, která byla volně zaměňována. Důsledkem je nejistá platnost některých druhových jmen. Později byl ale koncept používání jmen ustálen a *Endamoeba* je považována za samostatný rod (Patterson et al. 2000).

Typový druh *Amoeba blattae* Bütschli, 1878 je améba poprvé izolovaná ze švába *Periplaneta orientalis*, kterou o rok později od prvního nálezu Leidy přeřadil do rodu *Endamoeba* (Leidy 1879). Přestože jsou známy i další druhy, doposud nebyla ze žádného z nich publikována sekvenční data. Přesná pozice rodu *Endamoeba* na fylogenetickém stromě archaméb proto zůstává neznámá.

3. 8 Rod *Endolimax* Kuenen & Swellengrebel, 1917

Trofozoiti rodu *Endolimax* svojí morfologií připomínají rod *Entamoeba*. Navzájem se tyto dva rody liší strukturou jádra, kdy u rodu *Endolimax* nenalezneme periferní chromatinové granule. Jedná se o bezbíčíkaté améby, které žijí obvykle jako komenzálové trávicího traktu obratlovců i bezobratlých živočichů (Wenyon 1926). Kvůli zmiňované morfologické podobnosti s entamébami byl rod *Endolimax* tradičně považován za jejich blízkého příbuzného, a byl proto řazen do čeledi Entamoebidae. Fylogenetické analýzy však ukázaly, že tomu tak není a že je ve skutečnosti vnitřní skupinou bičíkatého, volně žijícího rodu *Mastigamoeba* (Cavalier-Smith et al. 2004; Fiore-Donno et al. 2010; Shadwick et al. 2009).

Typový druh *Endolimax nana* Wenyon & O'Connor, 1917 je jedním z nejčastěji se vyskytujících střevních prvoků člověka s nejvyšší prevalencí výskytu v tropickém a subtropickém podnebném pásu (Shah et al. 2012). Na rozdíl od *Entamoeba histolytica* není schopný napadnout okolní tkáně střeva a obvykle přežívá v trávicím traktu jako neškodný komenzál (Wenyon 1926). Výjimku tvoří imunosuprimovaní pacienti, pro které je *E. nana* patogenní a způsobuje u nich průjmová onemocnění. V poslední době jsou ale diskutovány možné projevy infekce jako např. chronické průjmy také u jinak zdravých jedinců (Shah et al. 2012).

3. 9 Rod *Iodamoeba* Dobell, 1919

Rod *Iodamoeba*, konkrétně typový druh *Iodamoeba butschlii* (Dobell, 1919), byl poprvé izolován z člověka. Jeho améby se tvarem a přítomností většího množství trávicích vakuol podobají menším jedincům *Entamoeba coli*, od kterých se ale na první pohled liší jádrem, které stejně jako jádro rodu *Endolimax* postrádá periferní chromatinové granule (Zaman et al. 1998). Trofozoiti rodu *Iodamoeba* mají také podobnou velikost jako améby druhu *Endolimax nana*, se kterými by se tak daly snadno zaměnit. *E. nana* má ale odlišnou především morfologii cyst (Dobell 1919). Detailní ultrastruktura jádra rodu *Iodamoeba* byla pozorována právě u cyst, kde je přítomný nukleolus, obklopený několika shluky elektrondenzního materiálu (Zaman et al. 1998). Cysty se vyznačují typickou inkluzí různé velikosti, která se po ponoření do roztoku jódu obarví. Jedná se o masu glykogenu, která nemusí být v cystě vždy přítomna a jejíž velikost se může v průběhu několika dnů měnit (Dobell 1919). Tento útvar se často chybně označuje jako jodoformní vakuola, přestože není obalen membránou (Zaman et al. 1998). Z rodu *Iodamoeba* dlouhou dobu neexistovala žádná DNA data, a byla proto na základě morfologické podobnosti spolu s rodem *Endolimax* řazena mezi entaméby. Na základě první získané sekvence DNA se ukázalo, že rod *Iodamoeba* je blízce příbuzný rodu *Endolimax* a spolu s ním tvoří vnitřní linii rodu *Mastigamoeba* (Ptáčková et al. 2013; Stensvold et al. 2012; Zadrobílková et al. 2015a, b).

3. 10 Rod *Rhizomastix* Alexeieff, 1911

Rod *Rhizomastix* zahrnuje bičíkaté améby, které nalezneme především jako komenzály bezobratlých i obratlovců. Buňky tohoto rodu se vyznačují přítomností tzv. rhizostylu, který vychází z bazálního tělíska bičíku a pokračuje dále často až k zadnímu konci buňky. Typické je pro rod *Rhizomastix* také jádro s velkým centrálním jadérkem a periferním heterochromatinem (Alexeieff 1911, Mackinnon 1913, Cepicka 2011). Rod *Rhizomastix* byl původně považován za blízkého příbuzného rodu *Cercomonas*, se kterým sdílí právě podobnou strukturu jádra a cytoplasmy (Alexeieff 1911, Mackinnon 1913). Již Kudo (1939) a později Cepicka (2011) upozorňují, že velmi podobnou morfologii jádra nalezneme také u rodu *Entamoeba* a že i další znaky jako je anaerobiosa, jeden přední bičík, hyalinní cytoplasma a tvorba eruptivních panožek sdílí *Rhizomastix* s archamébami. Fylogenetická analýza archaméb zahrnující vůbec první získaná sekvenční data z volně žijícího *R. libera* ukázala, že by mohl být rod *Rhizomastix* dokonce blízce příbuzný parazitickým entamébám (Ptáčková et al. 2013). Tuto hypotézu však vyvrátila první multigenová analýza archaméb, podle které se tento rod řadí mezi volně žijící mastigaméby (Pánek et al., *in press*).

Typový druh *Rhizomastix gracilis* Alexeieff, 1911 byl poprvé izolován ze střeva axolotla. Další nálezy byly zaznamenány zejména z hmyzu (Bhaskar Rao 1963, Mackinnon 1913, Zadrobílková et al. 2015b), ale jsou známy také volně žijící druhy popsané ze znečištěné vody nebo sladkovodního sedimentu (Ptáčková et al. 2013; Zadrobílková et al. 2015b; Zhang a Yang 1990).

4. Fylogeneze a evoluce

4. 1 Amoebozoa a Conosa

Améby a amébovité organismy byly dlouhou dobu považovány za jednu monofyletickou skupinu. Později se ale ukázalo, že tomu tak pravděpodobně není a že ve skutečnosti se amébovitý způsob života objevil u eukaryot několikrát nezávisle na sobě. Amébovité organismy dnes nalezneme v mnoha eukaryotických liniích, které jsou na sobě evolučně nezávislé. Tři z těchto linií ale zahrnují převážnou většinu všech amébovitých organismů. Jedná se o Heterolobosea, Rhizaria a Amoebozoa. Poslední jmenovaná představuje poměrně velkou skupinu amébovitých organismů, která je ale doposud jen velmi málo probádaná a je z ní k dispozici, vzhledem k její předpokládané velikosti, jen málo sekvenčních dat (viz Cavalier-Smith et al. 2015). Amoebozoa jsou evolučně poměrně zajímavou skupinou eukaryot, protože jsou blízce příbuzná říši Opisthokonta, kde nalezneme mimo jiné mnohobuněčné organismy, jako jsou živočichové a houby (Cavalier-Smith et al. 2004).

Amoebozoa se tradičně dělí na bezbičíkatá Lobosa a Conosa, která mají buňky opatřené jedním nebo více bičíky nebo jsou bezbičíkatá (Berney et al. 2015; Bolivar et al. 2003; Cavalier-Smith et al. 2004; Fahrni et al. 2003; Nikolaev et al. 2006). Conosa dostala název podle mikrotubulárního koše, který je typický pro všechny bičíkaté zástupce této skupiny. Morfologická data byla podpořena také molekulárními daty, kdy byla na základě analýzy 123 genů prokázána monofylie skupiny (Bapteste et al. 2002). Mezi Conosa se řadí parafyletický taxon Variosea, který sdružuje morfologicky velmi různorodé organismy, aerobní Macromycetozoa neboli tzv. „pravé hlenky“, vyskytující se ve formě améb nebo améboflagelátů, a obvykle anaerobní jednobičíkatá Archamoebae (Berney et al. 2015). Mononofylie archaméb nebyla vždy jistá. Na vině byl především nízký počet známých sekvencí DNA, které bylo možné zahrnout do fylogenetické analýzy, ale také přítomnost sekvencí, které byly chybně přiřazené jinému organismu (Hinkle et al. 1994; Edgcomb et al. 2002; Milyutina et al. 2001; Ptáčková et al. 2013; Zadrobílková et al. 2015a). Až na základě výrazného rozšíření datasetu DNA sekvencí o především volně žijící, ale i nové druhy parazitických archaméb byla jednoznačně potvrzena monofylie skupiny (Ptáčková et al. 2013; Zadrobílková et al. 2015a, b). Monofylii archaméb potvrzuje také zatím nejucelenější multigenová analýza (Pánek et al., *in press*).

4. 2 Přínosy a problémy metod rekonstrukce fylogeneze archaméb

Především kvůli absenci molekulárních dat se archaméby zprvu dělily na parazitické entaméby, které zahrnovaly rody *Entamoeba*, *Endamoeba*, *Endolimax* a *Iodamoeba*, a volně žijící pelobionty s rodem *Pelomyxa*, na základě kterého dostala tato skupina jméno, a dále s rody *Mastigamoeba*, *Mastigella* a *Mastigina*. Nízká prosekvenovanost skupiny a zprvu také nedostatečné metody rekonstrukce fylogeneze způsobovaly jak chybné postavení archaméb na eukaryotickém stromě (Cavalier-Smith 1993), tak nejednoznačné fylogenetické vztahy v rámci celé skupiny (Hinkle et al. 1994; Milyutina et al. 2001; Silberman et al. 1999). Až na základě pozdějších fylogenetických analýz se ukázalo, že původní členění podle způsobu života je umělé (Cavalier-Smith et al. 2004; Fiore-Donno et al. 2010; Shadwick et al. 2009). Dalším problémem se zdála být samotná délka sekvence SSU rDNA, která je dosud nejvíce využívána k rekonstrukci evolučních vztahů uvnitř řady eukaryotických skupin. Její délka se u archaméb pohybuje od cca 2000 bp u entaméb (Silberman et al. 1999), přes cca 2700 bp u *Mastigamoeba balamuthi* (Hinkle et al. 1994) až po cca 3500 bp dlouhou sekvenci u rodu *Pelomyxa* (Milyutina et al. 2001). To může přinášet problémy nejen během samotné amplifikace, ale také při následném alignmentu. V neposlední řadě zejména rody *Pelomyxa* a *Entamoeba* tvoří na fylogenetických stromech dlouhé větve, což je pravděpodobně způsobeno zvýšenou rychlostí, kterou probíhala jejich molekulární evoluce. Dlouhé větve mohou v analýzách vést k artefaktu přitahování dlouhých větví (LBA), kdy se evolučně vzdálené taxony s podobnou substituční rychlostí mylně jeví jako blízce příbuzné a zároveň se tyto větve posouvají blíže ke kořeni stromu (Stiller a Hall 1999). V některých fylogenetických analýzách se tak zdály být rody *Pelomyxa* a *Entamoeba* blízce příbuzné (Milyutina et al. 2001; Ptáčková et al. 2013), přestože si jsou ve skutečnosti poměrně evolučně vzdálené (Zadrobíková et al. 2015a). Kvůli extrémní délce a odlišnosti někteří autoři sekvenci SSU rDNA rodu *Pelomyxa* do analýz dokonce vůbec nezahrnovali (Fiore-Donno et al. 2010).

Zajímavým druhem, který dlouhou dobu vytvářel chaos ve fylogentických stromech archaméb, je *Breviata anathema*, původně zaměňovaná za druh *Mastigamoeba invertens*. Jak samotné jméno napovídá, byl tento druh původně řazen mezi archaméby, protože svojí morfologií (amébovité tělo, jeden přední bičík) i ekologií (volně žijící anaerob) připomínala rod *Mastigamoeba*. Ve fylogenetických analýzách ale tento druh nespadal do skupiny Archamoebae, ale spíše představoval izolovanou linii eukaryot (Bolivar et al. 2001; Edgcomb et al. 2002; Milyutina et al. 2001). Ve starších studiích se dokonce *M. invertens* větvila mezi dalšími prvoky postrádající klasické mitochondrie, původně považované jako amitochondriální, čímž podporovala teorii Archezoa (Stiller et al. 1998). Na základě elektron-mikroskopické studie ale bylo mimo jiné ukázáno, že bičíkatý aparát *M. invertens* obsahuje dvě bazální tělíska, nikoliv jedno, a že se tedy ve skutečnosti nejedná o rod *Mastigamoeba*. Organismus byl nově pojmenován jako *Breviata anathema* (Walker et al. 2006). Dnes se ví, že rod *Breviata* společně s rody *Subulatomonas* a *Pygsuia* představuje samostatnou linii eukaryot, Breviatea, blízce příbuznou opistokontům (Brown et al. 2013; Katz et al. 2011).

4. 3 Fylogeneze archaméb

Výše zmíněné problémy metod rekonstrukce fylogeneze se v poslední době daří docela dobře překonávat. K tomu přispěl i výrazný nárůst počtu molekulárních dat získaných především z volně žijících zástupců archaméb, díky kterému mimo jiné došlo k objasnění vzájemných evolučních vztahů mezi hlavními liniemi skupiny. V současné době se Archamoebae dělí na čtyři čeledi, kterými jsou Entamoebidae, Pelomyxidae, Rhizomastixidae a Mastigamoebidae (Ptáčková et al. 2013). Čeleď Entamoebidae zahrnuje pouze rod *Entamoeba* a již v minulosti vyslovil Cavalier-Smith (1991) hypotézu, že entaméby tvoří samostatnou větev, sesterskou ostatním archamébám. Tuto myšlenku potvrzuje multigenová analýza, kde čeleď Entamoebidae představuje hlubokou linii skupiny Archamoebae (Pánek et al., *in press*). Do čeledi Pelomyxidae patří kromě rodu *Pelomyxa* také *Mastigella*. Nově se totiž ukázalo, že rod *Pelomyxa* ve skutečnosti vytváří vnitřní větev rodu *Mastigella* (Zadrobílková et al. 2015a; Pánek et al., *in press*). Oba rody sdílí některé společné znaky, jako je podobná morfologie buňky a uspořádání heterochromatinu v jádře. Pohyb bičíku obou rodů je na rozdíl od ostatních bičíkatých zástupců archaméb poměrně pomalý nebo je bičík zcela nepohyblivý a v neposlední řadě u některých druhů těchto rodů nalezneme v buňce více než jedno jádro (Zadrobílková et al. 2015a). Stejně jako čeleď Entamoebidae obsahuje čeleď Rhizomastixidae pouze jediný rod, v tomto případě rod *Rhizomastix*. Původně se zdálo, že by mohly být obě tyto čeledi blízce příbuzné (Ptáčková et al. 2013), což se ale později nepotvrdilo. Rhizomastixidae je ve skutečnosti pravděpodobně sesterská s Mastigamoebidae (Pánek et al., *in press*). Čeleď Mastigamoebidae je tvořena dvěma nezávislými liniemi Mastigamoebidae A a Mastigamoebidae B (Ptáčková et al. 2013). První jmenovaná zahrnuje např. známý modelový organismus *Mastigamoeba balamuthi* nebo druh *M. punctachora*. Do linie Mastigamoebidae B náleží např. *Mastigamoeba simplex*, *M. scholaia* nebo rody *Iodamoeba* a *Endolimax* (Ptáčková et al. 2013). Z nejnovějších fylogenetických dat vyplývá, že archaméby lze ve skutečnosti rozdělit na převážně parazitická Entamoebida, která zahrnují čeledeď Entamoebidae, a na obvykle volně žijící Pelobiontida s čeleděmi Pelomyxidae, Rhizomastixidae a Mastigamoebidae (Pánek et al., *in press*). Původní členění na entaméby a pelobionty se tak zdá být s menšími změnami platné.

4. 4 Parazitismus v rámci skupiny Archamoebae

Na základě jednoho z nejstarších dělení archaméb podle způsobu života zahrnovala parazitická skupina Entamoebidae výše jmenované střevní améby rodů *Entamoeba*, *Endamoeba*, *Endolimax*, *Iodamoeba*, a navíc také rod *Dientamoeba* (Chatton 1925). Ultrastrukturní studie ale ukázala, že rod *Dientamoeba* je ve skutečnosti aberantní trichomonáda (Camp et al. 1974). Přestože se rod *Endolimax* ve starších analýzách obvykle jevil jako součást entaméb (Fahrni et al. 2003; Silberman et al. 1999), v některých případech měl tendenci se větvit poblíž rodu *Mastigamoeba*, avšak bez statistické podpory (Silberman et al. 1999). Jeho blízká příbuznost s mastigamébami byla s jistotou potvrzena až později (Cavalier-Smith et al. 2004). Za předpokladu, že poslední společný předek všech archaméb byl volně žijící, znamenalo to, že se v rámci této skupiny vyvinul parazitismus nejméně dvakrát nezávisle na sobě. Získání DNA sekvence z dalšího parazitického rodu *Iodamoba* pouze potvrdilo předchozí hypotézu, kdy se na fylogenetickém stromě tento rod spolu s rodem *Endolimax* větvil jako sesterský taxon k volně žijícímu druhu *Mastigamoeba simplex* (Stensvold et al. 2012).

Záhadným rodem s nejistou fylogenetickou pozicí byl dlouho rod *Rhizomastix*. Tento převážně parazitický rod se strukturou jádra velmi podobnou rodu *Entamoeba* se na základě první získané DNA sekvence spolu s rodem *Pelomyxa* umístil na fylogenetickém stromě blízko parazitických entaméb (Ptáčková et al. 2013). Již zmíněná multigenová studie ale možnou společnou evoluční historii druhů *Rhizomastix* a *Entamoeba* vyvrátila, protože ukázala, že je rod *Rhizomastix* ve skutečnosti sesterský s mastigamébami (Pánek et al., *in press*). Podle studie zaměřené na diverzitu tohoto rodu je jasné, že se větví na dvě izolované linie. Jedna představuje výhradně volně žijící zástupce a druhá zahrnuje parazitické a nejméně jeden potenciálně parazitický druh (Zadrobílková et al. 2015b). Pokud shrneme nejnovější data o skupině Archamoebae, tak je patrné, že se zde parazitismus objevil ve skutečnosti nejméně třikrát nezávisle na sobě, u společného předka entaméb, u společného předka rodů *Iodamoeba* a *Endolimax* a u předka parazitické linie rodu *Rhizomastix* (Pánek et al., *in press*). To vše platí za předpokladu, že se volně žijící způsob života většiny archaméb nevyvinul sekundárně.

Z rodu *Endamoeba* doposud chybí sekvenční data, a proto je jeho zařazení mezi Entamoebidae stále založeno pouze na morfologické podobnosti. Stejně tak chybí jakákoliv DNA sekvence z dalších parazitických rodů nebo druhů, které morfologicky nespadají do čeledi Entamoebidae. Prvním z nich je *Mastigamoeba bovis*, která byla nalezena v žaludku krav (Liebetanz 1910). Samotný popis ale postrádá bližší detaily, navíc nebyl tento druh od svého původního popisu už víckrát pozorován. Proto jeho zařazení nebo samotná existence vůbec zůstávají otázkou. Druhým druhem je parazit trávicího traktu žab *Tricholimax hylae*, Becker (1925). Některé jeho znaky, jako je fontánovité proudění cytoplasmy a přítomnost nepohyblivého bičíku s aberantním počtem mikrotubulů, nalezneme také u rodu *Pelomyxa* (Brugerolle 1982; Griffin 1988). Proto ho někteří autoři řadí do čeledi Pelomyxidae, i když často pod chybným označením *Mastigina hylae* (Brugerolle a Patterson 2000). Druhy *Mastigamoeba bovis* nebo *Tricholimax hylae* tak mohou představovat další nezávislé parazitické linie archaméb.

5. Metody kultivace archaméb

Archaméby obecně se v kulturách příliš často neudružují. Především starší popisy nových druhů tak byly provedeny pouze na základě několika málo, často pouze na jediném, pozorování, a nikoli na dlouhodobém sledování. I přesto je známo několik metod kultivace, které závisí především na způsobu života jednotlivých izolátů, tedy na prostředí, ze kterého byly získány. Velmi frekventovanou metodou kultivace volně žijících archaméb je odebrat sediment z lokality nálezu konkrétního organismu a ten pak využít při přípravě média (Bernard et al. 2000; Chystyakova et al. 2012; Griffin 1988; Frolov et al. 2004, 2005, 2006; Simpson et al. 1997; Walker et al. 2001). Podobný postup, kdy se buňky několik měsíců udržují v hermeticky uzavřených lahvích, naplněných vodou se sedimentem nebo médiem podle Lozina-Lozinsky, se v některých případech aplikuje na rod *Pelomyxa* (Chistyakova a Frolov 2011; Frolov et al. 2011). Nejjednodušší a pro udržení většího množství izolátů také nejefektivnější se zdá být nově zavedená metoda kultivace, kdy jsou přibližně 2 ml vzorku s původním substrátem inokulovány do média. K tomuto účelu se pro volně žijící izoláty osvědčilo parameciové médium ATCC 802 podle Tracey Sonneborn nebo 3% LB médium a pro mořské vzorky se obvykle používá modifikované tzv. mořské 802 médium ATCC 1525 (Ptáčková et al. 2013; Zadrobílková et al. 2015a, b). Jak už název napovídá, parameciové médium podle Sonnebornové, stejně jako Lozina-Lozinsky médium, bylo původně určeno především ke kultivaci nálevníků rodu *Paramecium* (Sonneborn 1970; Zalizniak et al. 2006).

Velkou potíží rodu *Pelomyxa* zůstává jeho obtížná kultivace, kdy obvykle tento rod do několika týdnů od založení kultury vymizí. Zatím pouze jediný izolát druhu *Pelomyxa schiedti* úspěšně přežívá již několik let (SKADARSKE, Zadrobílková et al. 2015a). Naproti tomu kultivace druhu *Mastigamoeba balamuthi* se zdá být rutinní záležitostí. Tento druh se obvykle pěstuje na médiu PY, které je poměrně bohaté na živiny, nebo se *M. balamuthi* axenizuje, tedy zbavuje veškerých prokaryot, a následně převádí do výživově ještě bohatšího média PYGC (Chávez et al. 1986; Nývltová et al. 2013, 2015).

Z parazitických zástupců archaméb se nejčastěji kultivuje *Entamoeba histolytica*. Pro její kultivaci je nejdříve zapotřebí aby se vzorek, nejčastěji se jedná o stolici, ustálil v xenické kultuře (Clark a Diamond 2002). K tomu slouží především dvojfázové médium Dobell-Laidlaw, jehož tekutá fáze obsahuje vaječný bílek (Dobell a Laidlaw 1926), nebo poměrně často využívané jednofázové médium TYSGM-9 (Diamond 1982). Toto médium se dnes používá ke kultivaci širokého spektra střevních prvoků. Pro úspěšnou kultivaci *E. histolytica* je důležité přidávat do xenických médií rýžový škrob, který je významným zdrojem potravy (Clark a Diamond 2002). Pro axenickou kultivaci je nejčastěji používaným médiem TYI-S-33, které je zdrojem všech důležitých složek potravy jako jsou peptidy a aminokyseliny, nukleové kyseliny, cukry, tuky a vitamíny. Ostatní druhy entaméb a rod *Endolimax* lze pěstovat podobným způsobem jako druh *E. histolytica* (Clark a Diamond 2002). Endobiotické druhy rodu *Rhizomastix* se nejúspěšněji kultivují na médiu Dobell-Laidlaw (Zadrobílková et al. 2015b).

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ORIGINAL PAPER

Evolution of Archamoebae: Morphological and Molecular Evidence for Pelobionts Including *Rhizomastix*, *Entamoeba*, *Iodamoeba*, and *Endolimax*

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The archamoebae form a small clade of anaerobic/microaerophilic flagellates or amoebae, comprising the pelobionts (mastigamoebids and pelomyxids) and the entamoebae. It is a member of the eukaryotic supergroup Amoebozoa. We examined 22 strains of 13 species of *Mastigamoeba*, *Pelomyxa* and *Rhizomastix* by light-microscopy and determined their SSU rRNA gene sequences. The SSU rRNA gene sequences of *Pelomyxa palustris* and *Mastigella commutans* in GenBank are shown to belong to *P. stagnalis* and *Mastigamoeba punctachora*, respectively. Five new species of free-living archamoebae are described: *Mastigamoeba abducta*, *M. errans*, *M. guttula*, *M. lenta*, and *Rhizomastix libera* spp. nov. A species of *Mastigamoeba* possibly living endosymbiotically in *Pelomyxa* was identified. *Rhizomastix libera*, the first known free-living member of that genus, is shown to be an archamoeba. *R. libera* possesses an ultrastructure unique within archamoebae: a rhizostyle formed from a modified microtubular cone and a flagellum with vanes. While many nominal species of pelobionts are extremely hard to distinguish by light microscopy, transient pseudopodial characters are worthy of further investigation as taxonomic markers.

Key words: Archamoebae; *Mastigamoeba*; pelobionts; phylogeny; *Pelomyxa*; *Rhizomastix*.

Introduction

The archamoebae is comprised of mostly free-living flagellated pelobionts (mastigamoebids and pelomyxids) and endobiotic aflagellate entamoebae. The best known is *Entamoeba histolytica*, that causes amoebic dysentery of humans. All pelobionts and entamoebae are anaerobic/microaerophilic and lack normal mitochondria, Golgi stacks, plastids, and peroxisomal microbodies (Brugerolle 1982; El-Hashimi and Pitman

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1970; Frolov 2011; Rosenbaum and Wittner 1970; Simpson et al. 1997; Walker et al. 2001). They were once considered to include remnants of pre-mitochondriate eukaryotes, or even the most basal eukaryotes (Brugerolle 1993; Cavalier-Smith 1983; Griffin 1979, 1988; Margulis 1970; Patterson and Sogin 1992; Whatley 1976; Whatley and Chapman-Andresen, 1990). This hypothesis was falsified by the discovery of acristate mitochondrial homologues in some species (Clark and Roger 1995; Gill et al. 2007; Tovar et al. 1999), and by a better understanding of artefacts of phylogenetic analysis (Philippe and Germot 2000; Philippe et al. 2000). Multigene phylogenetic analyses show that anaerobic pelobionts and entamoebae are derived within the aerobic Amoebozoa (Cavalier-Smith 1997, 1998; Simpson and Roger 2004).

Archamoebae have a relatively simple microtubular cytoskeleton. Among the flagellated taxa (*Mastigamoeba, Mastigella, Mastigina, Pelomyxa*, *Tricholimax*, and *Rhizomastix* – this paper), the flagellar apparatus has one or more monokinetids – single, flagellated basal bodies, without a second "barren" basal body as in most other uniflagellated eukaryotes (Brugerolle 1982, 1991a; Cavalier-Smith 1991; Chávez et al. 1986; Chistyakova and Frolov 2011; Chystyakova et al. 2012; Frolov 2011; Frolov et al. 2004, 2005a, b, 2006, 2011; Griffin 1988; Simpson et al. 1997; Walker et al. 2001). The basal body is usually associated with a cone of radiating microtubules and a single flattened ribbon of microtubules emerging from the side of the basal body below a fibrillar sheet. The microtubular cone may be attached to the nucleus in *Mastigamoeba*, *Mastigina*, and *Tricholimax* (Brugerolle 1982, 1991a, b; Chystyakova et al. 2012; Frenzel 1897; Simpson et al. 1997; Walker et al. 2001), or be independent of the nucleus, as in *Mastigella* (Frenzel 1897; Goldschmidt 1907a; Walker et al. 2001). The flagellum is at least the length of the cell body in many mastigamoebid flagellates, and usually displays a characteristic languid movement that may reflect the absence of outer dynein arms in the flagellar axoneme (Walker et al. 2001). In *Mastigamoeba schizophrenia* the basal body is composed of microtubular doublets rather than triplets (Simpson et al. 1997). In some species, for example *Tricholimax hylae* (Brugerolle 1982) and *Pelomyxa palustris* (Griffin 1988; Seravin and Goodkov 1987), the flagellum is short and non-motile, often with disorganized axonemal microtubules and no dynein arms. Members of *Pelomyxa* may develop into giant amoeboid cells with multiple separate monokinetids (Griffin 1979, 1988). The genera *Entamoeba*, *Endamoeba*, *Endolimax*, and *Iodamoeba*, have no flagellar apparatus (El-Hashimi and Pitman 1970; Morris 1936; Rosenbaum and Wittner 1970; Zaman et al. 1998, 2000).

Cavalier-Smith (1998) proposed a relationship of mastigamoebae, *Pelomyxa*, entamoebae, and mycetozoan slime moulds, as the Conosa. This was based on a proposed shared ultrastructural identity as some Mycetozoa have a single flagellum and a fan of microtubules that superficially resembles the cone in pelobionts. Recent molecular phylogenetic results suggest that the "conosan" flagellar apparatus may include plesiomorphic characters that are found in all amoebozoan flagellates (Shadwick et al. 2009) and therefore invalidating the 'Conosa'.

The name archamoebae was introduced and used by Cavalier-Smith (1983, 1987a, b) and Cavalier-Smith et al. (2004) to group the pelomyxids, entamoebae and mastigamoebae. The concept has been used at ranks of Infraphylum and Class (Cavalier-Smith 1998; Cavalier-Smith et al. 2004) and has been compositionally unstable (Cavalier-Smith 1991, 1997; Cavalier-Smith and Chao 1995). In its most recent incarnations (e.g. Cavalier-Smith et al. 2004; Smirnov et al. 2011) this group has included two clades, one comprising the pelomyxids and entamoebae, the other with the mastigamoebids and *Endolimax*. The grouping of entamoebae, pelomyxids and mastigamoebids has been supported with phylogenetic analyses employing parameter-rich models, computationally-intensive methods, and with increased taxon sampling (Edgcomb et al. 2002; Cavalier-Smith et al. 2004; Kudryavtsev et al. 2005; Milyutina et al. 2001; Nikolaev et al. 2006; Stensvold et al. 2012).

The Order Pelobiontida was introduced (Page 1976) originally to include only *Pelomyxa* (Page 1976, 1987), and has occasionally been used at other ranks, e.g. Class Pelobionta (Krylov et al. 1980). Griffin (1988) revised the Order Pelobiontida to include mastigamoebids, on the basis of the ultrastructural evidence for flagella in *Pelomyxa* (Griffin 1979, 1988). The term pelobiont has since been used to encompass mastigamoebae and pelomyxids (the genera *Mastigamoeba, Mastigella, Mastigina* and *Pelomyxa*) to the exclusion of entamoebae. It includes free-living flagellated amoebae with distinctive hyaline cytoplasm, a monokinetid flagellar apparatus with a cone of microtubules, and reduced mitochondria (Bernard et al. 2000; Brugerolle 1991b; Brugerolle and Patterson 2000; Frolov 2011; Griffin 1979, 1988; Larsen and Patterson 1990; Patterson 1999; Simpson et al. 1997; Walker et al. 2001, 2011). Pelobionts in this sense are typically assigned the taxonomic rank of order (Pelobiontida: Bernard et al. 2000; Brugerolle 1991b; Griffin 1988; Larsen and Patterson 1990; Simpson et al. 1997) or more rarely class (Peloflagellata by Goodkov and Seravin 1991; Peloflagellatea by Goodkov et al. 2004). This concept has existed in the literature for more than a century, with Schulze (1875) pointing to the light-microscopical similarities of *Pelomyxa* and *Mastigamoeba*. As its trophic form being a large amoeba, *Pelomyxa* was until recently more usually classified with lobose amoebae, (e.g. Bovee 1972; Bütschli 1880; Chatton 1925, 1953; Page 1976; Reichenow 1952; Siemensma 1987). Bütschli (1880) and Kudo (1939, 1977) included *Dinamoeba mirabilis*, now usually considered a synonym of *Mastigamoeba aspera* (Chystyakova et al. 2012; Page 1970; Penard 1936; Schulze 1875). Prior studies (Cavalier-Smith 1987a, b; Cavalier-Smith et al. 2004; Stensvold et al. 2012) suggest that entamoebae are not sister to the clade that contains pelomyxids and mastigamoebids, but are derived within it. If this is confirmed then the pelobionts and archamoebae are compositionally identical, and the terms are synonymous. They have been used interchangeably (Cavalier-Smith et al. 2004). The use of two terms for the same clade is confusing. Confusion arising from synonymy at lower taxonomic ranks is addressed by the nomenclatural principle of priority as this protects nomenclatural stability. In the event that entamoebae are derived from within the pelobionts (i.e. are themselves pelobionts), then the correct name for the ordinal taxon will be 'Pelobiontida' and the same root would be used for higher-ranked taxa. The term archamoebae would be abandoned under these circumstances. Until the sister group for the entamoebae is confirmed, the term 'archamoebae' can applied to the pelobionts and entamoebae.

The pelobionts are usually divided into two families, Pelomyxidae and Mastigamoebidae, currently containing 9 nominal genera and 248 nominal species (see Table S1). A brief description of the genera discussed in this paper is given later, and a taxonomic revision of all archamoebae is in preparation (Walker et al., unpublished).

The genus *Mastigamoeba* was introduced to describe an amoeboid organism with hyaline cytoplasm and pseudopodia different from those of *Cercomonas*, a long flagellum, and bacteria over the outside of the cell body (Frenzel 1897; Schulze 1875). Subsequent descriptions regarded the bacteria as a specific feature of *Mastigamoeba aspera*, and used *Mastigamoeba* for amoeboid organisms with hyaline cytoplasm, and a long flagellum connected to the nucleus (Goldschmidt 1907b; Kent 1880; Klebs 1892; Schulze 1875b; Stokes 1886, 1888, 1890). *Mastigella* was introduced to describe a mastigamoebid organism with multiple long flagella extended from the cell body on small "necks", that wandered over the cell body and were not attached to the nucleus (Frenzel 1897). The genus *Mastigella* is currently considered as those mastigamoebids without a connection between the flagellum and nucleus (Goldschmidt 1907a, b). Following from Goldschmidt's (1907a, b) informal group "Mastigamöben", *Mastigamoeba* and *Mastigella* were grouped as the Mastigamoebidae by Chatton (Chatton 1925; Kudo 1939, 1977).

Pelomyxidae was named as a (monotypic) family by Schulze (1877). *Pelomyxa* was first described using the pre-occupied name *Pelobius* (Greeff 1866) and then redescribed as *Pelomyxa palustris* (Greeff 1874). It was reported as a large multinucleate amoeba, with a division of the cytoplasm into an inner layer containing organelles displaying fountain-flow movement and a clear hyaline outer layer from which pseudopodia can "roll" out; and with a posterior uroid attaching the amoeba to the substrate. Later reports extended the description to refer to prokaryotes that co-exist endosymbiotically in the cell (van Bruggen et al. 1988), and to non-motile flagella (Griffin 1979, 1988; Seravin and Goodkov 1987). The number of species increased considerably (discussed in Whatley and Chapman-Andresen 1990; Frolov 2011). The phylogenetic position of *Pelomyxa* within the archamoebae remains unclear, but most analyses group it with *Entamoeba*, albeit with varying levels of support (Cavalier-Smith et al. 2004; Stensvold et al. 2012).

Entamoebae are aflagellated endobiotic members of the archamoebae that were, until recently, taxonomically separated from the pelobionts. They have traditionally been classified as members of the family Entamoebidae that included the genera *Entamoeba*, *Endamoeba*, *Endolimax*, *Iodamoeba*, and sometimes several genera of uncertain phylogenetic position, such as *Schizamoeba*, *Hydramoeba* and *Malpighamoeba* (Patterson et al. 2002). Cavalier-Smith et al. (2004) removed *Endolimax* from Entamoebidae on the basis of molecular phylogenetic analyses (see below) and erected the family Endolimacidae to contain it.

The sampling of archamoebae in phylogenetic analyses remains poor. SSU rRNA gene sequences are known from six taxa outside the medically important genus *Entamoeba* (Edgcomb et al. 2002; Hinkle et al. 1994; Milyutina et al. 2001; Silberman et al. 1999). A close relationship between *Endolimax nana* and *Mastigamoeba simplex* has been noted (Cavalier-Smith et al. 2004; Fiore-Donno et al. 2010; Shadwick et al. 2009). Recently, Stensvold et al. (2012) showed that *Iodamoeba*, another aflagellated endobiotic taxon, is closely related to *Endolimax*.

Archamoebae form the only anaerobic lineage of Amoebozoa. Several other anaerobic amoeboid flagellates with a single anterior flagellum exist. Two (*Breviata anathema* and *Subulatomonas tetraspora*) appear to be unrelated to archamoebae; and one (*Rhizomastix*) is shown here to be an archamoeba. *Breviata anathema* was originally identified in its ATCC culture as the pelobiont *Mastigamoeba invertens* (as reported in Stiller et al. 1998). It did not group with pelobionts in SSU rRNA gene trees (Edgcomb et al. 2002; Stiller and Hall 1999); and because of its distinctive ultrastructure, having two basal bodies and a complex system of microtubular roots different from pelobiont ultrastructure, it was renamed *Breviata anathema* (Walker et al. 2006). Its affinities remain unresolved, but arguments have been presented for and against affinities with Amoebozoa (Minge et al. 2009; Walker et al. 2006; Zhao et al. 2012), apusomonads (Heiss et al. 2013), excavates, and *Subulatomonas tetraspora* (Katz et al. 2011; Shadwick et al. 2009). The recently-described amoeboflagellate *Subulatomonas tetraspora* was shown to be closely related to *B. anathema*, but no ultrastructural information on the flagellar apparatus is available for this species (Katz et al. 2011). It has a "neck" joining the cell body to the flagellum, but this is much longer and more flexible than the necks seen in *Mastigella commutans* (Frenzel 1897) or *Mastigamoeba scholaia* (Klug 1936). Both *Breviata* and *Subulatomonas* have filose and branching pseudopodia that appear more like those of cercomonads than those of pelobionts. *Rhizomastix* was classified with mastigamoebids by Kudo (1939, 1977) and Cepicka (2011) suggested it might be related to pelobionts. In the absence of sequence and ultrastructural data, its position has remained uncertain until the present study. The name Rhizomastigidae has historically been used for today's Mastigamoebidae (e.g. Bütschli 1880, 1884; Lepsi 1965; Reichenow 1952). The name was created by Bütschli (1884) as Rhizomastigina and later standardized to Rhizomastigidae by Calkins (1901). However, as it was not based on and often did not include *Rhizomastix*, Rhizomastigidae is regarded by some as a nomen nudum (Loeblich and Tappan 1961). The composition of Rhizomastigidae has always been very confused.

This study aimed to investigate the morphological and molecular diversity of free-living archamoebae (pelobionts) and to elucidate the phylogenetic position of *Rhizomastix*. We isolated 22 strains of 13 species of *Mastigamoeba*, *Pelomyxa* and *Rhizomastix*, examined their light-microscopic morphology and determined their SSU rRNA gene sequences. Our phylogenetic analyses show that *Rhizomastix* is an archamoeba. We describe five new species of free-living archamoebae including the first known free-living member of *Rhizomastix*. The distinctive ultrastructure of *Rhizomastix* is described for the first time.

Results

New Strains

Twenty two isolates of free-living archamoebae were obtained from freshwater micro-oxic sediments (Table 1); 17 were established in culture. Most cultures were monoeukaryotic. A few strains were contaminated with other eukaryotic microorganisms, mainly ciliates (*Metopus* and *Trimyema*), euglenids (*Distigma* and *Rhabdomonas*), diplomonads (*Trepomonas*), or unidentified stramenopiles.

Morphology

Species are here identified as groups of isolates that share morphological characters and have sequences that are identical or form terminal monophyletic groups when all known pelobiont sequences are compared. The morphological characters that we use to distinguish species are similar to those used by previous authors (e.g. Bernard et al. 2000; Klug 1936). Because some sections of the SSU rRNA gene in pelobionts are extremely variable, and because we do not have a clear understanding of how ultrastructure, light-microscopical morphology, and sequence identity are related, it is not possible to provide species-level sequence identities for specific regions of the SSU rRNA gene.

The genus *Mastigamoeba*

We include species which have a single, long anterior flagellum and with the nucleus associated with the base of the flagellum (i.e. sensu Goldschmidt 1907b). Neither cysts nor multinucleate plasmodia were observed in any strain. All cells were uninucleate. Usually, several forms could be distinguished: 1. swimming elongated, flagellated cells;

Table 1. List of the strains included in the study.

Strain	Locality	Coordinates
3MLMA[a]	Bezručovo valley, Czech Republic	50°29′N, 13°20′E
CH2	Chile	35°05′S, 70°08′W
CHOM1[a]	Chomutov, Czech Republic	50°27′N, 13°21′E
GRUBER[a]	Gruberova studánka, Czech Republic	48°48′N, 15°38′E
HRAANM[a]	Hradiště peak, Czech Republic	50°27′N, 13°20′E
HRADANAN	Hradiště peak, Czech Republic	50°27′N, 13°20′E
HRN11[a]	Prague, Czech Republic	50°00′N, 14°30′E
IND5	Bhangarh, India	27°05′N, 76°17′E
IND8MA[a]	Bhangarh, India	27°05′N, 76°17′E
JAVA1[a, b]	Warna lake, Java, Indonesia	07°12′N, 109°54′E
LUH2NS4[a]	Vltava river alluvial plain, Czech Republic	48°48′N, 13°57′E
OLB6	Olbasee lake, Germany	51°16′N, 14°35′E
PSOVKA	Pšovka river, Czech Republic	50°23′N, 14°32′E
SEB4[a]	Prague, Czech Republic	50°00′N, 14°29′E
Pelomyxa belevskii	Osinovskoe lake, Sosnovo village, Leningrad Oblast, Russia	60°30′N, 30°30′E
Pelomyxa palustris 1	Osinovskoe lake, Sosnovo village, Leningrad Oblast, Russia	60°30′N, 30°30′E
Pelomyxa palustris 2	Plyussa river, Lyady village, Pskov Oblast, Russia	58°35′N, 28°55′E
Pelomyxa stagnalis	Sergievka park, St. Petersburg, Russia	59°53′N, 29°50′E
TEXEL[c]	Texel island, Netherlands	53°01′N, 04°44′E
VIT1AN	Kamenice, Czech Republic	49°54′N, 14°35′E
VIT7	Kamenice, Czech Republic	49°54′N, 14°35′E
VITSEDAN[d]	Kamenice, Czech Republic	49°54′N, 14°35′E

[a]monoeukaryotic culture. [b]derived from the same isolate as JAVA1 in Cepicka et al. (2010). [c]derived from the same isolate as TEXEL in Panek et al. (2012). [d]derived from the same isolate as VITSED in Panek et al. (2012).

2. elongated, flagellated cells gliding attached on the surface, 3. irregular, flagellated or aflagellated cells crawling slowly with eruptive pseudopodia, and 4. rounded or irregular flagellated or aflagellated resting forms. The gliding form was morphologically stable and easily observed and photographed. It changed quickly into the swimming form and vice versa, usually without morphological changes. We emphasize this form in our comparisons. The strains differed by cell shape and size; shape, structure and position of the nucleus; and by the length of the flagellum. The irregular resting forms of different species were often indistinguishable.

Protargol was used to stain the cytoplasm, nucleus, and flagellum. The cone was stained in only some strains. The diameters of living and protargol-stained cells of the strains are summarized in Table 2.

Mastigamoeba punctachora

Strain SEB4 corresponded in appearance (Fig. 1A – C) to the original description of *M. punctachora* (Bernard et al. 2000). Actively moving cells were elongate. The apical nucleus was shaped like a tear drop and was associated with the flagellar base. A large, rounded nucleolus occupied the central part of the nucleus. A nuclear granule, the distinguishing feature of *M. punctachora*, was observed in the proximal part of the nucleus in many cells. Protargol-stained cells of strains SEB4 and JAVA1 were usually oval. A darkly-staining basal body was observed at the place of insertion of the flagellum (Fig. 1D – I); with a slightly-stained cone originating from the basal body and extending to the nucleus. A fiber originating from the basal body, presumably the microtubular root, was seen in some cells (“F” in Fig. 1G – I). Some cells produced fine pseudopodia (Fig. 1C, E).

Mastigamoeba simplex

The morphology of the strain CH2 corresponds with the description of *M. simplex* by Bernard et al. (2000). Gliding cells were elongate (Fig. 2A – C). The pyriform nucleus occupied the anterior third to one half of the cell body and contains a large central nucleolus (Fig. 2A – C). The cells possessed a single anterior flagellum. The association between the nucleus and flagellum was not conspicuous, and the bulk of the nucleus appeared removed from the basal body. The anterior part of the cell was hyaline. Many cells produced one or a few posterior

Table 2. Dimensions (in μm) of living and protargol-stained specimens of pelobiont strains. Average of 30 specimens (12 in case of living cells of VIT7) ± standard deviation (smallest – largest value). CL – cell length; CW – cell width; CL/CW – cell length/cell width ratio; FL – length of flagellum; FL/CL – length of flagellum/cell length ratio; LIV – living cells; PTG – protargol-stained cells.

Species	Strain	CL LIV	CW LIV	CL/CW LIV	FL LIV	FL/CL LIV	CL PTG	CW PTG	CL/CW PTG	FL PTG	FL/CL PTG
Mastigamoeba punctachora	SEB4	19.7 ± 5.6 (8.3 – 30.2)	6.9 ± 1.3 (4.3 – 9.7)	2.9	49.8 ± 12.0 (19.1 – 70.4)	2.5	10.2 ± 2.6 (6.4 – 16.4)	7.5 ± 1.6 (5.4 – 11.9)	1.4	46.6 ± 15.5 (22.1 – 96.6)	4.6
Mastigamoeba simplex	CH2	14.6 ± 2.6 (8.0 – 19.7)	4.9 ± 1.0 (2.8 – 7.1)	3.0	33.5 ± 7.2 (21.2 – 48.6)	2.3	5.0 ± 1.1 (3.1 – 7.2)	3.5 ± 0.6 (2.5 – 5.2)	1.4	37.1 ± 10.4 (8.1 – 49.6)	7.4
Mastigamoeba abducta sp. nov.	3MLMA	18.5 ± 3.1 (13.0 – 24.4)	5.5 ± 1.3 (3.9 – 9.3)	3.4	35.9 ± 4.3 (26.8 – 46.1)	1.9	6.7 ± 1.8 (4.0 – 12.6)	4.6 ± 1.0 (3.1 – 7.6)	1.5	35.7 ± 8.7 (20.0 – 52.2)	5.3
	CHOM1	14.2 ± 4.1 (7.3 – 23.2)	7.6 ± 1.6 (4.6 – 11.0)	1.9	29.5 ± 4.2 (21.3 – 41.8)	2.1	5.8 ± 1.5 (3.4 – 10.8)	4.4 ± 0.8 (3.0 – 7.1)	1.3	30.2 ± 11.5 (14.6 – 55.4)	5.2
	GRUBER	n.a.	n.a.	n.a.	n.a.	n.a.	7.9 ± 1.6 (5.4 – 12.7)	6.4 ± 1.0 (4.6 – 9.4)	1.2	27.0 ± 7.1 (15.1 – 49.0)	3.4
	HRN11	14.9 ± 4.1 (5.7 – 26.0)	7.3 ± 2.5 (3.7 – 15.8)	2.0	26.8 ± 4.9 (13.3 – 37.0)	1.8	6.8 ± 1.8 (4.6 – 11.8)	5.4 ± 1.9 (3.6 – 11.4)	1.3	21. 0 ± 6.9 (12.5 – 38.2)	3.1
	PSOVKA	n.a.	n.a.	n.a.	n.a.	n.a.	7.7 ± 1.5 (4.9 – 10.6)	5.6 ± 0.9 (3.8 – 6.9)	1.4	30.3 ± 9.8 (13.0 – 48.5)	3.9
Mastigamoeba guttula sp. nov.	HRADANAN	9.3 ± 1.8 (5.6 – 13.4)	6.4 ± 6.4 (4.5 – 8.6)	1.5	30.0 ± 6.4 (21.1 – 53.8)	3.2	6.1 ± 1.4 (4.0 – 10.6)	4.9 ± 1.3 (3.2 – 9.5)	1.2	23.5 ± 8.9 (9.3 – 43.1)	3.9
	LUH2NS4	7.8 ± 1.5 (5.8 – 11.2)	6.1 ± 1.0 (3.8 – 8.2)	1.3	25.8 ± 1.4 (19.4 – 35.3)	3.3	7.4 ± 1.8 (4.0 – 11.5)	5.5 ± 1.3 (3.2 – 8.8)	1.3	32.2 ± 7.0 (15.2 – 45.8)	4.4
Mastigamoeba scholaia	VITSEDAN	9.3 ± 2.5 (5.3 – 17.3)	6.8 ± 1.4 (4.4 – 10.0)	1.4	46.3 ± 8.6 (24.6 – 70)	5.0	5.4 ± 1.0 (3.8 – 8.0)	4.1 ± 0.7 (3.0 – 5.4)	1.3	n.a.	n.a.
	TEXEL	10.7 ± 3.1 (6.2 – 18.2)	9.6 ± 2.4 (6.4 – 15.0)	1.1	31.0 ± 5.7 (22.7 – 49.1)	2.9	7.5 ± 2.3 (4.6 – 16.2)	6.4 ± 1.6 (3.8 – 9.3)	1.2	33.5 ± 6.4 (19.8 – 44.8)	4.5
Mastigamoeba lenta sp. nov.	VIT1AN	14.6 ± 1.8 (10.8 – 18.4)	9.7 ± 1.6 (6.8 – 13.7)	1.5	n.a.	n.a.	7.9 ± 1.9 (4.9 – 12.9)	5.4 ± 1.0 (3.7 – 8.1)	1.5	n.a.	n.a.
Mastigamoeba errans sp. nov.	HRAANM	14.7 ± 2.8 (9.6 – 22.7)	9.3 ± 1.4 (7.0 – 12.0)	1.6	n.a.	n.a.	6.4 ± 1.3 (4.3 – 9.4)	4.4 ± 0.8 (3.1 –5.9)	1.5	n.a.	n.a.
	WAC-6	14.6 ± 3.7 (9.2 – 23.2)	10.4 ± 2.7 (7.6 -18.7)	1.4	n.a.	n.a.	7.6 ± 2.0 (4.2 – 10.7)	5.9 ± 1.7 (3.0 – 9.7)	1.3	n.a.	n.a.
Mastigella sp.	VIT7	52.1 ± 20.2 (44.7 – 120.3)	52.6 ± 14.3 (34.2 – 91.3)	1.0	26.0 ± 6.4 (12.5 – 41.8)	0.5	n.a.	n.a.	n.a.	n.a.	n.a.
Rhizomastix libera sp. nov.	IND8MA	10.1 ± 2.2 (5.4 – 14.1)	4.2 ± 0.9 (2.8 – 6.7)	2.4	18.9 ± 3.3 (10.9 – 25.0)	1.9	3.9 ± 0.6 (2.6 – 5.0)	3.3 ± 0.7 (2.3 – 5.9)	1.2	12.1 ± 3.2 (7.5 – 21.3)	3.1

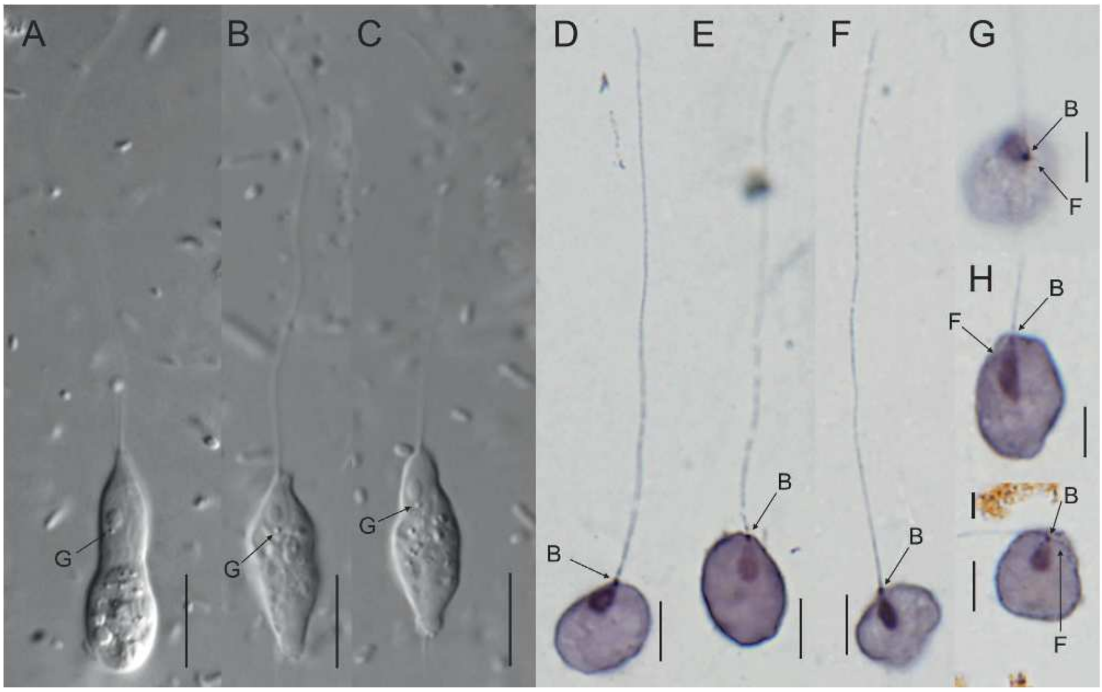

Figure 1. Morphology of *Mastigamoeba punctachora*. (**A – C**) Gliding cells of the strain SEB4. (**D – G**) Protargol-stained cells of the strain SEB4. (**H, I**) Protargol-stained cells of the strain JAVA1. B – basal body; F – cytoskeletal fiber; G – perinuclear granule. Scale bars = 10 μm for A – C, 5 μm for D – I. DIC (A – C) or bright field (D – I).

thorn-like pseudopodia which appeared to be a distinguishing feature of cells in culture (Fig. 2A, B, E, F, G). As well as the elongated form, a few rounded and uniflagellated, or irregular and aflagellated cells were observed (Fig. 2D, E). The aflagellated cells moved slowly with eruptive hyaline lobopodia. *M. simplex* can be distinguished from the other species because the body of the nucleus is removed from, yet attached to, the flagellar base, and by the thorn-like posterior pseudopodia.

Mastigamoeba abducta sp. nov.

Strains 3MLMA, CHOM1, GRUBER, HRN11, and PSOVKA resemble *Mastigamoeba simplex* but the single posterior thorn-like pseudopodium typical of *M. simplex* (Bernard et al. 2000) was not observed. Most actively moving cells were elongated and the pyriform nucleus containing a large central nucleolus was situated in the central part of the cell (Fig. 3A – D). The association between the nucleus and flagellum was usually difficult to observe in living cells. The anterior part of the cell was hyaline. Some gliding cells produced fine pseudopodia (Fig. 3B, D, H – J) from the anterior and posterior part of the cell. Besides the elongated form, a few rounded uniflagellated or irregular aflagellated cells were observed (Fig. 3E, F). The aflagellated cells moved slowly with eruptive hyaline lobopodia. No distinctive basal body was seen in protargol-stained cells (Fig. 3G – J). Some (Fig. 3G, H), but not all (Fig. 3I), protargol-stained cells had a connection between the nucleus and flagellum. *M. abducta* sp. nov. is distinguished from *M. simplex* by the lack of thorn-like posterior pseudopodia and from other *Mastigamoeba* species because the body of the nucleus is removed from, but still attached to, the flagellar base.

Mastigamoeba guttula sp. nov.

Gliding cells of strains LUH2NS4 and HRADANAN were mostly rounded (Fig. 4A, C, D), and a few cells were elongated (Fig. 4B). The rounded, teardrop-shaped nucleus was located subapically or centrally, contained a central nucleolus and was connected to the base of the flagellum (Fig. 4A – D). There was a thin layer of hyaline

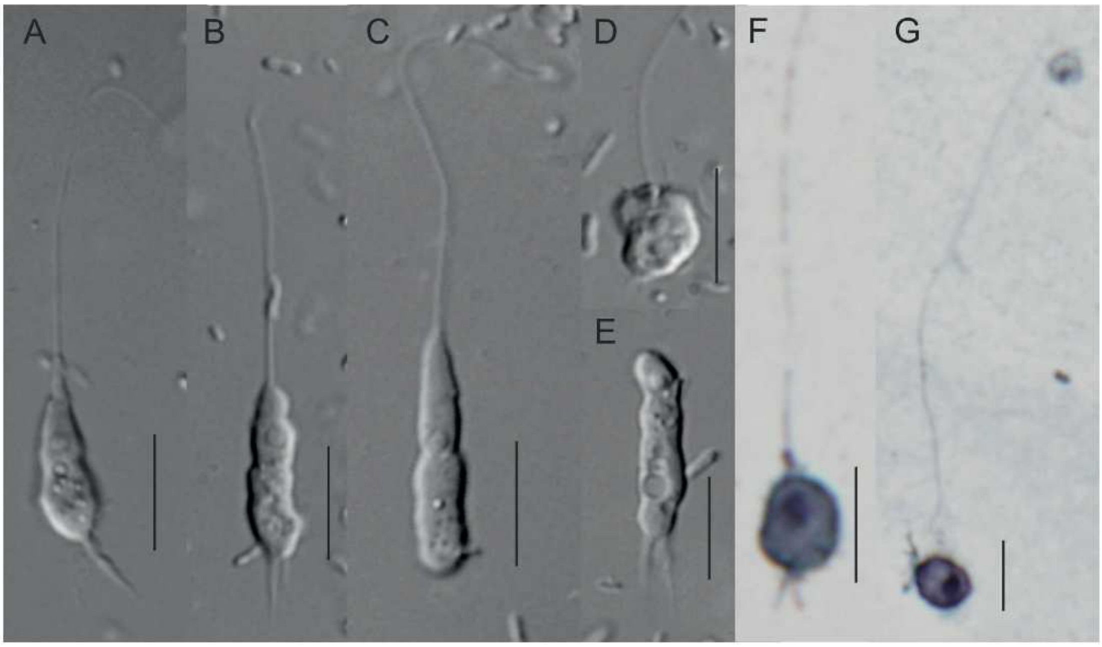

Figure 2. Morphology of *Mastigamoeba simplex* strain CH2. (**A – C**) Gliding cells. (**D**) Rounded cell. (**E**) Aflagellate crawling cell. (**F, G**) protargol-stained cells. Scale bars = 10 μm for A – E, 5 μm for F, G. DIC (A – E) or bright field (F, G).

cytoplasm anterior to the nucleus. Many cells produced numerous fine pseudopodia which were easily seen after protargol staining (Fig. 4F – I). A few aflagellate cells moved slowly with eruptive hyaline lobopodia (Fig. 4E). This species is distinguished from other species by its distinctive teardrop-shaped nucleus located apically to subapically.

Mastigamoeba scholaia

Cells of strains TEXEL and VITSEDAN were morphologically similar to those of *M. guttula* sp. nov. They differ in how the flagella insert; in *M. guttula* sp. nov. the flagellum inserts directly on the cell surface whereas the flagellar base in *M. scholaia* was supported by a small but distinct apical neck (Fig. 5A – C). The neck was preserved in a shrunken state in most protargol-stained specimens (Fig. 5E, G – I). Numerous fine pseudopodia were formed by most cells from the whole surface. Resting rounded aflagellate cells with numerous pseudopodia were observed (Fig. 5D). *M. scholaia* is distinguished from the other species by the neck at the base of the flagellum.

Mastigamoeba from *Pelomyxa belevskii*

Vacuoles in *Pelomyxa belevskii* contained aflagellated amoebae (Fig. 6A) which after being released from *P. belevskii*, produced a flagellum and started to swim. They died soon after. The swimming cells were oval with an apical nucleus which was associated with the base of the flagellum (Fig. 6B – D). There was a hyaline zone lateral to the nucleus. The morphology of these cells was not studied thoroughly due to the limited material available and further study is needed before it can be described formally.

Mastigamoeba lenta sp. nov.

Unlike other strains, most cells of strain VIT1AN were aflagellated (Fig. 7A – D, I, J). The cells crawled slowly, using a single hyaline lobopodium. Some cells formed thin uroidal filaments (Fig. 7A, G). The nucleus was teardrop-shaped, suggesting that a microtubular cone was present. There was a prominent nucleolus in the central part of the nucleus. Fewer than 5% of cells had a single flagellum (Fig. 7E – H) of various lengths. The nucleus of uniflagellated cells was apical as in other

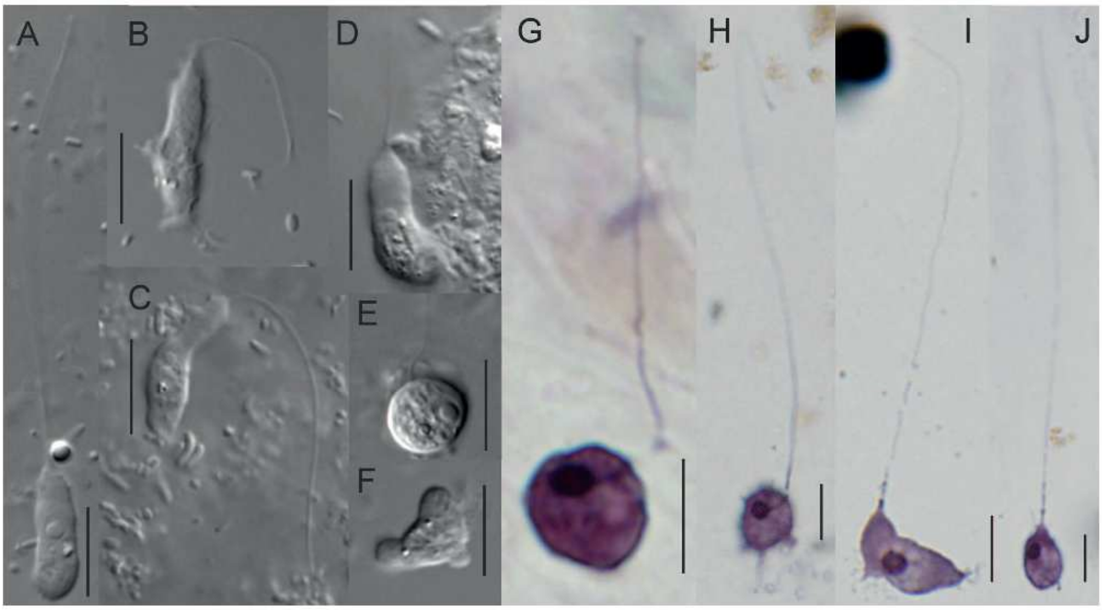

Figure 3. Morphology of *Mastigamoeba abducta* sp. nov. (**A**) Gliding cell of the strain 3MLMA. (**B, C**) Gliding cell of the strain HRN11. (**D**) Gliding cell of the strain CHOM1. (**E**) Rounded cell of the strain CHOM1. (**F**) Aflagellate cell of the strain HRN11. (**G**) Protargol-stained cell of the strain 3MLMA. (**H, I**) Protargol-stained cells of the strain HRN11. (**J**) Protargol-stained cell of the strain CHOM1. Scale bars = 10 μm for A – F, 5 μm for G – J. DIC (A – F) or bright field (G – J).

members of *Mastigamoeba*. The connection between the flagellar base and nucleus was clearly visible in the only protargol-stained cell with a flagellum (Fig. 7H). The pseudopodium of aflagellate cells was preserved in the uniflagellated forms in various positions, including the posterior part of the cell (Fig. 7E). Both the flagellar beating and movement of uniflagellated cells were slow compared to other *Mastigamoeba* strains. This species can be distinguished by the dominance of aflagellated forms and by the extremely slow movement.

Mastigamoeba errans sp. nov.

As with *Mastigamoeba lenta* sp. nov., the strains HRAANM and WAC-6 consisted almost exclusively of aflagellate cells (Fig. 8A – D, H, I). The cells crawled slowly using a single lobopodium. The anterior hyaline zone was thicker than in *M. lenta* sp. nov. In addition, some cells formed fine pseudopodia from the surface of the lobopodium (Fig. 8D). The nucleus was rounded, suggesting that the cone was spread widely in flagellates (Fig. 8G) or very reduced in the aflagellate cells (Fig. 8B). The prominent central nucleolus was smaller in WAC-6 than in HRAANM. Flagellated cells were rare (fewer than 0.5%) in HRAANM (Fig. 8E – G). They possessed a long single flagellum associated with the apical nucleus. Both the flagellar beating and cell movement were fast in comparison to other *Mastigamoeba* strains. The uniflagellate form was short-lived as the cells were seen to attach quickly to the substrate, to start crawling, and to lose the flagellum after several minutes. The leading pseudopodium did not always form at the flagellar base, so in some cases the flagellum was directed posteriorly before it was lost. Flagellated cells of the strain WAC-6 were observed once. They were morphologically similar to that of the strain HRAANM. *M. errans* sp. nov. can be distinguished by the dominance of the aflagellated form with an anterior hyaline lobopodium, and the rounded nucleus.

The genus *Mastigella*

We include here those species that have a single, long anterior flagellum, and with the nucleus not associated with the base of the flagellum (i.e. *Mastigella* sensu Goldschmidt 1907b).

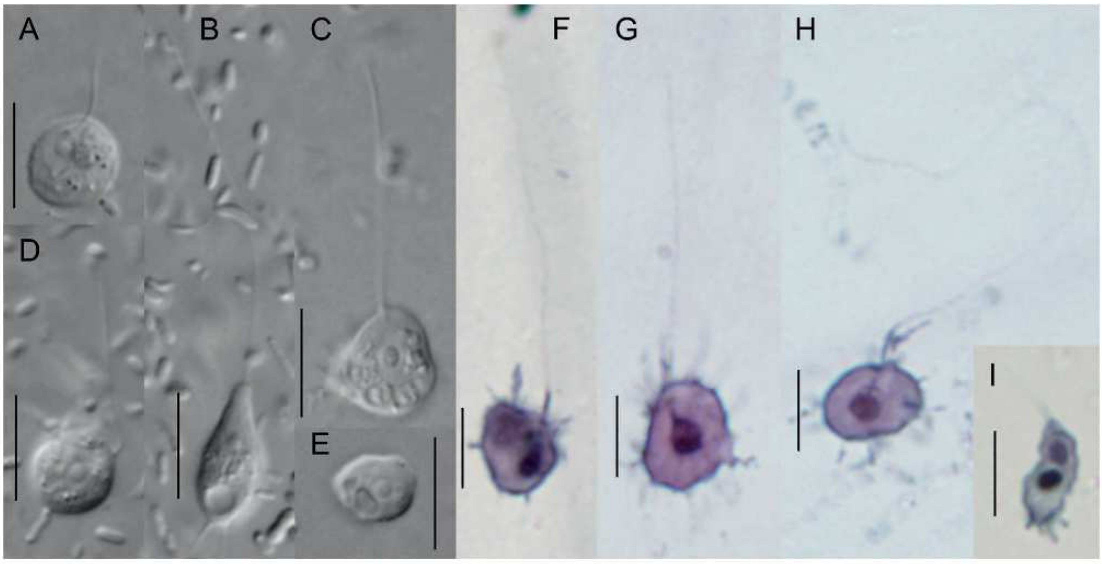

Figure 4. Morphology of *Mastigamoeba guttula* sp. nov. (**A, B**) Gliding cells of the strain LUH2NS4. (**C**) Gliding cell of the strain HRADANAN. (**D**) Gliding cell of the strain LUH2NS4. (**E**) Afflagellate cell of the strain LUH2NS4. (**F – H**) Protargol-stained cells of the strain LUH2NS4. (**I**) Protargol-stained cell of the strain HRADANAN. The oval structure in the center of the cell is an artifact of staining. Scale bars = 10 μm for A – E, 5 μm for F – I. DIC (A – E) or bright field (F – I).

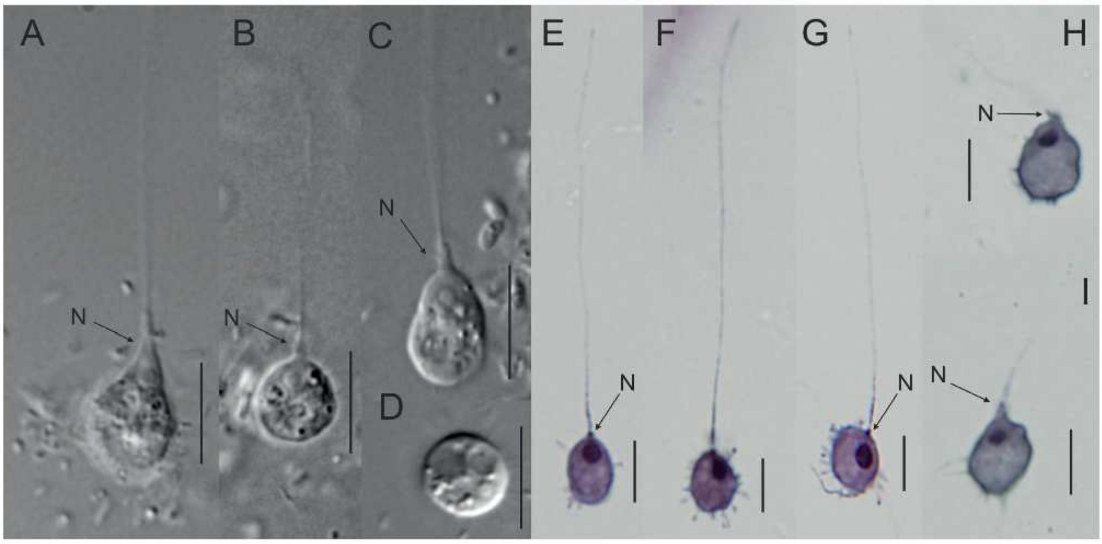

Figure 5. Morphology of *Mastigamoeba scholaia*. (**A, B**) Gliding cells of the strain TEXEL. (**C**) Gliding and aflagellate cell of the strain VITSEDAN. (**D**) Aflagellate cell of the strain TEXEL. (**E – G**) Protargol-stained cells of the strain TEXEL. (**H, I**) Protargol-stained cells of the strain VITSEDAN. N – neck. Scale bars = 10 μm for A – D, 5 μm for E – I. DIC (A – D) or bright field (E – I).

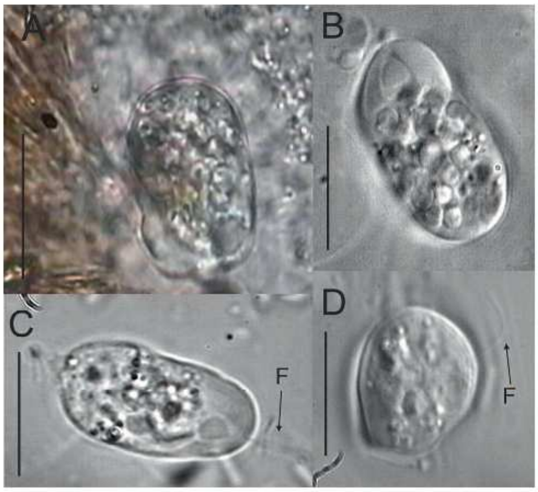

Figure 6. Morphology of *Mastigamoeba* sp. obtained from *Pelomyxa belevskii* (**A – D**). F – flagellum. Scale bars = 50 µm. Bright field (A) or DIC (B – D).

Mastigella sp.

Cells of the strain VIT7 were rare. They were larger (ca. 50 µm long) than cells of *Mastigamoeba*, which averaged about 15 µm long (Fig. 9; Table 2). About half of the cells were uniflagellate (Fig. 9A, B). The flagellum was shorter than the cell body; its movement was slow, ineffective, and did not significantly participate in the movement of cells. The cells crawled slowly using long hyaline finger-like pseudopodia (Fig. 9B – E). The cell body had tiny “dots” – possibly vacuoles or some kind of inclusion body near to the surface – lending the cell a refractive appearance under DIC optics. Pseudopodia were clear and hyaline. Although we were unable to observe the nucleus in the living cells, the flagellar base did not seem to be associated with a particular cell structure and seemed to move freely in the subsurface cytoplasm as the cell moved. Strain VIT7 was therefore identified as a *Mastigella*. The only protargol-stained cell was aflagellate (Fig. 9F). The strain VIT7 was lost before it could be characterized more thoroughly.

The genus *Pelomyxa*

Members of *Pelomyxa* are multinuclear amoeboid organisms that produce a broad leading pseudopodium during movement. The cells have numerous immotile flagella on the surface (except for the area of the leading pseudopodium). The amoeba undergoes growth and multiplication of nuclei. Cysts are known from P. *palustris.* Most morphological descriptions emphasize the traits seen in the trophic, locomotive form. At the light microscopical level, pelomyxids are characterized by the organization of the peripheral zone of

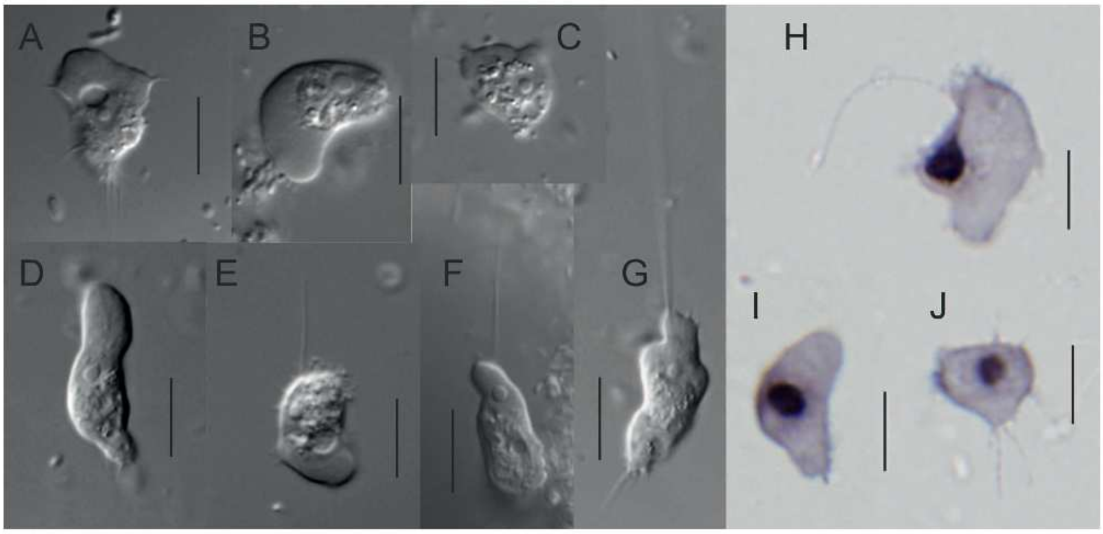

Figure 7. Morphology of *Mastigamoeba lenta* sp. nov. (**A – D**) Aflagellate cells. (**E – G**) Uniflagellate cells. (**H**) Protargol-stained uniflagellate cell. (**I, J**) Protargol-stained aflagellate cells. Scale bars = 10 µm for A – G, 5 µm for H – J. DIC (A – G) or bright field (H – J).

Figure 8. Morphology of *Mastigamoeba errans* sp. nov. (**A, B**) Aflagellate cells of the strain HRAANM. (**C, D**) Aflagellate cells of the strain WAC-6. (**E**) Swimming cell of the strain HRAANM. (**F, G**) Gliding cells of the strain HRAANM. (**H, I**) Protargol-stained aflagellate cells of the strain WAC-6. Scale bars = 10 µm for A – G, 5 µm for H, I. DIC (A – G) or bright field (H, I).

hyaline cytoplasm, the presence and shapes of hyaline pseudopodia, the uroid and the structure of the nuclei. *Pelomyxa* species can differ in cell colour largely determined by the content of digestive vacuoles.

Pelomyxa palustris

Cells of P. *palustris*, especially early-growth-stage specimens, were very mobile. They were oval or cigar-shaped. Large individuals could measure 2 mm and more (Fig. 10A). A bulb-shaped uroid was usually seen in the posterior part of the cell. The cytoplasm was intensely vacuolised (Fig. 11A), grey in colour and contained a lot of mineral particles. There were as many as several hundred small (12 – 16 µm in diameter) round nuclei (Fig. 11A). Numerous small spherical nucleoli were situated at the periphery of the nuclei (Fig. 11B) except in cysts in which the nuclear diameter was up to 30 µm. Cells were covered with a filamentous glycocalyx about 50 nm thick; the filaments were perpendicular to the cell membrane. The basal part of the flagellar apparatus was small, and the basal body difficult to see among the microtubules of the cone. The cone was made up of radial microtubules that formed a bundle parallel to the cell surface. The number of radial microtubules was quite small (Fig. 11C).

Pelomyxa stagnalis

Cells were usually sedentary; locomotive forms could exceed 800 µm long (Fig. 10B). Cells were oval or pyriform, usually with a bulb-shaped uroid in the posterior part, and covered by an amorphous glycocalyx 20 – 30 nm thick. Their cytoplasm was greenish-brown in most cases. Digestive vacuoles of this species were small and generally contained detritus whose mineral component mostly consisted of diatom frustules. There were 30 – 50 round nuclei per cell, each 25 – 30 µm in diameter. The nuclear envelope consisted of several layers: a multilamellar layer adjacent to the nuclear membrane with a layer of small vesicles often filled with electron-dense material next to it (Fig. 11G). The nucleolus was central and round, but sometimes consisted of 2 – 3 irregular lobes, formed by several intertwining fragments (Figure 11H). Nucleoli contained distinctive corpuscles similar to Cajal bodies in animals (Fig. 11H). Flagellar kinetosomes were short with a bundle of a few radial microtubules running parallel to the cell surface (Fig. 11I).

Pelomyxa belevskii

P. belevskii cells were ovoid, motionless, and usually smaller than 500 µm (Fig. 10C). No uroid was discerned. The cytoplasm was transparent or

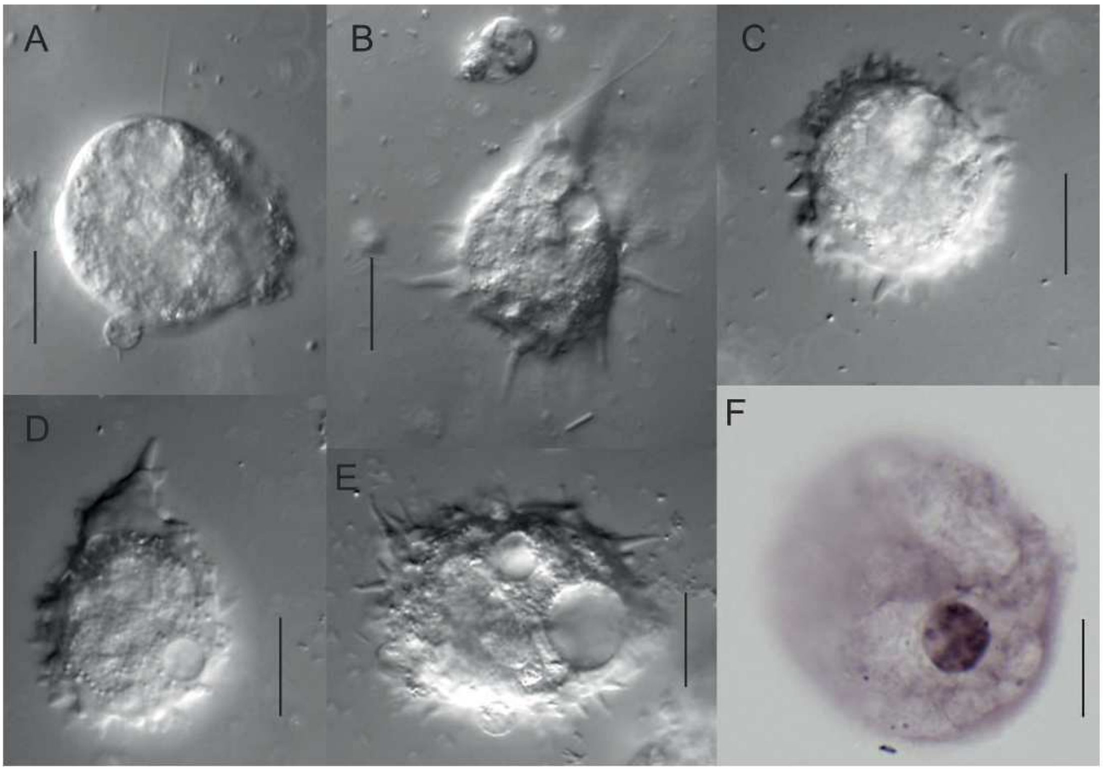

Figure 9. Morphology of *Mastigella* sp. (**A**) Flagellate resting cell. (**B**) Flagellate crawling cell. (**C**) Aflagellate resting cell. (**D, E**) Aflagellate crawling cells. (**F**) Protargol-stained aflagellate cell. Scale bars = 20 μm for A – E, 10 μm for F. DIC (A – E) or bright field (F).

variously orange. There were several big digestive vacuoles with fragments of vascular plant tissues in the cytoplasm. Around the periphery of the cells was a layer of hyaline cytoplasm. The cell surface bore multiple short conical projections, often palmatipartite. There were 50 – 60 large (up to 30 μm), and ovoid nuclei per cell (Fig. 11D). Numerous small nucleoli were located at the periphery of the nucleus. The glycocalyx was filamentous, similar to that of *P. palustris*. Nucleoli

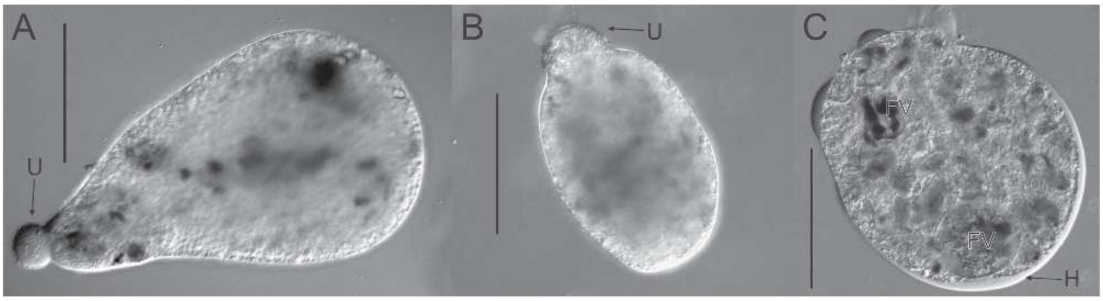

Figure 10. Gross morphology of *Pelomyxa* spp. (**A**) *P. palustris*. (**B**) *P. stagnalis*. (**C**) *P. belevskii*. FV – food vacuole; H – superficial layer of hyalopasm; U – uroid. Scale bars = 400 μm for A, B, 300 μm for C. DIC.

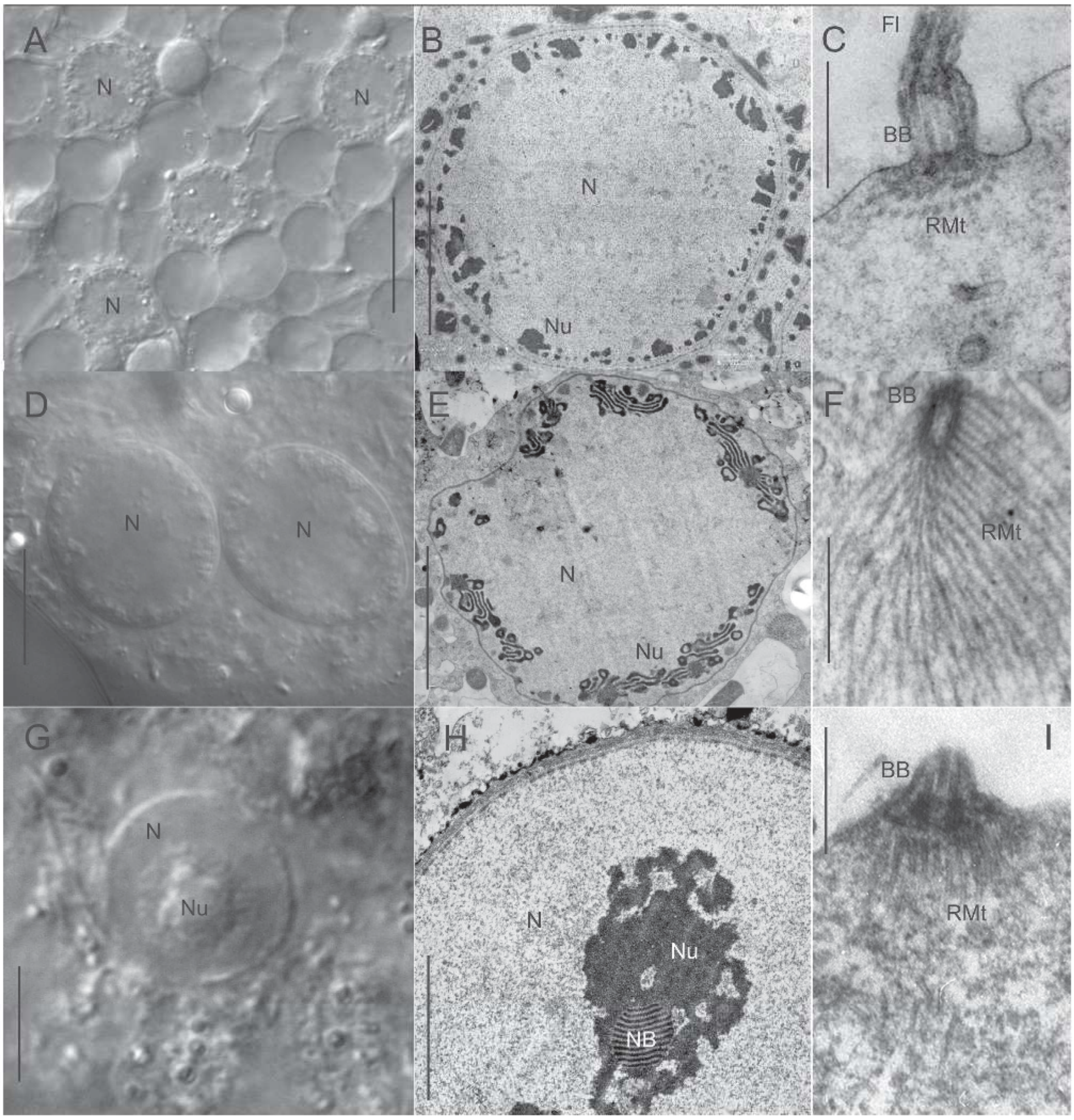

Figure 11. Nuclear morphology, nuclear ultrastructure, and structure of flagellar apparatus of *Pelomyxa* spp. (**A – C**) *P. palustris*, (**D – F**) *P.belevskii*, (**G – I**) *P. stagnalis*. BB– basal body; Fl – flagellum; N – nucleus; NB – nucleolar body; Nu – nucleolus; RMt – radial microtubules. Scale bars = 25 μm for A, 20 μm for D, G, 5 μm for B, H, 15 μm for E, 400 nm for C, I, 1 μm for F. DIC (A, D, G) or TEM (B, C, E, F, H, I).

had a characteristic appearance as bundles of electron-dense vermiform bodies and rings (Fig. 11E). The flagellar apparatus of *P. belevskii* included a relatively long basal body associated with numerous radial microtubules (Fig. 11F) that were directed proximally.

The genus *Rhizomastix*

The flagellate genus *Rhizomastix* can be distinguished from other pelobionts by light-microscopy by the thick fibre or rhizostyle that runs from the flagellar base through the cell and around the

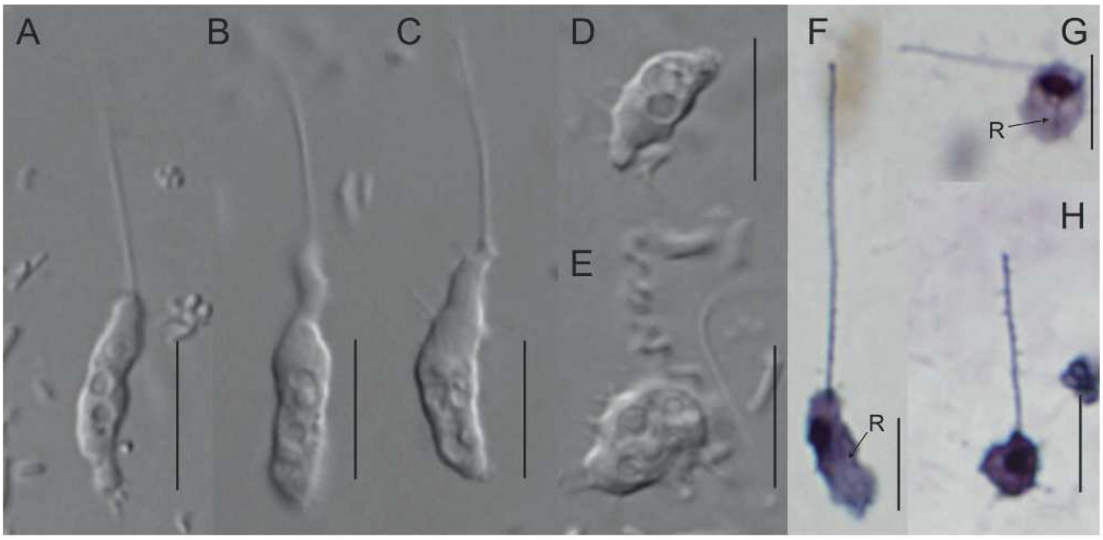

Figure 12. Morphology of *Rhizomastix libera* sp. nov. (**A**) Swimming cell of the strain IND8. (**B, C**) Gliding cells of the strain IND8. (**D**) Aflagellate cell of the strain IND8. (**E**) Resting uniflagellate cell of the strain IND8. (**F – H**) Protargol-stained cells of the strain IND8. R – rhizostyle. Scale bars = 10 μm for A – E, 5 μm for F – H. DIC (A – E) or bright field (F – H).

nucleus. It is a tapering bundle of microtubules. The term "rhizostyle" (see Cepicka 2011) has been used for some other cytoskeletal structures of pelobionts, such as the lateral flagellar root (e.g. Becker 1925; Brugerolle 1982). It is visible in protargol-stained cells. The typical motion of living cells is a remarkably fast and somewhat jerky movement. The rounded nucleus with a prominent nucleolus occupies a central or subapical place in the cell. Under light-microscopy, there is no visible connection between the flagellar base and nucleus in living cells, but the nucleus seems to be somehow fixed in its place in the cytoplasm.

Rhizomastix libera sp. nov.

Cells were elongate (see Table 2). The anterior part of living cells in front of the nucleus is hyaline. There was a cone-like uroid in some swimming cells (Fig. 12A). Crawling cells produced fine pseudopodia (Fig. 12D, E). A few aflagellated cells were observed in the culture (Fig. 12D). Swimming cells of the strain IND8MA were elongated (Fig. 12A – C).The "rhizostyle" is visible in many protargol-stained cells (Fig. 12F, G). Its proximal part was associated with the flagellar base and it continued to the posterior part of the cell along the nucleus. The nucleus was heavily stained and its internal structure could not be observed. The flagellum was thicker than in other pelobionts and had fine projections on its surface (Fig. 12H). *R. libera* sp. nov. can be distinguished from the other members of *Rhizomastix* by its small size, short flagellum, and fast movement.

R. libera sp. nov. was examined by transmission electron microscopy (Figs 13 and 14). The longitudinal axis of the cell is defined as running from the apical flagellar apparatus through the nucleus and the flagellar root runs laterally to the right.

In axial longitudinal sections (Figs 13A, 14B), the body is amoeboid, with no fixed shape, no theca nor cytoskeletal structures supporting the cell membrane. There is a central nucleus, with an electron-dense nucleolus (Figs 13A, E, 14D, F), surrounded by endoplasmic reticulum (Figs 13A, E, 14D), food vacuoles (Fig. 13A) and vacuoles containing endosymbiotic prokaryotes (Fig. 13A, D, E). A multimembrane structure (Fig. 13B) is present, positioned close to the flagellar apparatus. Mitosome-like, acristate, double-membrane-bound organelles (Fig. 13C) with diameter less than 200 nm are present in the cytoplasm.

The flagellar apparatus (Figs 13A, 14A – P) consists of a flagellum, a single basal body, a flagellar root of 8 microtubules, and a rhizostyle extending proximally into the cell. The flagellum has a normal 9+2 doublet structure of microtubules. Two vanes that vary in size along the length of the axoneme

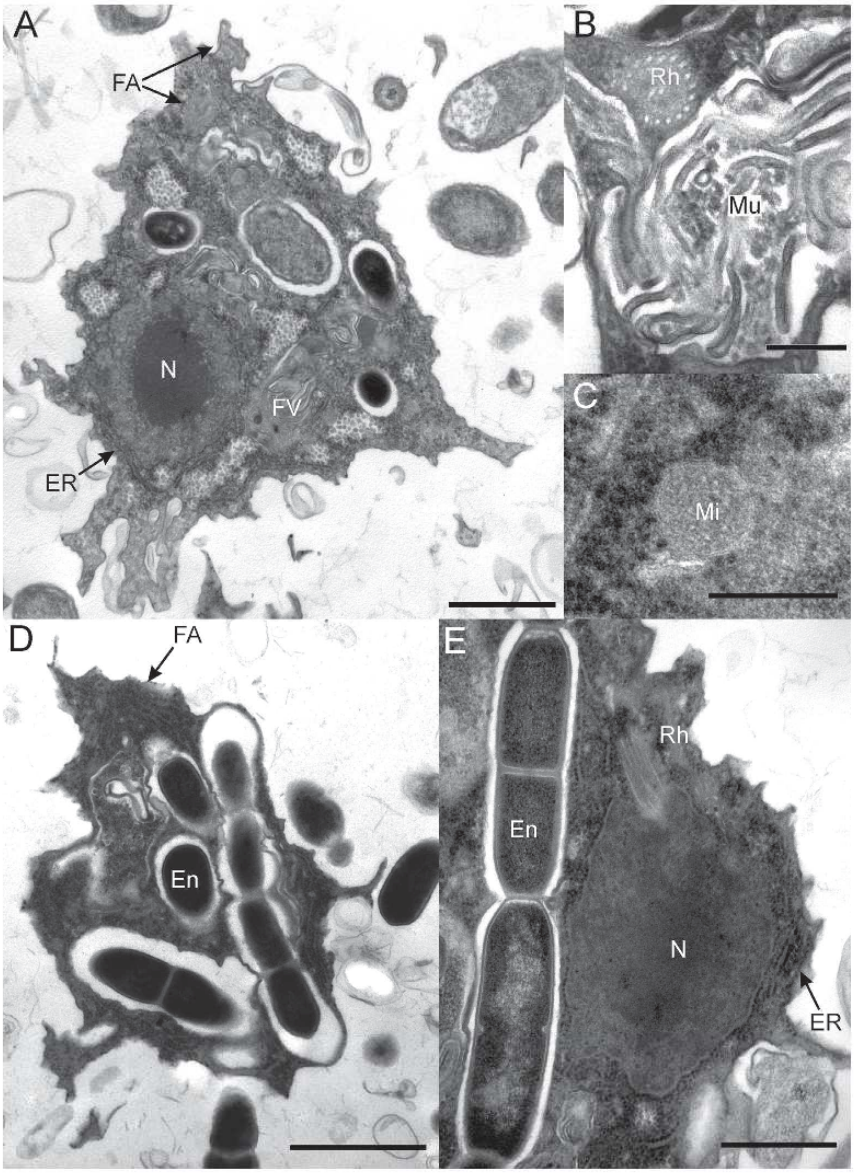

Figure 13. Ultrastructure of *Rhizomastix libera* sp. nov. **(A)** Axial longitudinal section of the cell, showing the amoeboid body, with no fixed shape; a central nucleus, surrounded by endoplasmic reticulum, containing an electron-dense nucleolus, and vacuoles containing food or endosymbionts. **(B)** Multi-membrane structure reminiscent of Golgi apparatus, positioned close to the top of the rhizostyle. **(C)** Mitosome-like, acristate, double-membrane-bound organelle. **(D)** Sagittal section through side of cell showing vacuoles containing endosymbiotic prokaryotes. **(E)** Section through nucleus showing connection of the rhizostyle above and below, endoplasmic reticulum and a vacuole containing an endosymbiont. En – prokaryotic endosymbiont; ER – endoplasmic reticulum; FA – flagellar apparatus; FV – food vacuole; Mi – mitochondrion-like organelle; Mu – multimembrane organelle; N – nucleus; Rh - rhizostyle. Scale bars = 1 μm for A, D, 200 nm for A, C, and 500 nm for E.

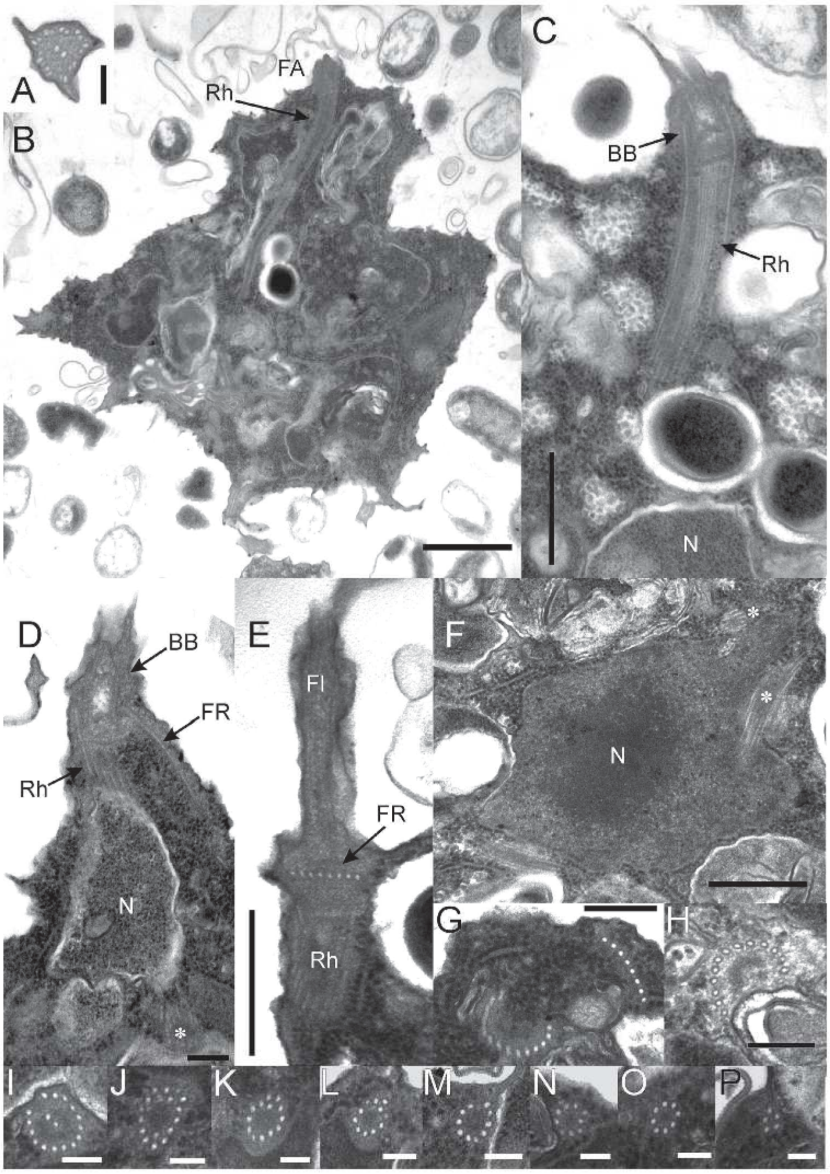

Figure 14. Ultrastructure of the flagellar apparatus of *Rhizomastix libera* sp. nov. (**A**) Transverse section of the flagellum, showing two flagellar vanes and standard 9+2 structure of microtubules. (**B**) Axial longitudinal section through the cell, showing the rhizostyle extending from the flagellar apparatus to the posterior of the cell. (**C**) Flagellar apparatus in longitudinal section, showing the basal body giving rise to the rhizostyle. (**D, E**) Flagellar apparatus in longitudinal section, showing the flagellar root arising from the side of the basal body; in (D) with the rhizostyle folding around the nucleus, and shown below the nucleus (asterisk). (**F**) Rhizostyle folding around the nucleus (top right and bottom left). (**G, H**) Transverse section of the rhizostyle close to the flagellar apparatus, with root microtubules to the right in (G). (**I – P**) Progression of the rhizostyle through the cell, showing the reduction in number of central and peripheral microtubules. BB – basal body; FA – flagellar apparatus; Fl – flagellum; FR – flagellar root; N – nucleus; Rh, asterisk – rhizostyle. Scale bars = 100 nm for A, I – P, 1 μm for B, 500 nm for C, E, F, and 200 nm for D, G, H.

(Figs 13A, 14A) account for its thick appearance by light-microscopy. Figure 14E shows a “ruffled” appearance of the flagellar membrane with a vane moving in and out of the plane of section on the right.

It was not possible to determine whether or not dynein arms were present in the axoneme, due to the quality of the fixation. The basal body appears to have a normal triplet structure, but clear pictures were not obtained; a second basal body was never observed. One flagellar root (Fig. 14D, E, G) consists of a flat ribbon of eight microtubules and arises about halfway up the basal body (Fig. 14E), descending laterally into the cytoplasm. The rhizostyle is a cylindrical root of microtubules that extends proximally from the base of the basal body (Fig. 14B – E) and wraps around the nucleus, and turns at an angle of greater than 90 degrees (Fig. 14D, F, asterisks). The number of microtubules varies within and between individuals. At its origin there are 13 – 15 microtubules in a circle around a central dense area (Fig. 14G, H), with a central pair of microtubules arising slightly proximally to the basal body (Fig. 14I). The central pair moves towards the side of the rhizostyle (Fig. 14J – L) away from the central dense area (Fig. 14I – O) and end somewhere near where the rhizostyle makes first contact with the nucleus. The rhizostyle then gets narrower (Fig. 14B) and the number of microtubules decreases as it extends proximally (Fig. 14G – P), with five microtubules being the fewest seen (Fig. 14P).

Phylogenetic analyses

The archamoebae showed extraordinary interspecies variability of SSU rRNA gene sequences. Most variability was found in sites corresponding to hypervariable regions of the SSU rRNA molecule (Wuyts et al. 2000). These parts of SSU rRNA gene were virtually unalignable even among closely related species, and were almost wholly trimmed from the data set prior to the phylogenetic analysis. The SSU rRNA gene without the hypervariable regions distinguish species of archamoebae.

Strains of species may exhbit intra-strain sequence variability. The variability of strains 3ML, HRN11, PSOVKA and of *Pelomyxa belevskii* was negligible (< 1%); those of strain LUH2NS4 differed in up to 2.3% of positions, while strains CHOM1 and IND8 varied by 7.0% and 6.4%, respectively. The vast majority of differences were located in the hypervariable regions, which were trimmed during the data set preparation. A preliminary analysis did not lead to differences in phylogenetic position of different clones of a strain, so we included only the sequence of a single clone of each strain in the final analysis.

The phylogenetic tree of archamoebae based on the first data set (see Methods) (Fig. 15) has Amoebozoa split into several lineages without resolved interrelationships consistent with previous studies (Fiore-Donno et al. 2010; Kudryavtsev et al. 2011; Shadwick et al. 2009). The archamoebae formed a well-supported clade with four robust lineages: (1) species of *Entamoeba*, (2) species of *Pelomyxa*, (3) *Rhizomastix libera* sp. nov., and (4) the Mastigamoebidae comprised of *Mastigamoeba*, *Endolimax*, *Iodamoeba*, and several environmental sequences. *Entamoeba* and *Pelomyxa* formed a clade; with *Rhizomastix libera* sp. nov. as sister to it but without statistical support. SSU rRNA gene sequences of the two new *Pelomyxa palustris* isolates were almost identical. The GenBank sequence AF320348 for *P. palustris* was almost identical with the sequence obtained by us from *P. stagnalis*. The Mastigamoebidae split into two clades –A and B. Mastigamoebidae A split into three branches without resolved interrelationships: I. *Mastigamoeba errans* sp. nov. and environmental sequence GU919401, II. Environmental sequence AM114798, III. *Mastigamoeba balamuthi*, *M. punctachora*, *M.* sp. obtained from *Pelomyxa* spp., strain VIT7, *Mastigella commutans*, and environmental sequence AM114799. The sequence AF421219, designed as *Mastigella commutans*, was almost identical with strains of *Mastigamoeba punctachora*. The clade of Mastigamoebidae B split into three branches without resolved interrelationships: I. *Mastigamoeba lenta* sp. nov., II. *Endolimax nana* and *Iodamoeba* sp., III. *Mastigamoeba simplex*, *M. guttula* sp. nov., *M. scholaia*, *M. abducta* sp. nov., and environmental sequence GU921440.

To further examine relationships between *Entamoeba*, *Rhizomastix*, and *Pelomyxa*, we created three additional data sets in which one of the genera was omitted. When *Pelomyxa* sequences were removed (data set 2), the topology and node supports remained similar to the analysis of the first data set with two important exceptions (Fig. 16A). The value of bootstrap support for the archamoebae being monophyletic increased slightly, and *Rhizomastix* and *Entamoeba* formed sister lineages with a relatively strong support. When *Entamoeba* spp. were removed (data set 3), *Pelomyxa* formed the basal branch of the archamoebae, *Rhizomastix* formed a sister branch to the Mastigamoebidae, though without strong support, and support for archamoebal monophyly

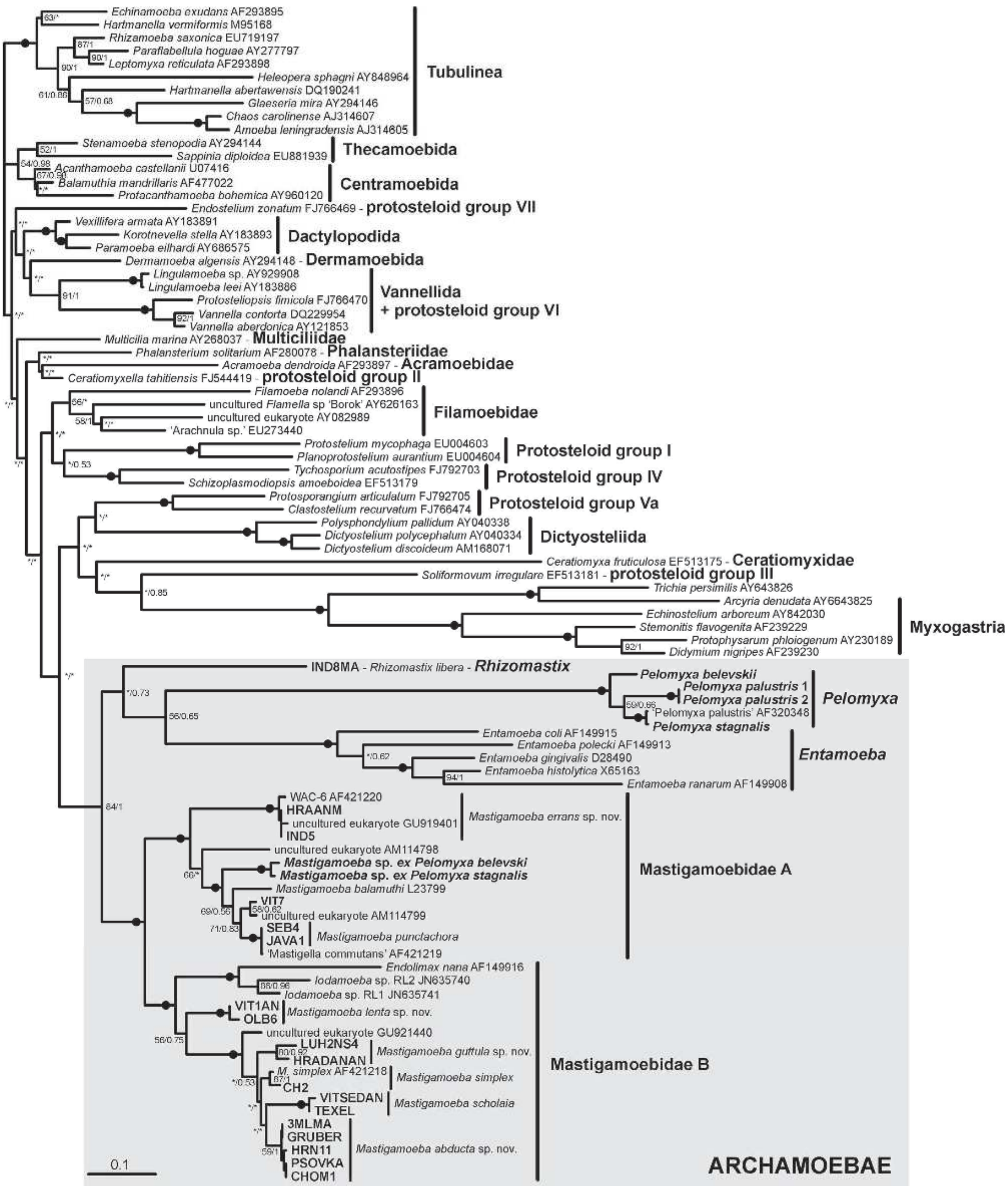

Figure 15. Unrooted phylogenetic tree of Amoebozoa based on SSU rRNA gene sequences. The tree was constructed by the maximum likelihood method (GTR+I+Γ model). The values at the nodes represent statistical support in maximum likelihood bootstrap values/Bayesian posterior probabilities. Support values below 50%/.50 are represented by an asterisk (*). New sequences are in bold. Higher taxa of Amoebozoa are named according to Shadwick et al. (2009) and Smirnov et al. (2011).

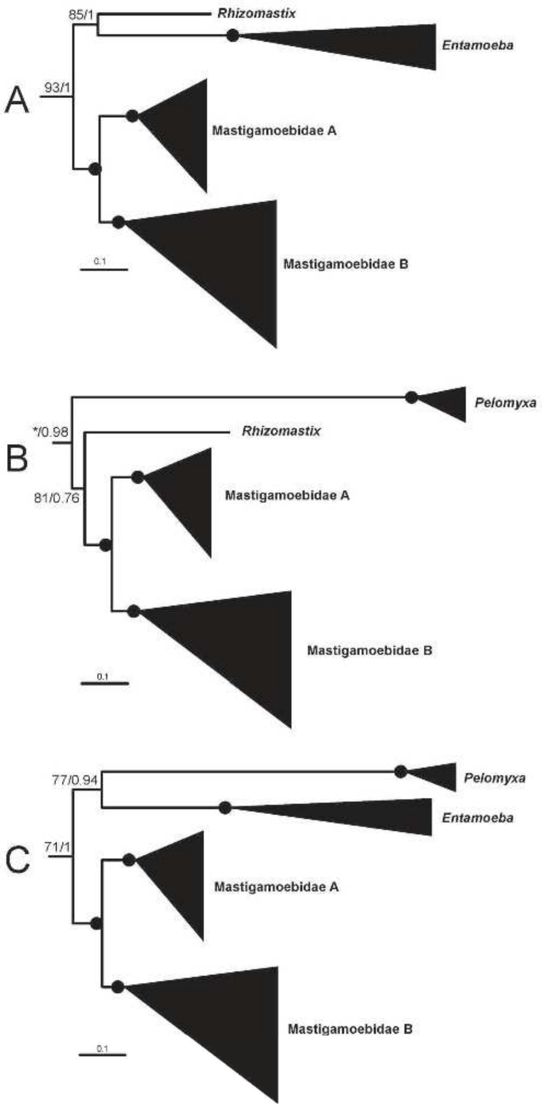

Figure 16. Phylogenetic trees of pelobionts based on SSU rRNA gene sequences, with exclusion of *Pelomyxa* spp. (**A**), *Entamoeba* spp. (**B**), or *Rhizomastix libera* (**C**). The trees were constructed by the maximum likelihood method (GTR+I+Γ model) and were rooted with main lineages of non-pelobionts (outgroups not shown). The values at the nodes represent statistical support in maximum likelihood bootstrap values/Bayesian posterior probabilities. Support values below 50% are represented by an asterisk (*).

decreased (Fig. 16B). Finally, when *Rhizomastix* was removed from the analysis (data set 4), *Pelomyxa* and *Entamoeba* remained sister taxa with medium support, and support for a monophyletic archamoebae decreased (Fig. 16C).

Discussion

Species Identities of Strains

Based on morphological distinctiveness and placement in molecular trees, our isolates belong to 12 species. Amoeboid members of the genus *Pelomyxa* can be determined easily using a combination of light-microscopic morphology and ultrastructure (Chistyakova and Frolov 2011; Griffin 1988; Page 1981). Our phylogenetic analysis shows that the sequence AF320348 deposited in GenBank under the name *Pelomyxa palustris* (Milyutina et al. 2001) belongs to *P. stagnalis.* The morphology of cells of *P. belevskii* closely corresponds to Page's (1981) observations of *Pelomyxa palustris.* A description of the ultrastructure of *P. belevskii* is here presented for the first time. We show that it has a distinctive nucleus and flagellar apparatus.

Cells of all but two of the isolates that showed swimming behaviour are assigned to *Mastigamoeba* because there is a connection between the nucleus and flagellar base. The connection is most clearly shown in protargol-stained preparations. The taxonomy of *Mastigamoeba* is problematic. More than 50 nominal species have been described (see Table S1); most formal descriptions are brief and incomplete (see Bernard et al. 2000). Species have been distinguished mainly on the basis of cell size, flagellar length, and location of pseudopodial formation (Bernard et al. 2000; Brugerolle 1991b; Chatton 1925; Goldschmidt 1907a, b; Kudo 1939, 1977; Lemmermann 1914; Poche 1913; Reichenow 1928, 1952; Siemensma 1987; Walker et al. 2001). Given the highly variable nature of the species, and incompleteness of many descriptions, many cannot be reliably distinguished by light-microscopy and may represent invalid species, a situation not uncommon among protists (Thessen et al. 2012). In this study, where we supplemented light-microscopy with molecular features, we were able to confirm the significance of morphological differences between species. Species with distinctive light-microscopical appearances form clades in SSU rRNA gene phylogenetic analyses.

Our *Mastigamoeba* strains fall into eight distinct species, four of which are new. The nuclear granule in cells of strain SEB4 identifies it unequivocally as *Mastigamoeba punctachora* (Bernard et al. 2000). Cells of SEB4 stained by protargol show several features unobserved in other *Mastigamoeba* strains, namely the conspicuous darkly-staining basal body, cone, and putative microtubular root. These characters were also observed in cells of the strain JAVA confirming that it belongs to *M. punctachora* as well. Interestingly, SSU rRNA gene sequences of the two strains are almost identical with GenBank sequence AF421219 which had been ascribed to *Mastigella commutans* (Edgcomb et al. 2002). It is possible that the culture of *M. commutans* was contaminated with *M. punctachora* or cultures of the two species were confused, as both cultures were held in the same laboratory at the time that these sequences were generated. The morphology of strain CH2 is identical to that of *Mastigamoeba simplex* reported by Bernard et al. (2000). Importantly, the posterior thick pseudopodia were not observed in any other strain. Cells of *M. abducta* sp. nov. are morphologically similar to *M. simplex* with respect to the nucleus, which is subcentral in both species but is situated more centrally in *M. abducta* sp. nov. Cells of *M. abducta* sp. nov. do not form the thorn-like posterior pseudopodia.The two species separate clearly on molecular criteria. Cells of strains VITSEDAN and TEXEL have a persistent neck supporting the flagellar base, are often rounded, and have radiating pseudopodia. As far as we know, a distinct neck has been reported in *Mastigamoeba* only in *M. scholaia* (Klug 1936), and cells of this species are rounded with radiating pseudopodia. Therefore, we have identified these strains as *M. scholaia*. This small, distinct neck is about 1 μm long and has also been described in *Mastigella commutans* (Frenzel 1897); it differs from that recently described in *Subulatomonas*, which is considerably longer (about 5 -10 μm) and much more flexible (Katz et al. 2011). *M. guttula* sp. nov. is similar to *M. scholaia* because of the sun-like appearance of many cells, but it lacks the neck and the nucleus is subapical as opposed to subcentral in *M. guttula* sp. nov. The two species have distinct SSU rRNA gene sequences. The remaining two *Mastigamoeba* species, *M. errans* sp. nov. and *M. lenta* sp. nov. differ from other mastigamoebae by their predominantly amoeboid lifestyle. Besides their divergent phylogenetic position, the two species differ in swimming speed and lifespan of the flagellate stage (flagellates of *M. errans* sp. nov. swim rather quickly and are short-lived).

The amoebae that occurred in vacuoles of *Pelomyxa belevskii* emerged, produced flagella and started to swim. In their short life, they were similar to mastigamoebae. Our preliminary data suggest that *P. belevskii* may contain more than one species of eukaryotic endobionts. Because of this, we have not described unique correspondence between those cells and the 18S rRNA gene sequence obtained. The emergence of flagellates from much larger cells is consistent with descriptions of *Mastigina setosa* by Goldschmidt (1907a) and *Pelomyxa palustris* by Whatley and Chapman-Andresen (1990). No symbionts were observed in *P. stagnalis*, and there were no candidates for the *Mastigamoeba* sequence from these cultures, unless symbiotic mastigamoebae were overlooked.

Pelobiont strain VIT7 was identified as *Mastigella* because of the absence of a microtubular connection between the flagellar base and nucleus. Unfortunately, the strain VIT7 was lost before its morphology could be examined thoroughly. As with *Mastigamoeba*, *Mastigella* is taxonomically problematic in the sense that many nominal species have been described (see Table S1), most of them inadequately. It is difficult to unequivocally assign a *Mastigella* isolate to a nominal species. Strain VIT7 is most similar to *Mastigella vitrea* and *M. nitens*. In particular, the highly refractive glassy appearance with clear hyaline pseudopodia of VIT7 is reminiscent of Goldschmidt's description and drawing of *M. vitrea*. However, cells of VIT7 were mostly smaller (average 52 μm long: see Table 2) than those of *M. vitrea* (120-150 μm, see Goldschmidt 1907a). *M. nitens* cells have a hyaline border (Penard 1909) which was lacking in cells of VIT7.

The presence of the rhizostyle in cells of strain IND8MA identifies it unequivocally as *Rhizomastix*. *R. libera* sp. nov. is the first free-living member of the otherwise endobiotic genus *Rhizomastix* and its cells are considerably smaller than other species (Bhaskar Rao 1963, 1970; Cepicka 2011; Krishnamurthy 1969; Ludwig 1946; Mackinnon 1913; Sultana 1976; Yakimoff and Kolpakoff 1921). The rapid flagellar and cell movement of the new species differentiates it from *R. biflagellata* and *R. ranae*, the only other species whose movement has been recorded (Cepicka 2011; Krishnamurthy 1969).

Phylogeny of Archamoebae

Among the major lineages of protists, the Amoebozoa is relatively poorly studied. Analyses of the SSU rRNA gene have failed to resolve relationships among amoebozoan lineages (e.g. Brown et al. 2011; Fiore-Donno et al. 2010; Kudryavtsev et al. 2009, 2011; Shadwick et al. 2009; Stensvold et al. 2012). The actin gene has also been utilized in phylogenetic analyses of Amoebozoa, but also does not resolve deep-level relationships within the Amoebozoa (Lahr et al. 2011). The monophyly of higher taxa within Amoebozoa thus remains unsupported, with the exception of the Tubulinea, Dictyosteliida, Myxogastria and archamoebae. We analyzed 91 SSU rRNA gene sequences representing a broad diversity of Amoebozoa. Our results are in agreement with previous studies in that they provide poor resolution of the backbone of the amoebozoan tree. The archamoebae have relatively good support (maximum likelihood bootstrap support 84, Bayesian posterior probability 1). A clade consisting of Myxogastria, Dictyosteliida, *Ceratiomyxa*, and protosteloid groups III and Va appear as sister to archamoebae with weak support (for nomenclature of protostelid lineages see Shadwick et al. 2009). However, this clade's monophyly and internal topology remain uncertain.

The present study improves the taxon sampling of pelobiont SSU rRNA gene sequences. Before this, SSU rRNA gene sequences of only six non-*Entamoeba* archamoebal species were determined (Edgcomb et al. 2002; Hinkle et al. 1994; Milyutina et al. 2001; Silberman et al. 1999; Stensvold et al. 2012). We show that sequences AF320348 and AF421219 do not belong to *Pelomyxa palustris* and *Mastigella commutans* respectively as proposed by Milyutina et al. (2001) and Edgcomb et al. (2002), but to *Pelomyxa stagnalis* and *Mastigamoeba punctachora*, respectively. We added new SSU rRNA gene sequences for *Rhizomastix* and seven previously uncharacterized species of *Pelomyxa* and *Mastigamoeba*. These new sequences have a positive effect on the statistical support for a monophyletic archamoebae. Previously, some authors (Fiore-Donno et al. 2010) excluded the *Pelomyxa* SSU rRNA gene sequence (AF320348) of *P. stagnalis* (as *P. palustris*) because it is extremely long, divergent (Milyutina et al. 2001), and difficult to align. The SSU rRNA gene sequences of true *P. palustris* and *P. belevskii* are about 400 bp shorter than that of *P. stagnalis*. In our analysis which included only the sequence AF320348, the maximum likelihood bootstrap support for monophyletic archamoebae was 63; with the newly determined *Pelomyxa* sequences, the support increased to 84.

The archamoebae consist of four lineages, corresponding roughly with nominal families: Mastigamoebidae, Pelomyxidae, Entamoebidae, and Rhizomastixidae. The Mastigamoebidae appears robustly monophyletic and contains all the pelobionts with a motile flagellum associated with the microtubular cone and flagellar root arising from a single basal body, as well as two endobiotic taxa of uncertain ultrastructure. Mastigamoebidae robustly split into two clades, Mastigamoebidae A and B. Since the ultrastructures of only three *Mastigamoeba* species with known phylogenetic position have been characterized so far (Chávez et al. 1986; Walker et al. 2001), it is currently impossible to characterize lineages Mastigamoebidae A and B morphologically. Genera *Iodamoeba* and *Endolimax* seem to have completely lost any externally obvious microtubular cytoskeleton but robustly cluster within Mastigamoebidae B. The reduced flagellar apparatus in these taxa may reflect their parasitic lifestyle. Their flagellar loss is independent of that of *Entamoeba*. As information on ultrastructure of *Endolimax* and *Iodamoeba* is fragmentary (Zaman et al. 1998, 2000), the future may include discovery of remnants of their flagellar apparatus. The phylogenetic position of genera *Endolimax* and *Iodamoeba* within Mastigamoebidae is supported by their nuclear morphology. The nucleus of members of the two genera is devoid of peripheral chromatin (Zaman et al. 1998, 2000) as with *Mastigamoeba* spp. (Chávez et al. 1986; Frolov 2011; Simpson et al. 1997; Walker et al. 2001). On the other hand, the nucleus of *Entamoeba* possesses a peripheral layer of electron-dense granules (El-Hashimi and Pitman 1970; Rosenbaum and Wittner 1970).

Rhizomastix libera sp. nov. is clearly a free-living archamoeba as proposed by Cepicka (2011) and Kudo (1939, 1977), and is likely related to *Entamoeba* and *Pelomyxa*. As *Pelomyxa* spp. and *Entamoeba* spp. form long branches in tree, their relationship may be a long-branch attraction artefact. To explore this, we performed analyses in which *Pelomyxa*, *Entamoeba* or *Rhizomastix*, respectively, were excluded. The support values for a sister relation between *Rhizomastix* and *Entamoeba* when *Pelomyxa* spp. were removed from analyses were higher than the support values of a sister relationship of *Pelomyxa* and *Entamoeba* when *Rhizomastix* was removed from the analyses. However, AU tests could not reject topologies with *Rhizomastix* as a basal archamoeba. Since SSU rRNA gene analysis alone is obviously unable to elucidate the relationships between higher archamoebal taxa, it is necessary to perform multigene phylogenetic analyses. Moreover, there are several key members of the archamoebae from which DNA sequence data are currently unavailable (e.g. *Mastigamoeba aspera*, *Tricholimax hylae*, *Rhizomastix gracilis*, *Endamoeba blattae*, and species of *Mastigella* and *Mastigina*). Nevertheless, the possible close relationship between *Entamoeba* and *Rhizomastix* seems to be supported by nuclear morphology as most *Rhizomastix* species (though not *R. libera* sp. nov.) share the conspicuous peripheral chromatin arranged into granules with *Entamoeba* (Cepicka 2011). It is because of the uncertain relationships of *Rhizomastix* and entamoebae to the pelobionts that we use the term archamoebae to refer to the organisms studied (see Introduction).

Rhizomastix Has a Unique Ultrastructure

R. libera sp. nov. has many features of pelobionts such as the amoeboid flagellate habit, anaerobiosis coupled with reduction of mitochondria, a simple microtubular cytoskeleton consisting of a basal body supporting the flagellum and two microtubular roots. The ultrastructure of cells of *R. libera* sp. nov. is sufficiently different from other archamoebae as to justify the establishment of a separate family, Rhizomastixidae fam. nov.

Until now, the origin of the rhizostyle of *Rhizomastix* was unclear and various hypotheses had been proposed (Cepicka 2011). We can now see that it is a modified microtubular cone as found in other pelobionts. It may anchor the flagellar basal body firmly so allowing the swift movements of this species. The flagellar vanes of *Rhizomastix* are distinctive, are evident in protargol-stained preparations, and make the flagellum look thicker. The flagellum of *R. biflagellata* is thicker than flagella of *Trimitus* in Figure 2D in Cepicka (2011) suggesting that *R. biflagellata* bears flagellar vanes as well. The small mitochondrion-related organelle (MRO) of *R. libera* sp. nov. is similar in size to that of the other pelobionts (Gill et al. 2007; León-Avila and Tovar 2004; Mi-ichi et al. 2011;Walker et al. 2001). The multimembrane organelle in *R. libera* sp. nov. seems to be unique, but may be a fixation artefact. A similar, though bigger and more organized structure has been recently reported from *Pelomyxa flava* (Frolov et al. 2011). The multimembrane organelle of *R. libera* sp. nov. is to some extent reminiscent of Golgi apparatus. A stacked Golgi apparatus has not been found in the archamoebae so far, though related elements of the endomembrane system have been shown to be functionally present in *Entamoeba histolytica* (Bredeston et al. 2005) and *Mastigamoeba balamuthi* (Dacks et al. 2004).

According to our phylogenetic trees, the endobiotic life style emerged at least twice independently within the archamoebae. *Rhizomastix* and Entamoebidae are clearly phylogenetically distinct from *Iodamoeba* and *Endolimax*; and *Tricholimax* (endobiotic in frogs) is of yet-undetermined position, but shows similarities to both *Pelomyxa* and *Mastigamoeba* (Brugerolle 1982). The endobiotic members of the group typically have reduced flagellar apparatuses. The ancestrally anaerobic metabolism of archamoebae is likely to be an adaptation that allowed endobiont members to thrive within gut-ecosystems. *Rhizomastix libera* sp. nov. is the first known free-living member of the genus *Rhizomastix*. As the genus *Rhizomastix* contains both endobiotic and free-living species, it may become a useful model to study of the origin of parasitism within archamoebae.

Changes in Taxonomy of Archamoebae and Pelobionts

The taxonomy of archamoebae is confusing. The families Mastigamoebidae, Pelomyxidae and Entamoebidae are universally accepted. Recent classifications that bring these families together (e.g. Smirnov et al. 2011) rely on the relationships between entamoebae and pelobionts that we do not believe to be supported (yet) by our data. Until the relationships among the consitutent families are robustly confirmed, we prefer to refer to the entamoebae and pelobionts informally as archamoebae. In the event that entamoebae are found to be derived from within the pelobionts, then the correct name for this group using the principle of priority will be ‘Pelobiontida’. In the event of the entamoebae being shown to be sister to the pelobionts, then the correct name for the clade would be Archamoebida Cavalier-Smith, 1983.

Other families have been established with the archamoebae: Phreatamoebidae, Mastigellidae and Endolimacidae (Cavalier-Smith 1991; Cavalier-Smith et al. 2004). The former two are not recognized by most authors. *Phreatamoeba balamuthi*, the type and sole species of *Phreatamoeba*, was transferred to *Mastigamoeba* by Simpson et al. (1997). *Mastigella*, the type and sole genus of Mastigellidae, is usually considered to belong to the Mastigamoebidae (e.g. Cavalier-Smith et al. 2004; Chatton 1925; Frolov 2011). The family Endolimacidae was established by removing *Endolimax* from Entamoebidae to make each family monophyletic (Cavalier-Smith et al. 2004). Stensvold et al. (2012) recently showed that the genus *Iodamoeba* is closely related to *Endolimax*. The family-level classification of *Endolimax* within Endolimacidae and *Iodamoeba* within Entamoebidae disrupts the monophyly of the family Mastigamoebidae and we treat *Endolimax* and *Iodamoeba* as members of Mastigamoebidae. They form an internal branch of *Mastigamoeba*, making the genus *Mastigamoeba* paraphyletic. The full scope and character of *Mastigamoeba* is uncertain, as our analyses do not include most of the previously described taxa in *Mastigamoeba*, we lack electron-microscopical data for many taxa, and the phylogenetic position of *M. aspera*, the type species, is unknown. Until studies are conducted on the type species, it would be premature to make the genus *Mastigamoeba* monophyletic either by transferring species of *Endolimax* and *Iodamoeba* to it or breaking it up. We describe four new species of *Mastigamoeba*, *M. abducta*, *M. errans*, *M guttula*, and *M. lenta* spp. nov.

The genus *Rhizomastix* is transferred into archamoebae and for reasons given above we establish a new family to accommodate it. The name Rhizomastigidae Calkins, 1901 is a nomen nudum that was not based on and did not include *Rhizomastix* (Loeblich and Tappan 1961), and so is unavailable. To avoid homonymy, we follow Recommendation 29A of the International Code of Zoological Nomenclature and name the new family Rhizomastixidae fam. nov. We transfer *Pararhizomastix hominis* described by Yakimoff and Kolpakoff (1921) to *Rhizomastix* as we see no reason to place it in a separate genus. We add a new species of *Rhizomastix*, *R. libera* sp. nov.

Taxonomic Summary

The taxonomic scheme below summarizes only the taxa mentioned in this paper, and is not intended to be a taxonomic summary of all pelobiont or archamoebal taxa. Previously-used and current names of all pelobionts sensu stricto are given in Supplementary Table 1.

Archamoebae: Anaerobic/microaerophilic Amoebozoa with reduced mitochondria. May exist as amoebae or amoeboflagellates. Ancestrally with a single anterior flagellum, microtubular cone and flagellar root. Secondarily aflagellate or multiflagellate. Amoeboid movement with eruptive lobopodia. Free-living or endobiotic.

Remarks: To avoid confusion related to current usage (Adl et al. 2005, 2012; Walker et al. 2011) we use the term archamoebae to describe the group that contains mastigamoebids, pelomyxids, entamoebae, and *Rhizomastix*, and we use it informally.

Family Mastigamoebidae Chatton, 1925: Diagnosis: Archamoebae with trophozoites which are uninucleate to multinucleate, with single motile anterior flagellum associated with microtubular cone, or aflagellate. Amoebae flattened, amoeboid movement slow, typically with multiple pseudopodia. Free-living or endobiotic.

Type genus: *Mastigamoeba* Schulze, 1875.

Other genera: *Mastigella* Frenzel, 1897; *Mastigina* Frenzel, 1897; *Endolimax* Kuenen & Swellengrebel, 1917; *Iodamoeba* Dobell, 1919.

Remarks: Genus *Endolimax* Kuenen & Swellengrebel, 1917 is transferred here from Endolimacidae Cavalier-Smith, Chao & Oates, 2004. Genus *Iodamoeba* is transferred here from Entamoebidae Chatton, 1925. Members of these do not possess an external flagellar apparatus, so their generic assignment cannot be determined on the basis of morphological characters without further study; however, based on molecular phylogenetics they are assigned to Mastigamoebidae.

Genus *Mastigamoeba* Schulze, 1875: Diagnosis: Mastigamoebid with a uniflagellated trophic stage, in which the nucleus and flagellum are connected by a cone of microtubules that arises from the base and sides of the single (flagellated) basal body; a cylinder is present in the transition zone of the flagellum. A single ribbon of microtubules arises from the side of the basal body, and the ribbon has a bilaminar sheet on its anterior edge. Basal bodies usually have nine triplets of microtubules, but one taxon has nine doublets and this is regarded as derived. The flagellum has a conventional eukaryotic '9+2' arrangement of microtubules, but lacks a dynein arm on the outer side of each doublet. The flagellates may, at least in some species, transform to amoebae with one, few or many nuclei. Both forms may transform into cysts. Nuclei are usually single, but are paired in one species, and in another the nucleus contains a small extra-nucleolar "dot". The outside of the cell is usually naked but in some species there may be small bacteria-like bodies while other species have regular or irregular spines. Cells have been found in soils, and freshwater and marine habitats.

Type species: *Mastigamoeba aspera* Schulze, 1875

Remarks: This genus was the first created for mastigamoeboid flagellates to house species with a flagellum, an amoeboid body but with a hyaline cytoplasm dissimilar to that of other superficially similar taxa such as the cercomonads (Kent 1880; Klebs 1892; Schulze 1875; Stokes 1886, 1888, 1890). Frenzel (1897) created *Mastigella* as a vehicle for species with numerous similar characteristics (see Introduction) but Goldschmidt (1907b) widened the circumscription of *Mastigella* to simply having no (direct) connection between the nucleus and the flagellum. This had the effect of narrowing the circumscription of *Mastigamoeba* to include only mastigamoebids with a connection between the flagellum and the nucleus. Species were created on the basis of shape and size, pseudopodial form and contractile vacuole number and location. Many of the 53 nominal species and three further "taxa" which have not been given names cannot be unambiguously distinguished on the basis of their light-microscopical features. *Dinamoeba* Leidy, 1874 was introduced as a genus of amoeboid protists for species with many short, acute pseudopodia, some blunt pseudopodia, and a posterior uroid. The type of the genus, *Dinamoeba mirabilis*, has a mucous coat and small bodies adhering to the cell, and was distinguished from *Mastigamoeba aspera* by the absence of a flagellum (Schulze 1875). However, this argument is invalidated by observations by De Groot (1936) and Siemensma (1987) of flagellated forms of *Dinamoeba*. A recent paper (Chystyakova et al. 2012) describes the light-microscopic appearance and ultrastructure of *Mastigamoeba aspera* and concludes that it is the same as *Dinamoeba mirabilis*. The International Code of Zoological Nomenclature (International Commission for Zoological Nomenclature 1999) requires *D. mirabilis* Leidy, 1874 to take priority over *M. aspera* Schulze, 1875, making *Mastigamoeba* a junior synonym of *Dinamoeba*. As *M. aspera* is the type species of *Mastigamoeba*, priority would undermine the current and wide use of *Mastigamoeba* as well as the name of the family. About 21 publications have included studies of, or reference to *Dinamoeba* in the last 130 years, compared to 160 that have dealt with *Mastigamoeba* in the same period. In order to protect the current usage, we recommend the use of *Mastigamoeba* Schulze, 1875 over *Dinamoeba* Leidy, 1874, while a formal taxonomic case is being prepared for conservation of *Mastigamoeba*, in the context of a full taxonomic revision of archamoebae (Walker et al., unpublished).

***Mastigamoeba abducta* sp. nov. Diagnosis**: *Mastigamoeba*, trophozoite is predominantly uniflagellate with a single almost central nucleus. Flagellar insertion is not supported by a persistent neck. Living gliding cells elongate, 15.9 (5.7 – 26.0) µm long and 6.8 (3.7. – 15.8) µm wide. Anteriorly directed flagellum 30.7 (13.3 – 46.1) µm long. Protargol-stained cells 7.0 (3.4 – 12.7) µm long and 5.3 (3.0 – 11.4) µm wide with flagellum 28.9 (12.5 – 55.4) µm long.

Type locality: Bezručovo valley, Czech Republic, 50°29′N, 13°20′E.

Syntype slides: protargol preparations of the monoeukaryotic strain 3MLMA, deposited in the Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, catalogue numbers 6/29 – 6/31.

Habitat: free-living, isolated from fresh-water microoxic sediments.

Etymology: *abducta* [Latin] – detached, taken away, removed – refers to the difficulty of seeing a connection of the flagellar base and nucleus in living cells.

***Mastigamoeba errans* sp. nov. Diagnosis**: *Mastigamoeba*, trophozoites are predominantly amoeboid. Flagellates rare, uniflagellate, elongate, fast, short-lived, and with apical nucleus. Living amoebae 14.6 (9.2 – 23.2) µm long and 9.9 (7.0 – 18.7) µm wide. Protargol-stained amoebae 7.0 (4.2 – 10.7) µm long and 5.2 (3.0 – 9.7) µm wide.

Type locality: Hradiště peak, Czech Republic, 50°27′N, 13°20′E.

Syntype slides: protargol preparations of the monoeukaryotic strain HRAANM, deposited in the Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, catalogue numbers 8/14 – 8/16.

Habitat: free-living, isolated from fresh-water microoxic sediments.

Etymology: *errans* [Latin] – straying, wandering. Most cells of the strain VIT1AN were amoeboid and move slowly and seemingly randomly.

***Mastigamoeba guttula* sp. nov. Diagnosis**: *Mastigamoeba*, trophozoite predominantly uniflagellate with a single subcentral nucleus. Cells often produce radiating thin pseudopodia. Living gliding cells rounded to slightly elongate, 8.6 (5.6 – 13.4) µm long and 6.3 (3.8. – 8.6) µm wide. Anteriorly directed flagellum 27.9 (19.4 – 53.8) µm long. Protargol-stained cells 6.8 (4.0 – 11.5) µm long and 5.2 (3.2 – 9.5) µm wide with flagellum 27.9 (9.3 – 45.8) µm long.

Type locality: Hradiště peak, Czech Republic, 50°27′N, 13°20′E.

Syntype: protargol preparations of monoeukaryotic strain HRADANAN, deposited in the Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, catalogue numbers 7/6 and 7/7.

Habitat: free-living, isolated from fresh-water microoxic sediments.

Etymology: *guttula* [Latin] – droplet. Named after the teardrop-shaped nucleus.

***Mastigamoeba lenta* sp. nov. Diagnosis**: *Mastigamoeba*, trophozoite predominantly amoeboid. Flagellates uncommon, uniflagellate, elongate, slow, and with subapical nucleus. Living amoebae 14.6 (10.8 – 18.4) µm long and 9.7 (6.8 – 13.7) µm wide. Protargol-stained amoebae 7.9 (4.9 – 12.9) µm long and 5.4 (3.7 – 8.1) µm wide.

Type locality: Kamenice, Czech Republic, 49°54′N, 14°35′E.

Syntype slides: protargol preparations of the strain VIT1AN with *M. lenta* sp. nov. and *Trimymea* sp., deposited in the Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, catalogue numbers 8/1 – 8/3.

Habitat: free-living, isolated from fresh-water microoxic sediments.

Etymology: *lenta* [Latin] – slow, sluggish. Most cells of the strain VIT1AN were amoeboid and move very slowly.

Family Endolimacidae Cavalier-Smith, Chao & Oates, 2004 Remark: Endolimacidae was established by Cavalier-Smith et al. (2004) for *Endolimax*. According to its phylogenetic position, *Endolimax* Kuenen & Swellengrebel, 1917 is here transferred to Mastigamoebidae Chatton, 1925.

Family Entamoebidae Chatton, 1925 Diagnosis: Aflagellate archamoebae. Flagellar apparatus completely reduced. Amoeboid movement typically monopodial and relatively fast.

Type genus: *Entamoeba* Casagrandi & Barbagallo, 1897.

Other genus: *Endamoeba* Leidy, 1879.

Remark: *Iodamoeba* Dobell, 1919 is transferred to Mastigamoebidae Chatton, 1925.

Family Rhizomastixidae fam. nov. *Non* Rhizomastigina Buetschli, 1884 = Rhizomastigidae Calkins, 1901 (emendation) *et auct.* = *nomina nuda* (not based on and not including *Rhizomastix*).

Diagnosis: Amoeboflagellate archamoebae. Trophozoites with single anterior flagellum. Microtubular cone modified into the “rhizostyle”. Amoeboid movement slow.

Type genus: *Rhizomastix* Alexeieff, 1911.

Etymology: Rhizomastix- (considered as the stem of the name) + -idae.

Genus *Rhizomastix* Alexeieff, 1911 Diagnosis: As for family Rhizomastixidae.

Type species: *R. gracilis* Alexeieff, 1911.

Other species: *R. hominis* (Yakimoff & Kolpakoff, 1921) comb. nov.; *R. periplanetae* Bhaskar Rao, 1963; *R. ranae* Krishnamurthy, 1969; *R. gryllotalpae* Bhaskar Rao, 1970; *R. dastagiri* Sultana, 1976; *R. biflagellata* Cepicka, 2011; *R. libera* sp. nov.

***Rhizomastix libera* sp. nov. Diagnosis**: *Rhizomastix* whose trophozoite is predominantly uniflagellate with a single central nucleus. Movement rapid and jerky. Living cells rounded to elongate, 10.1 (5.4 – 14.1) µm long and 4.2 (2.8. – 6.7) µm wide. Anteriorly directed flagellum 18.9 (10.9 – 25.0) µm long. Protargol-stained cells 3.9 (2.6 – 5.0) µm long and 3.3 (2.3 – 5.9) µm wide with flagellum 12.1 (7.5 – 21.3) µm long.

Type locality: Bhangarh, India. 27°05′N, 76°17′E.

Syntype slides: protargol preparations of the monoeukaryotic strain IND8MA, deposited in the Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic, catalogue numbers 6/24 – 6/26.

Habitat: free-living, isolated from fresh-water microoxic sediments.

Etymology: *libera* [Latin] – free, unrestricted. The newly described species is the first known free-living member of the genus *Rhizomastix*.

***Rhizomastix hominis* (Yakimoff & Kolpakoff, 1921) comb. nov.** *Pararhizomastix hominis* Yakimoff & Kolpakoff, 1921.

Family Pelomyxidae Schulze, 1877 Diagnosis: Amoeboflagellate archamoebae. Trophozoites uninucleate to multinucleate, with single or numerous nonmotile flagella. Each flagellum associated with microtubular cone. Cells cylindrical. Amoeboid movement typically monopodial and relatively fast.

Type genus: *Pelomyxa* Greeff, 1874.

Remark: No taxonomic changes are made in this family.

Methods

Organisms: All strains were isolated from fresh-water anoxic/microoxic sediments of lakes, pools and rivers. Approximately 2 ml of the samples was inoculated into Sonneborn's *Paramecium* medium (ATCC medium 802). The strains except for that of *Pelomyxa* spp. and mastigamoebae obtained from cells of *Pelomyxa* were cultivated at room temperature with transfers occurring once per week. The strain IND8MA of *Rhizomastix libera* sp. nov. was cultivated in 3% LB medium under the same conditions after approximately 50 initial passages in the ATCC 802 medium. The strain JAVA1 of *Mastigamoeba punctachora* was derived from the same isolate as the trichomonad JAVA1 in Cepicka et al. (2010). All strains were grown in polyxenic cultures with unidentified bacteria. For isolation of *Pelomyxa* spp. silt samples from lake, pond or backwater sediments were collected in 500 ml plastic containers. Then the samples were poured into Petri dishes and examined with stereo microscope MBS-1 (LOMO). *Pelomyxa* cells were picked with micropipette and gathered in 5 ml vials containing the water filtered from original samples. Strains 3MLMA, CH2, CHOM1, HRAANM, HRADANAN, HRN11, IND8MA, LUH2NS4, SEB4, TEXEL, VIT1AN, and VITSEDAN are deposited in the culture collection of the Department of Parasitology of Charles University in Prague, Czech Republic.

Light microscopy: Living and protargol-stained cells were examined under a microscope BX51 (Olympus) or Leica DM 2500, using DIC optics for living cells. Protargol-stained preparations were prepared as follows: moist films spread on cover slips were prepared from pelleted cultures obtained by centrifugation at 500 *g* for 8 minutes. The films were fixed in Bouin-Hollande's fluid for 10 hours, washed with 70% ethanol, and stained with 1% protargol (Bayer, I. G. Farbenindustrie) following Nie's (1950) protocol.

Transmission electron microscopy: Cells of well-grown culture of the stain IND8MA were pelleted by centrifugation, were resuspended in a solution containing 2.5% glutaraldehyde (Polysciences) and 5 mM $CaCl_2$ in 0.1 M cacodylate buffer (pH 7.2), and fixed at room temperature for 4 hours. After washing in 0.1 M cacodylate buffer (three times per 15 minutes), the cells were postfixed with 2% OsO_4 in 0.1 M cacodylate buffer for 3 hours. After washing with an excess volume of 0.1 M cacodylate buffer (three times per 15 minutes) the fixed cells were dehydrated in acetone and embedded in Epon resin (Poly/Bed 812, Polysciences). The ultrathin sections were stained with lead citrate and uranyl acetate (2 – 3%) and examined using a TEM JEOL 1011 transmission electron microscope. For TEM of *Pelomyxa* spp. 10 cells of each species were fixed with a cocktail of 5% glutaraldehyde and 0.5% OsO_4 in 0.1 M cacodylate buffer. Fixation was performed on melting ice in the dark for 4 hours, with the complete replacement of the fixator 15 min after the beginning of the fixation. Then the amoebae were washed for 15 min in 0.1 M cacodylate buffer and postfixed with 2% OsO4 in 0.1 M cacodylate buffer in the dark on melting ice (1 h). After a transition through a graded ascending alcohol series the material was embedded in Epon-Araldite mixture. In order to facilitate the preparation of ultrathin sections the objects embedded in the resin were treated with 10% solution of hydrofluoric acid. Ultrathin sections were cut with a Reichert ultratome (Reichert Microscope Services) and viewed in the Tesla BS_300 electron microscope (Tesla).

DNA extraction, amplification, cloning and sequencing: The genomic DNA of most isolates was isolated from the cultures using the DNeasy Blood and Tissue Kit (Qiagen) and the ZR Genomic DNA II Kit™ (Zymo Research). For DNA isolation of *Pelomyxa* spp. 50 – 70 cells of each species (washed individually three times in distilled water prior to the DNA isolation) were collected in tubes with 0.5 ml solution containing 1% SDS and 50 mM EDTA. Then the samples were subjected to salt extraction according to the protocol of Aljanabi and Martinez (1997). Universal eukaryotic primers MedlinA (CGTGTTGATCCTGCCAG) and MedlinB (TGATCCTTCTGCAGGTTCACCTAC) (Medlin et al. 1988) were used to amplify almost-complete SSU rRNA gene of strains 3MLMA, CH2, CHOM1, GRUBER, HRAANM, HRADANAN, HRN11, IND8MA, JAVA1, LUH2NS4, SEB4, and VIT1AN. Pelobiont-specific primers PeloSSU59F (GTGTTAAAGATTAAGCCATGCATG) and PeloSSU750R (GTATTTGTCGTCACTACCTCG) were used to amplify a shorter SSU rRNA gene fragment of strains IND5, OLB6, PSOVKA, TEXEL, VIT7, and VITSEDAN. PCR amplification of SSU rRNA gene of *Pelomyxa* spp. and their symbiotic mastigamoebae was performed as described by Milyutina et al. (2001). The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), Zymoclean™ GEL DNA Recovery Kit (Zymo Research), or GFX PCR DNA and Gel Band Purification kit (GE Healthcare). The purified PCR products were either directly sequenced or cloned into the pGEM®-T EASY vector using the pGEM®-T EASY VECTOR SYSTEM I (Promega), or into the pTZ57R/T vector using the InsTAclone™ PCR Cloning Kit (Fermentas). Both PCR products and clones were bidirectionally sequenced by primer walking. To confirm the origin of the obtained sequences, SSU rRNA gene of most strains was partially resequenced (directly from PCR products amplified with primers PeloSSU59F and PeloSSU750R) approximately a year after the original sequencing. Sequence data reported in this paper are available in GenBank under accession numbers JX157632-JX157666.

Phylogenetic analyses: Four data sets containing SSU rRNA gene sequences were created. The first data set consisted of 13 sequences of archamoebae retrieved from GenBank, 24 new sequences, 4 sequences of uncultured eukaryotes (GenBank accession numbers AM114799, AF421219, GU919401, and GU921440), and 50 SSU rRNA gene sequences representing the main non-archamoebal amoebozoan lineages used as the outgroup. The sequences were aligned using the MAFFT method (Katoh et al. 2002) with the help of the MAFFT 6 server http://align.bmr.kyushu-u.ac.jp/mafft/online/server/ with G-INS-i algorithm at default settings. The alignment was manually edited using BioEdit 7.0.9.0 (Hall 1999). The final data set of unambiguously aligned characters consisted of 1299 positions. The second data set was derived from the first data set by removing sequences of the genus *Pelomyxa*. The third data set was derived from the first data set by removing sequences of the genus *Entamoeba*. The fourth data set was derived from the first data set by removing sequence of *Rhizomastix libera*. Phylogenetic trees were constructed by maximum likelihood (ML) and Bayesian methods. ML analysis was performed in Phyml 3.0 (Guindon and Gascuel 2003) under the GTR+I+Γ model (four discrete categories) which was selected by Akaike criterion implemented in Modeltest 3.7 (Posada and Crandall 1998). Node support was assessed by ML analysis of 1000 bootstrap data sets. Bayesian analysis was performed using MrBayes 3.1.2. (Ronquist and Huelsenbeck 2003) using the GTR+I+Γ+covarion model with four discrete categories. Four MCMCs were run for 10 million generations (17 million generations for the fourth data set), with a sampling frequency of 100 generations (until average standard deviation of split frequencies was lower than 0.01). First 25% of trees were removed as burn-in.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.protis.2012.11.005.

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7. 2. Zadrobílková et al. 2015a

Zadrobílková E, Walker G, Čepička I (2015a) Morphological and molecular evidence support a close relationship between the free-living archamoebae *Mastigella* and *Pelomyxa*. Protist **166:** 14-41

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ORIGINAL PAPER

Morphological and Molecular Evidence Support a Close Relationship Between the Free-living Archamoebae *Mastigella* and *Pelomyxa*

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Members of the archamoebae comprise free-living and endobiotic amoeboid flagellates and amoebae that live in anoxic/microoxic habitats. Recently, the group has been divided into four separate families, Mastigamoebidae, Entamoebidae, Pelomyxidae, and Rhizomastixidae, whose interrelationships have not been completely resolved. There still are several key members of the archamoebae, notably the genus *Mastigella*, from which sequence data are missing. We established 12 strains of 5 species of *Mastigella* and *Pelomyxa* in culture, examined their morphology and determined their actin gene sequences. In addition, we examined the ultrastructure of three strains and determined and analyzed SSU rDNA sequences of two strains. Our data strongly suggest that *Mastigella* is specifically related to *Pelomyxa*, and it is transferred into the family Pelomyxidae. Surprisingly, *Mastigella* is likely paraphyletic with *Pelomyxa* forming its internal branch. The two genera share several morphological features that point to their common evolutionary history. Three new species of *Mastigella* are described: *M. erinacea* sp. nov., *M. rubiformis* sp. nov. and *M. ineffigiata* sp. nov.

Key words: Archamoebae; *Mastigella*; *Pelomyxa*; phylogeny.

Introduction

The Archamoebae is a phylogenetically-delineated group of free-living and endobiotic protists that display distinct flagellar ultrastructure, hyaline eruptive pseudopodia, reduced mitochondrial organelles, and endosymbiotic bacteria. They inhabit anoxic/microoxic freshwater and marine sediments or live as commensals or pathogens in the intestine of invertebrates and vertebrates, including humans. Members of Archamoebae lack normal mitochondria, Golgi stacks, peroxisomes, and plastids. This apparently simple ultrastructure originally led to their placement in the now-defunct Archezoa. More recently, it has been shown that they have mitochondrial remnants (reviewed in Barberà et al. 2007; Hampl and Simpson 2007).

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While many individual members of the group have been described in detail since the end of the 19th century, the systematics of the Archamoebae as a whole is currently in a state of flux. For example, it has been recently shown that the aflagellate, endobiotic members of the group, genera *Entamoeba, Endolimax*, and *Iodamoeba*, formerly classified together in the family Entamoebidae, do not form a clade (Constenla et al. 2013; Ptáčková et al. 2013; Stensvold et al. 2012). There still remain genera from which DNA sequences are undescribed, such as *Mastigina*, *Tricholimax*, and *Endamoeba*. In a recent phylogenetic study the Archamoebae splits into four lineages representing separate families: (1) Mastigamoebidae with genera *Mastigamoeba*, *Endolimax*, and *Iodamoeba*; (2) Entamoebidae comprising species of *Entamoeba*; (3) Pelomyxidae with the genus *Pelomyxa*; (4) Rhizomastixidae represented by several species of *Rhizomastix* (Ptáčková et al. 2013). However, the relative phylogenetic position of each of the four major lineages remains questionable, probably because of long branches formed by members of *Pelomyxa* and *Entamoeba* (Ptáčková et al. 2013), and it remains to be established precisely how the taxa defined by molecular phylogenetics correspond to the taxonomic concepts represented by the formal family names, particularly in the cases of Mastigamoebidae and Pelomyxidae.

Members of the group usually possess a single flagellum (*Mastigamoeba*, *Mastigella*, *Mastigina*, *Rhizomastix*, *Tricholimax*), or they are secondarily aflagellate (*Entamoeba*, *Endamoeba*, *Endolimax*, *Iodamoeba*), or they are multiflagellate (*Pelomyxa*). The canonical arrangement of the flagellar apparatus in Archamoebae, as inferred to be the ancestral state for the group, is composed of a single basal body giving rise distally to a flagellum, and proximally to a microtubular cone and lateral root (Simpson et al. 1997; Walker et al. 2001). The position of the cone in relation to the nucleus is important for the identification of particular genera: the cone extends from the basal body to the nuclear membrane in *Mastigamoeba* and *Tricholimax* (Brugerolle 1982, 1991, 1993; Chystyakova et al. 2012; Frenzel 1897; Simpson et al. 1997; Walker et al. 2001) while in *Mastigella*, as it has been defined since 1907, the cone extends into the cytoplasm but does not connect with the nucleus (Goldschmidt 1907; Walker et al. 2001). The ultrastructural account of *Mastigina hylae* (Brugerolle 1982) is of a species originally and currently placed in *Tricholimax*, so no data exists for the flagellar apparatus in *Mastigina.* The flagellar apparatus as seen in *Pelomyxa* is highly variable and can be divided into at least two main groups (Chystjakova et al. 2014). In *Rhizomastix* the microtubular cone has probably been modified to a rhizostyle, a flagellum-like microtubular bundle that extends to the posterior of the cell (Ptáčková et al. 2013).

Although the axonemes of *Mastigella, Rhizomastix* and *Mastigamoeba* have the typical eukaryotic (9 + 2) arrangement of doublets and central microtubules, the outer dynein arms between the microtubular doublets are missing (Ptáčková et al. 2013; Walker et al. 2001). The axonemes of *Pelomyxa* and *Tricholimax* lack dynein arms and have variable numbers and organization of central and peripheral microtubules (Brugerolle 1982; Chistyakova and Frolov 2011; Frolov et al. 2005, 2006, 2007, 2011; Griffin 1988). The flagella of *Tricholimax* and *Pelomyxa* do not contribute to cell movement (Brugerolle 1982; Chistyakova and Frolov 2011; Frolov et al. 2005, 2006, 2007, 2011; Griffin 1988).

On the basis of their similar morphology, the genera *Mastigamoeba* and *Mastigella* have traditionally been placed together in the family Mastigamoebidae (Adl et al. 2012; Cavalier-Smith et al. 2004; Chatton 1925; Goldschmidt 1907; Griffin 1988; Ptáčková et al. 2013). Although 158 nominal species of *Mastigella* were described between 1897 and 1979 (see Supplementary Material table S1 in Ptáčková et al. 2013), only a single DNA sequence had apparently been determined to date (Edgcomb et al. 2002). This SSU rDNA sequence has been presented in numerous published phylogenetic trees of Archamoebae, showing *Mastigella commutans* as a sister to *Mastigamoeba balamuthi* with relatively high support (Cavalier-Smith et al. 2004; Edgcomb et al. 2002; Lahr et al. 2011; Nikolaev et al. 2006; Stensvold et al. 2012). However, it was recently shown that the sequence is almost identical with the sequences of *Mastigamoeba punctachora*, and we assume that it is from a misidentified culture, as both organisms were held in culture by the same lab and sequenced at the same time (see Ptáčková et al. 2013). In view of the fact that there is no DNA sequence from *Mastigella* in the published literature, it is possible that the genus might represent a separate lineage within Archamoebae.

In order to determine the phylogenetic position of *Mastigella*, we isolated 12 strains of 5 species of *Mastigella* and *Pelomyxa*, examined their light-microscopic morphology, and determined their actin gene sequences and SSU rRNA gene sequences of two *Mastigella* strains. Our phylogenetic analyses suggest that *Mastigella* and

Pelomyxa are closely related, but *Mastigella* is possibly paraphyletic. We also describe three new species of *Mastigella* on the basis of light-microscopic morphology and/or ultrastructure.

Results

New Strains

Thirteen new strains obtained from micro-oxic sediments were established in culture (Table 1). The cells were always found at the bottom of the culture tubes, suggesting they were anaerobic/microaerophilic. Two strains, TRECIME and KBEL2C, were lost before their morphology could be examined in detail. However they exhibited typical morphology of *Mastigella*, and we were able to obtain their DNA before the loss of the cultures. Most cultures also contained other eukaryotes, which are very easily distinguished morphologically and phylogenetically from archamoebae, usually ciliates (*Metopus* spp.), diplomonads (*Trepomonas*, *Hexamita* spp.) or heteroloboseans (*Psalteriomonas lanterna*). Strain HRAAN of *Mastigella rubiformis* sp. nov. was obtained from the same sample as strain HRAANM of *Mastigamoeba errans* (see Ptáčková et al. 2013), and both species were present in the culture. Strain OLB6AN of *M. ineffigiata* sp. nov. was cultured with *Rhizomastix libera*. The dimensions of living cells of the strains of *Mastigella* and *Pelomyxa* are summarized in Table 2. All of our strains were characterized using light-microscopy. Due to low density of cultures and problems with specimen preparation, we have not presented here electron-microscopic pictures of *Mastigella eilhardi* and *Mastigella erinacea* sp. nov.

Morphology

Mastigella eilhardi Bürger, 1905

Gliding cells of the strains ATCC 50342 and GO7 were mostly elongated, averaging approximately 50 μm in length (Table 2); and possessed a single flagellum that was about 0.6 times the length of the cell. Its beating was faster than in other *Mastigella* species, but the movement of the cell was generally slow. Flagellar movement of crawling cells was very slow. The flagellar base was supported by an elongated, hyaline neck, which contained a thin cone not connecting the nucleus to the flagellum; this cone was sometimes visible in protargol-stained specimens and under the light microscope (Fig. 4D, M, N). Both strains produced a few lobate pseudopodia (Figs 1B, C, K, 2A – I) around the cell, with large pseudopodia sometimes being formed anteriorly (Fig. 2G – I). Posteriorly, finger-shaped pseudopodia (Fig. 2J) or a villous area (Fig. 1A, B, J, K) could form, or a feather-like mix of finger-shaped and villous pseudopodia (Fig. 1H); a lobate uroid was occasionally formed (Fig. 2B). The posterior villous area could extend to the anterior of the cell, leaving a thin hyaline neck (Fig. 1J). A single nucleus, containing a spherical nucleolus that appeared toroidal in some planes of section (Figs 1E – G, 2A – C) was situated in the central or posterior part of the cell, behind the anterior hyaloplasm. Although the cells usually possessed one nucleus, 2 – 20% of them were binucleate (Fig. 2J, M). The cytoplasm was hyaline in the anterior, but contained food vacuoles, contractile vacuoles (Fig. 2C, G – I), small refringent granules (Figs 1F, G, J, K, 2A), and endosymbiotic bacteria (Fig. 1H).

M. eilhardi Bürger, 1905 is distinguished by having conical cells with an extremely long, hyaline neck and a villous posterior end, as seen in Figure 1A, J, K, and in Plate VI, figs 1a – d, of Bürger (1905).

Mastigella erinacea sp. nov.

Cells of strains KORISSION, LARNAKA2N, and TOLEDO were elongated, rounded or irregularly shaped, averaging approximately 44 μm in length. Strains KORISSION and LARNAKA2N often lacked any conspicuous pseudopodia. When pseudopodia were present they could be rounded (Figs 3A, B, 4B), palmatipartite (Fig. 3B), very short villous (Figs 3B, C, 4B), thin and short (Figs 3F, 4C), or thin and long, finger-shaped (Figs 3G, H, 4C). Strain TOLEDO displayed extreme variation in pseudopodial morphology. Its pseudopodia included fine needle-like (Fig. 5D – F), villous (Fig. 5B, C), finger-shaped (Fig. 5A, B), irregular finger-shaped (Fig. 5H), palmatipartite (Fig. 5I), and round and eruptive (Fig. 5C) shapes. Amoeboid movement of the cells of *Mastigella erinacea* sp. nov. was almost nonexistent or extremely slow, with the locomotive form producing an anterior hyaline or non-hyaline lobopodium (Figs 3D, 5H). The cells of all strains were aflagellate, rarely flagellate, with the flagellum emerging straight from the cell (i.e. without a “neck”). When a cell of *M. erinacea* sp. nov. moved using the flagellum, it rotated along its anteroposterior axis but remained in approximately the same place: flagellar movement thus seemed ineffective. A villous area was sometimes

Table 1. List of the strains included in the study. n.a. – not available. [a]strain isolated by Cavalier-Smith in 1990 (unpublished) and deposited in the American Type Culture Collection under the name *Mastigella radicula* (Moroff) Goldschmidt; [b]strains isolated by Ptáčková et al. (2013).

Species	Strain	Locality	Habitat	Coordinates
Pelomyxa schiedti Schaeffer, 1918	KIEL3	Behrensdorf, Germany	fresh-water sediments	54°21′N 10°36′E
	SKADARSKE	Skadar, lake Skadar, Albania	fresh-water sediments	42°3′N 19°29′E
	TIWI	Tiwi valley, Oman	fresh-water sediments	22°47′N 59°13′E
	WACT07	Cusco region, Peru	fresh-water sediments	n.a.
Mastigella erinacea sp. nov.	KORISSION	Lake Korission, Corfu, Greece	brackish sediments	39°27′N 19°52′E
	LAR2N	Larnaka, Cyprus	brackish sediment	34°51′N 33°37′E
	TOLEDO	Toledo, Castilia - La Mancha, Spain	salt marsh	39°58′N 3°39′W
Mastigella eilhardi Bürger, 1905	ATCC 50342[a]	Yorkshire, Stairfoot Quarry, United Kingdom	fresh-water	n.a.
	GO7	Monis Toplous - Vai, Crete, Greece	fresh-water sediments	35°14′N 26°14′E
Mastigella rubiformis sp. nov.	HRAAN	Hradiště peak, Czech Republic	fresh-water sediments	50°27′N 13°20′E
Mastigella ineffigiata sp. nov.	OLB6AN	Olbasee lake, Germany	fresh-water sediments	51°16′N 14°35′E
Mastigella sp.	KBEL2C	Kbely, Prague, Czech Republic	sewage disposal plant	50°07′N 14°33′E
	TRECIME	Tre Cime, Italy	fresh-water sediment	46°35′N 12°15′E
Mastigamoeba abducta Ptáčková et al., 2013	3ML[b]	Bezručovo valley, Czech Republic	fresh-water sediment	50°29′N 13°20′E
	CHOM1[b]	Chomutov, Czech Republic	fresh-water sediment	50°27′N 13°21′E
Rhizomastix libera Ptáčková et al., 2013	IND8[b]	Bhangarh, India	fresh-water sediment	27°05′N 76°17′E

observed, occasionally with the flagellum emerging from it (Figs 3C, 5I). Cells were mainly binucleate (Figs 3B – D, F, 4E, F), sometimes uninucleate (Figs 3A, 5B), and occasionally tetranucleate (Fig. 3E). The nucleolus was central and rounded, and contained a central granular ball of chromatin (Fig. 3A, D). The cytoplasm of *M. erinacea* sp. nov. was markedly non-hyaline and filled with refringent granules. Elongated endosymbiotic bacteria were also observed (Fig. 4A).

M. erinacea sp. nov. can be distinguished by its origin from saline sediments. It is binucleate for almost all of its life cycle. Sometimes it has extremely variable pseudopodial shape, and the flagellum may emerge from an anterior villous area. The canonical appearance of *M. erinacea* sp. nov. is shown in Figures 3C, E, 5B – E, G.

Mastigella rubiformis sp. nov.

Cells of strain HRAAN were elongate or rounded, averaging ca. 30 µm in length; with a single flagellum of at least body length, with a slow beat, emerging either straight from the cell, or from an unpronounced triangular cytoplasmic protrusion, *i.e.* lacking a “neck” at the base of the flagellum. Aflagellate cells were occasionally observed (not shown). Cells moved very slowly. The anterior of the cell was hyaline during the movement with the flagellum extended, with lateral, finger-shaped pseudopodia (Fig. 6F). During amoeboid movement, there was a distinct hyaline layer around the cell (Fig. 6A – E), with an anterior leading pseudopodium (Fig. 6E), eruptive pseudopodia being formed anteriorly (Fig. 6B), lobate pseudopodia laterally (Fig. 6D), and tuft-like fine pseudopodia formed posteriorly (Fig. 6D, E). Lobate, eruptive pseudopodia were very pronounced in swimming forms (Fig. 6G – J), and this is canonical for this species. The cells contained one or rarely two nuclei, with a small nucleolus, and characteristic ultrastructure of peripheral chromatin clumps, visible both by light-microscopy (Fig. 6A) and electron-microscopy (Fig. 7I), but not after

Table 2. Dimensions (in μm) of living specimens of *Mastigella* and *Pelomyxa* strains. Average of 30 specimens ± standard deviation (smallest – largest value). CL – cell length; CW – cell width; CL/CW – cell length/cell width ratio; FL – length of flagellum; FL/CL – length of flagellum/cell length ratio; n. a. – not available.

Species	Strain	CL	CW	CL/CW	FL	FL/CL
Pelomyxa schiedti Schaeffer, 1918	KIEL3	n.a.	n.a.	n.a.	n.a.	n.a.
	SKADARSKE	51.7 ± 12.9 (35.2 – 92.4)	28.4 ± 4.6 (20.0 – 38.0)	1.8 ± 0.3 (1.3 – 2.5)	n.a.	n.a.
	TIWI	n.a.	n.a.	n.a.	n.a.	n.a.
	WACT07	95.5 ± 27.2 (52.1 – 155.8)	49.5 ± 14.3 (27.5 – 74.1)	2.1 ± 0.7 (1.2 – 4.0)	n.a.	n.a.
Mastigella erinacea sp. nov.	KORISSION	49.1 ± 10.2 (26.5 – 64.3)	26.4 ± 6.3 (15.6 – 41.2)	1.9 ± 0.5 (1.1 – 3.5)	58.8 ± 18.2 (25.9 – 88.9)	1.2 ± 0.4 (0.5 – 2.0)
	LAR2N	35.9 ± 5.2 (25.7 – 45.5)	21.2 ± 3.4 (16.4 – 28.5)	1.7 ± 0.3 (1.3 – 2.5)	n.a.	n.a.
	TOLEDO	45,9 ± 10.7 (23.1 – 75.5)	25.8 ± 5.2 (15.9 – 35.5)	1.8 ± 0.5 (1.3 – 3.5)	78.8 ± 22.4 (36.1 – 115.3)	1.8 ± 0.5 (0.8 – 3.3)
Mastigella eilhardi Bürger, 1905	ATCC50342	49.5 ± 13.4 (30.8 – 72.2)	13.6 ± 4.4 (8.3 – 29.9)	4.0 ± 1.8 (1.7 – 7.6)	21.3 ± 5.6 (8.8 – 30.5)	0.5 ± 0.2 (0.2 – 0.8)
	GO7	56.5 ± 18.4 (10.3 – 97.1)	10.9 ± 3.6 (6.4 – 24.8)	5.6 ± 2.0 (0.4 – 10.7)	20.2 ± 9.0 (10.7 – 61.8)	0.5 ± 1.0 (0.2 – 6.0)
Mastigella rubiformis sp. nov.	HRAAN	30.8 ± 6.5 (21.0 – 45.0)	24.1 ± 5.2 (17.5 – 40.0)	1.3 ± 0.4 (0.7 – 2.3)	n.a.	n.a.
Mastigella ineffigiata sp. nov.	OLB6AN	68.9 ± 15.0 (40.6 – 122.2)	38.4 ± 10.3 (20.9 – 63.1)	1.9 ± 0.5 (1.0 – 3.2)	n.a.	n.a.

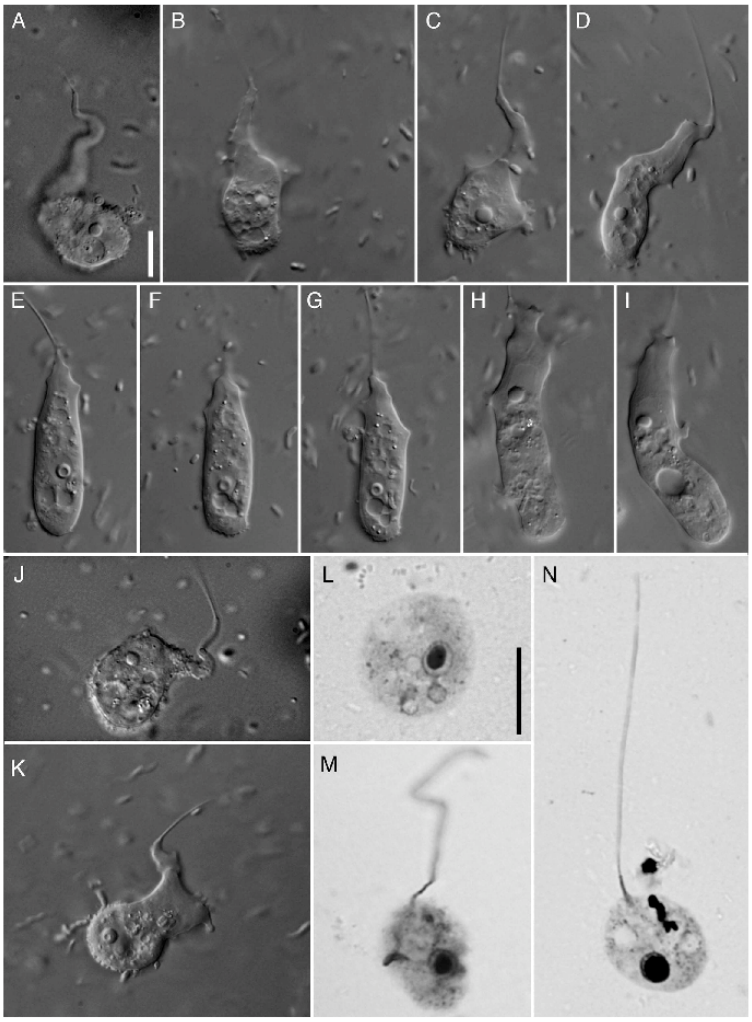

Figure 1. Light-microscopical morphology of *Mastigella eilhardi* strain GO7, showing the characteristic swan-like long "neck" and posterior villous pseudopodia; with a hyaline anterior, nucleus containing a "hollow" or "donut-shaped" nucleolus, and endosymbiotic prokaryotes. **A – K** Gliding cells. **L – N** Protargol-stained cells. Scale bar in A, L = 10 μm. DIC (A – K) or bright field (L – N). Arrows show a microtubular cone in D and endosymbiotic bacteria in H.

protargol staining (Fig. 6K, L). The non-hyaline areas of the cytoplasm of the cell were filled with very prominent oval-shaped endosymbiotic bacteria (Fig. 6A – C), as well as refringent granules, vacuoles and contractile vacuoles.

Transmission electron microscopy confirmed numerous endosymbiotic bacteria (Fig. 7F) and the lack of a microtubular connection between the flagellar base and nucleus (Fig. 7F, H, J). Mitochondrion-related, acristate, double-membrane-bound organelles, 400 – 600 nm in diameter, were sometimes observed (Fig. 7G). The single flagellum showed standard eukaryotic 9+2 arrangement of doublets, and no outer dynein arms were visible (Fig. 7A). A cone of microtubules arose from the base of the flagellum, and a microtubular root extended laterally from the basal body, below a fibrillar root sheet (Fig. 7H, J).

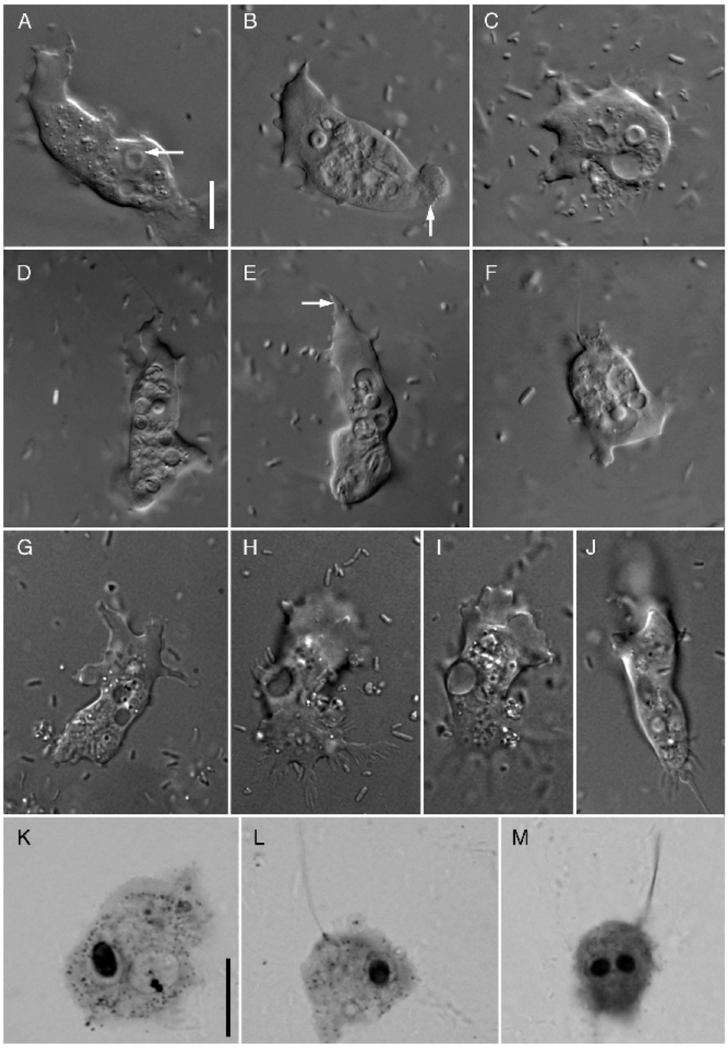

Figure 2. Light-microscopical morphology of *Mastigella eilhardi* strain ATCC 50342, showing pseudopodial variation, nucleus with "hollow", "donut-shaped" nucleolus, and endosymbiotic bacteria. **A – F** Gliding cells. **G – J** Crawling cells. **K – M** Protargol-stained cells. Scale bar in A, K = 10 µm. DIC (A – J) or bright field (K – M). Arrows show donut-shaped nucleolus in A; lobate uroid in B; flagellar neck in E.

Mastigella rubiformis sp. nov. is described here as a new species, distinguished by being uninucleate, with a nucleus with chromatin in clumps around its periphery, resembling the nuclei of some *Pelomyxa* species (Fig. 6A); and by its swimming form with numerous small lobed pseudopodia giving it a mulberry-like appearance (Fig. 6I).

Mastigella ineffigiata sp. nov.

The cells of the strain OLB6AN were approximately 70 µm long and did not hold a specific, fixed shape, varying between rounded and rectangular. The single flagellum showed a slow beat and did not appear to contribute to cell movement; it arose from a non-pronounced triangular protrusion, i.e. lacking

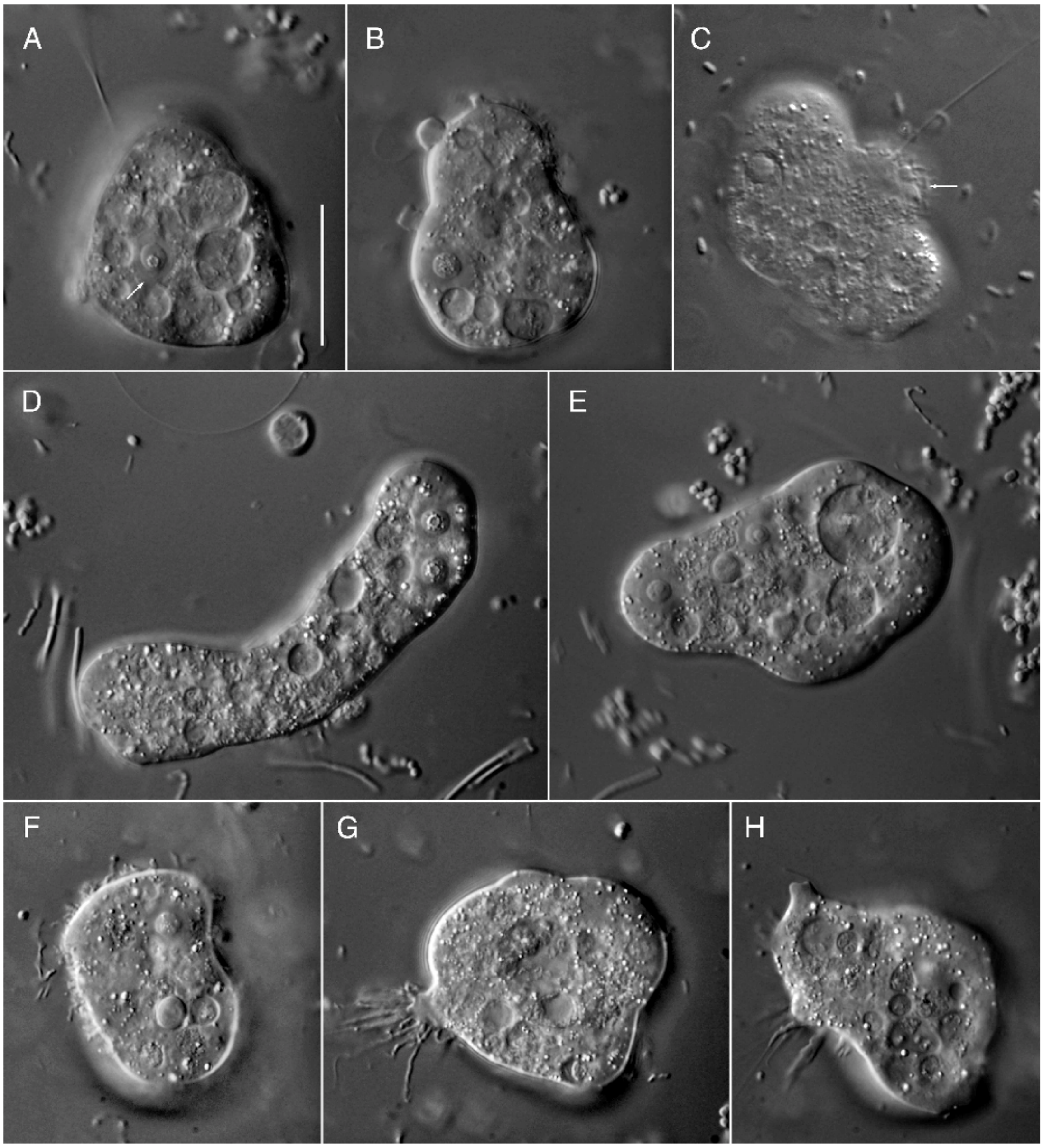

Figure 3. Light-microscopical morphology of *Mastigella erinacea* sp. nov. strain KORISSION, showing binucleate or quadrinucleate cells with distinctive "fried-egg" nucleus with a granular nucleolus, and villous or finger-shaped pseudopodia. **A – C** Gliding cells. **D – H** Aflagellate crawling cells. Scale bar in A = 20 µm. DIC (A – H). Arrow in A shows nucleus; in C it shows anterior villous area from which the flagellum may originate in some cells.

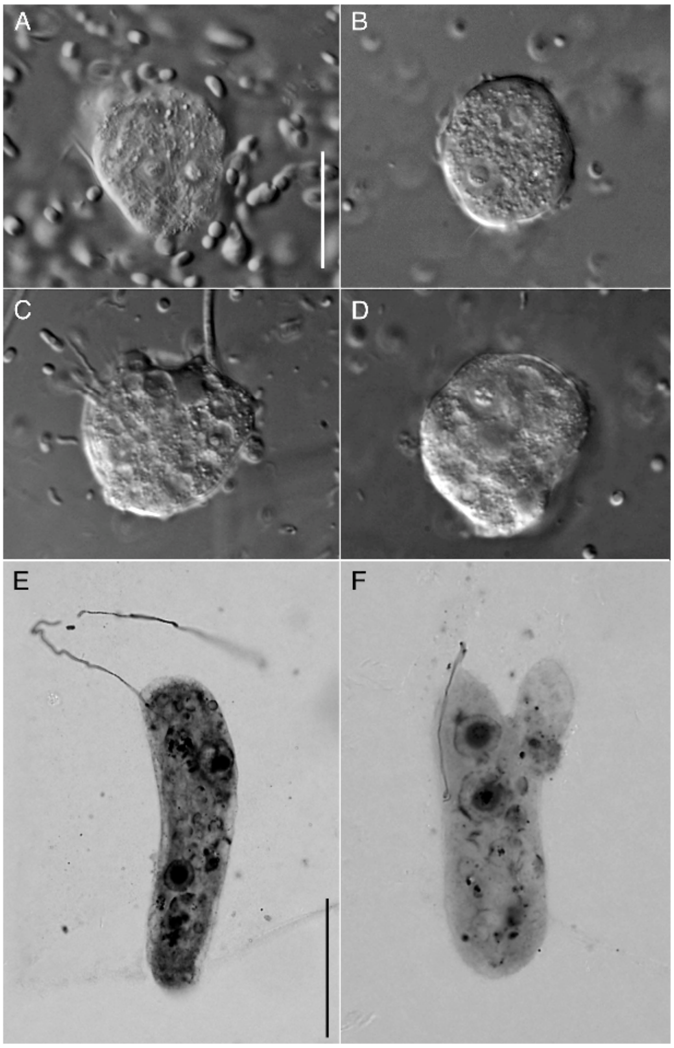

Figure 4. Light-microscopical morphology of *Mastigella erinacea* sp. nov. strain LARNAKA2N, showing binucleate cells with distinctive "fried-egg" nucleus and granular nucleolus, endosymbiotic bacteria, and villous or finger-shaped pseudopodia. **A – D** Gliding cells. **E, F** Protargol-stained cells. Scale bar in A, E = 20 μm,. DIC (A – D) or bright field (E, F).

a cytoplasmic "neck". Pseudopodia were hyaline and were formed by eruption at the anterior end during amoeboid movement (Fig. 8A, B, D – F), and could include very short villous pseudopodia laterally or posteriorly (Fig. 8G). An inconspicuous uroid was rarely seen (Fig. 8E). The single nucleus was central, with a central, smoothly rounded nucleolus where a small "dent" was sometimes visible (Fig. 8F). Two nuclei were observed only in a single cell (Fig. 8H). The cytoplasm was filled with conspicuous endosymbionts as well as food and contractile vacuoles, and there was a hyaline area around the edge of the cell.

Transmission electron microscopy revealed oval-shaped cells with numerous invaginations of the cell membrane (Figs 8B, 9G). There was a central nucleus with an electron-dense nucleolus (Fig. 9G, J), surrounded by vacuoles containing oval-shaped endosymbiotic prokaryotes (Fig. 9G, H). Mitochondrion-related acristate organelles,

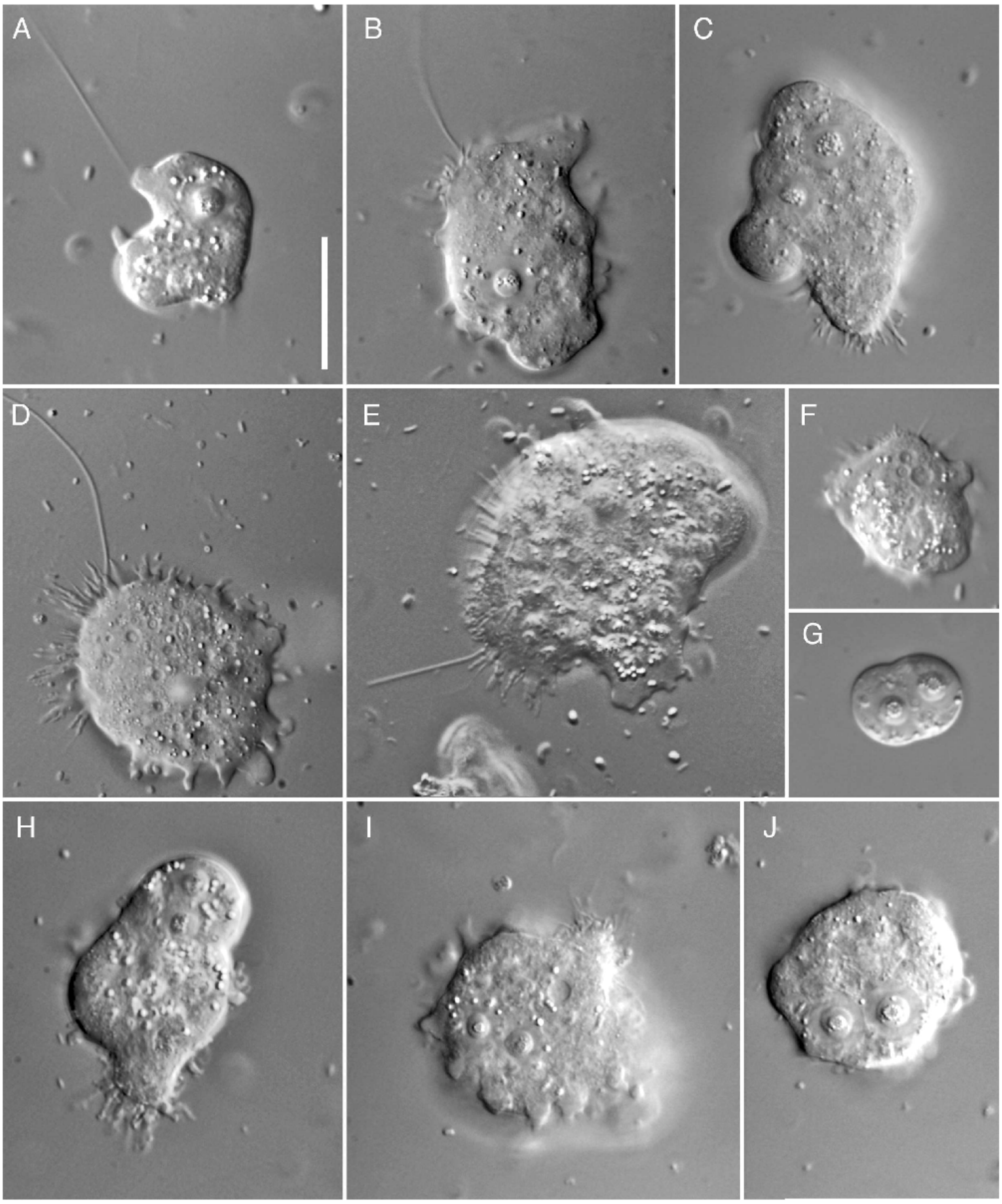

Figure 5. Light-microscopical morphology of *Mastigella erinacea* sp. nov. strain TOLEDO, showing binucleate cells with distinctive "fried-egg" nucleus and granular nucleolus, endosymbiotic bacteria, and highly variable villous, lobate or finger-shaped pseudopodia. **A, B, I, J** Gliding cells. **C – H** Crawling cells. Scale bar in A = 20 µm. DIC (A – J).

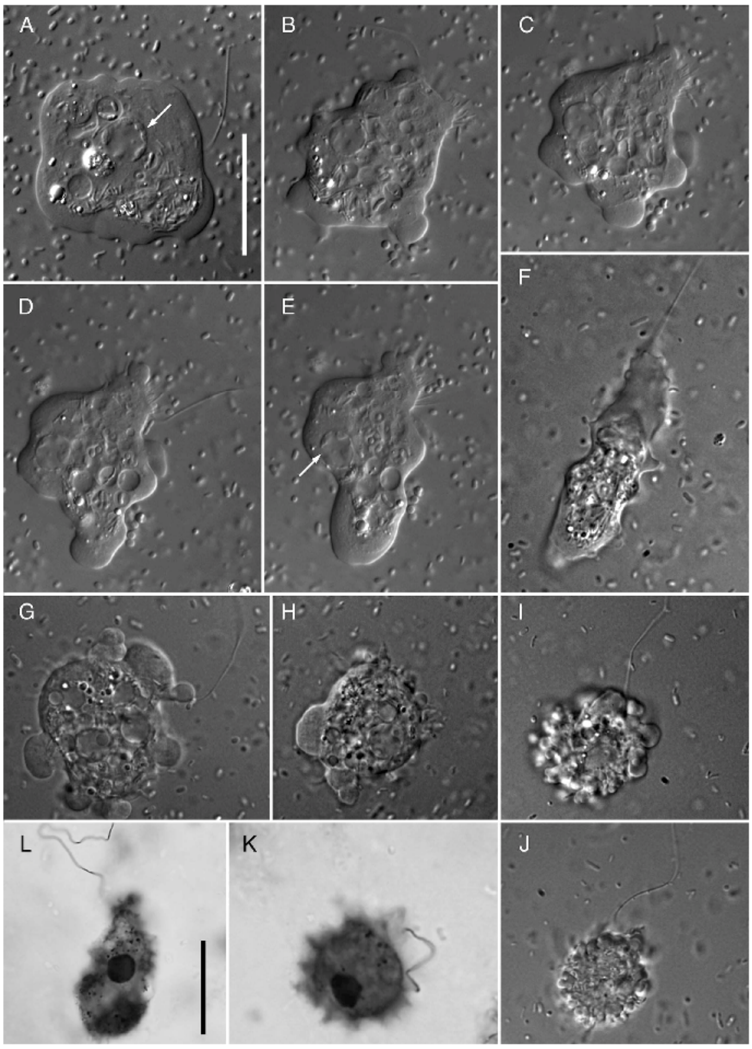

Figure 6. Light-microscopical morphology of *Mastigella rubiformis* sp. nov. strain HRAAN, showing cells with hyaline area, distinctive "*Pelomyxa*-like" nucleus, and prominent endosymbiotic bacteria. **F – J** Gliding cells. **A – E** Crawling cells. **K, L** Protargol-stained cells. Scale bar in A = 20 µm, in L = 10 µm. DIC (A – J) or bright field (K, L). Arrows show the nucleus with peripheral chromatin clumps in A and E.

enclosed by a double membrane, were approximately 300 nm in diameter and were positioned close to the endosymbionts (Fig. 9H, I).

Mastigella ineffigiata sp. nov. is described here as a new species, distinguished on the basis of its size, its formless appearance, the lack of a flagellar neck and with a small "dent" in the nucleolus. Its typical appearance is shown in (Fig. 8A, D, F, G).

Pelomyxa schiedti Schaeffer, 1918

Cells of strains SKADARSKE and WACT07 averaged 74 µm but ranged from 35 µm to 156 µm long. Strains KIEL3 and TIWI were lost before measurement of living and protargol-stained cells was carried out, however TIWI appeared to possess the smallest cells. Locomotive amoebae were oval-shaped. They moved very quickly, with

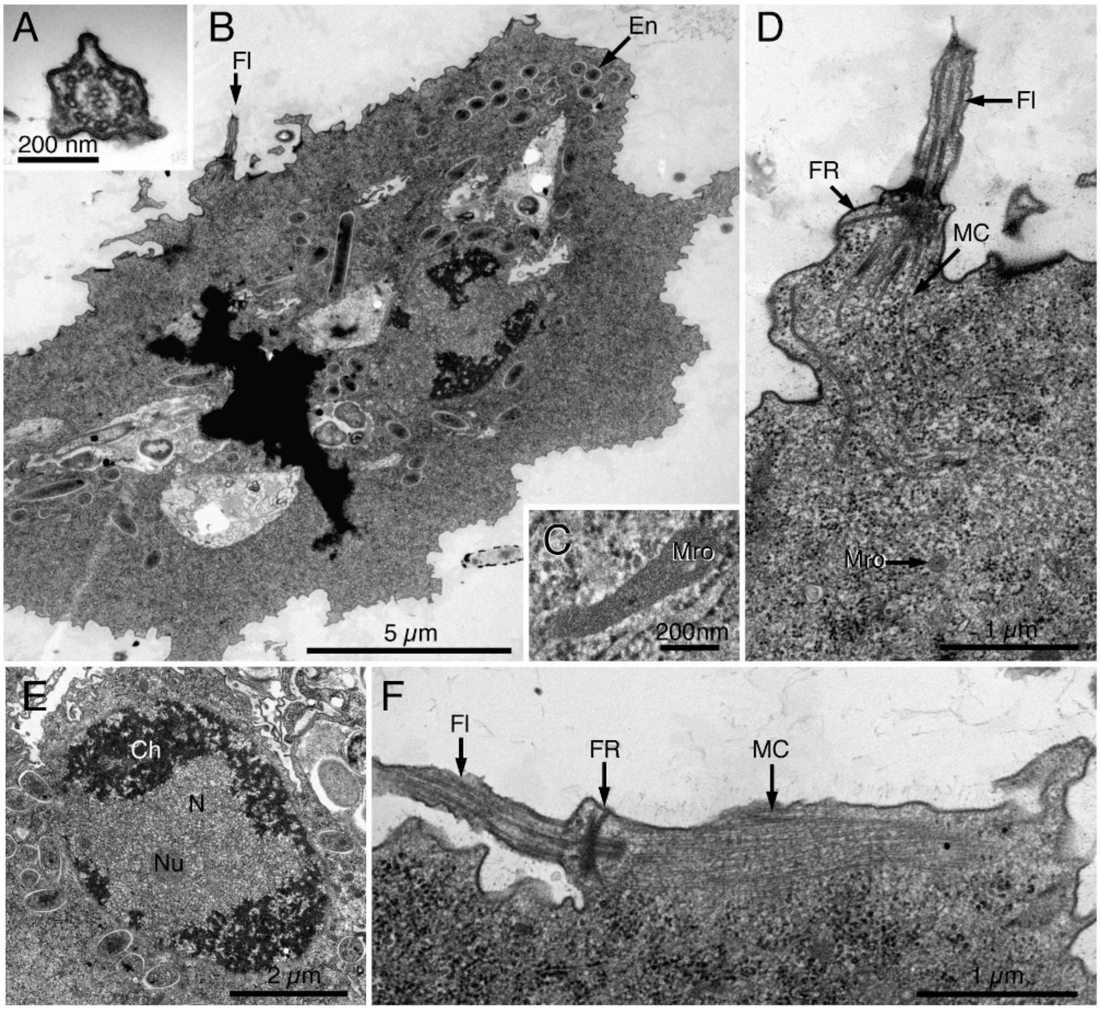

Figure 7. Ultrastructure of *Mastigella rubiformis* sp. nov. strain HRAAN. **A.** Transverse section of the flagellum, showing standard 9+2 structure of microtubules and absence of outer dynein arms. **B** Longitudinal section of the cell, showing the amoeboid body, single nucleus, endosymbionts, and flagellar apparatus. **C.** Mitochondrion-related organelle. **D, F** Flagellar apparatus in longitudinal section, showing the lateral microtubular root emerging laterally from the basal body, immediately posterior to the root sheet visible just to the left in F. **E** Section through the nucleus showing small nucleolus and peripheral clumps of chromatin. Ch – chromatin; En – endosymbionts; Fl – flagellum; FR – flagellar root; MC – microtubular cone; Mro – mitochondrion-related organelle; N – nucleus; Nu – nucleolus.

hyaline, eruptive anterior lobopodia (Fig. 10A). Lateral irregular finger-shaped pseudopodia occurred (Fig. 11B, C), and a lateral villous area could also be present (Fig. 12A, B). A spineolate or villous-bulbous uroid (see Smirnov and Brown 2004) was often present in locomotive cells (Figs 10A, 11B, C, 13A, B). Multiple immobile flagella were present, but poorly visible (Figs 10E, 11E); they emerged directly from the cell without a cytoplasmic "neck". Cells usually contained two nuclei, though some were uninucleate (e.g. Fig. 10D), and more rarely some cells were quadrinucleate (Fig. 13F). A characteristic peripheral ring of chromatin granules was visible under the light

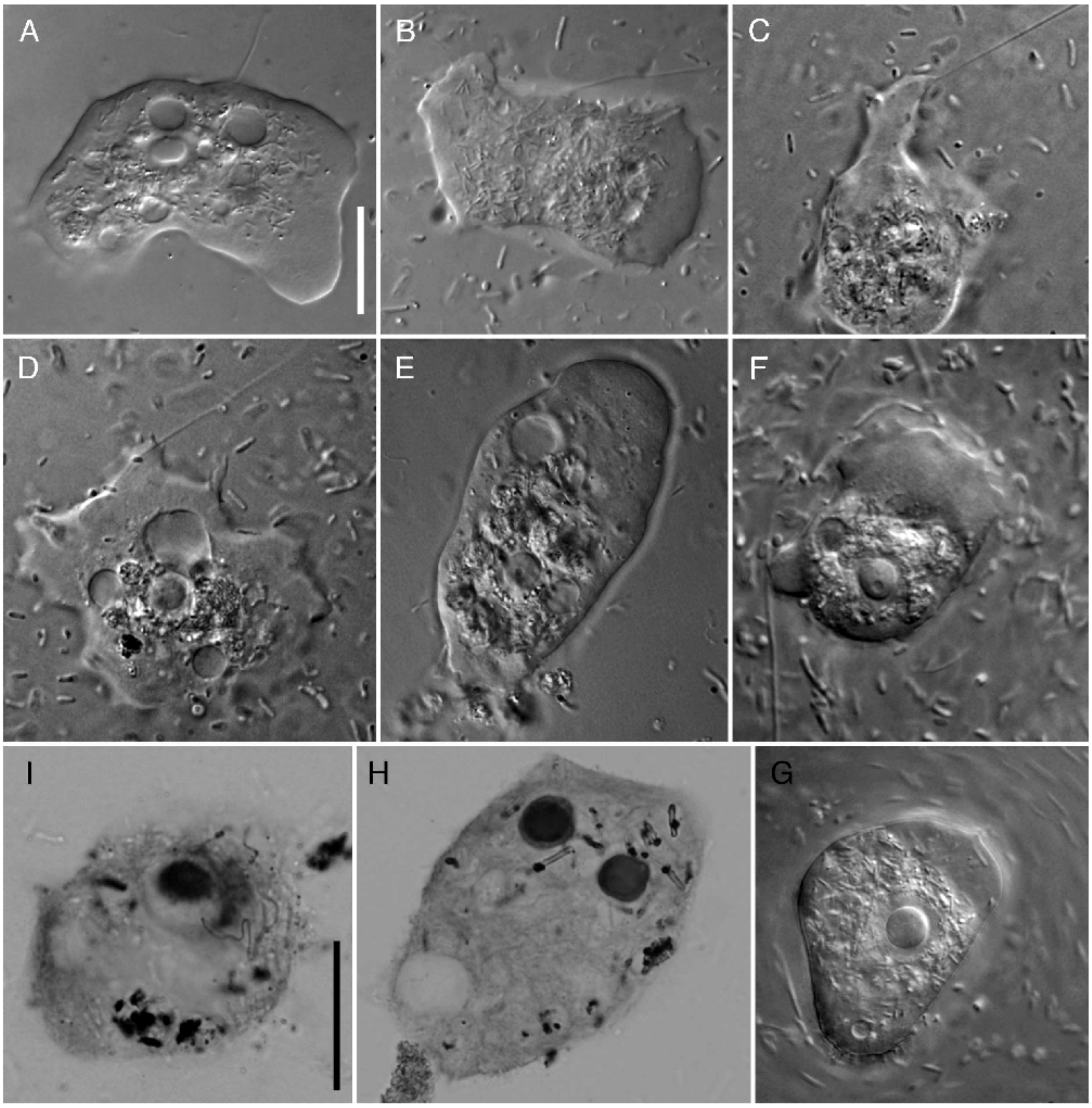

Figure 8. Light-microscopical morphology of *Mastigella ineffigiata* sp. nov. strain OLB6AN, showing “shapeless” morphology and prominent endosymbionts. **C** Gliding cell. **A, B, D – G** Crawling cells. **H, I** Protargol-stained cells. Scale bar in A, I = 20 μm. DIC (A – G) or bright field (H, I).

microscope. In protargol-stained specimens, the nuclei stained heavily and their internal structure could not be discerned. Endosymbiotic prokaryotes were present and could be conspicuous in some optical planes (Figs 10F, 12A, 13A, C); the cytoplasm was filled with endosymbionts, refringent granules, and some vacuoles (though it was not as vacuolated as *Pelomyxa palustris* or *P. belevskii*), and was markedly non-hyaline except for leading eruptive pseudopodia.

The strain WACT07 was examined by transmission electron-microscopy. In transverse section, the cell body was rounded, with no conspicuous invaginations of the cell membrane. The nucleus contained electron-dense peripheral chromatin and a small nucleolus (Fig. 14J), and

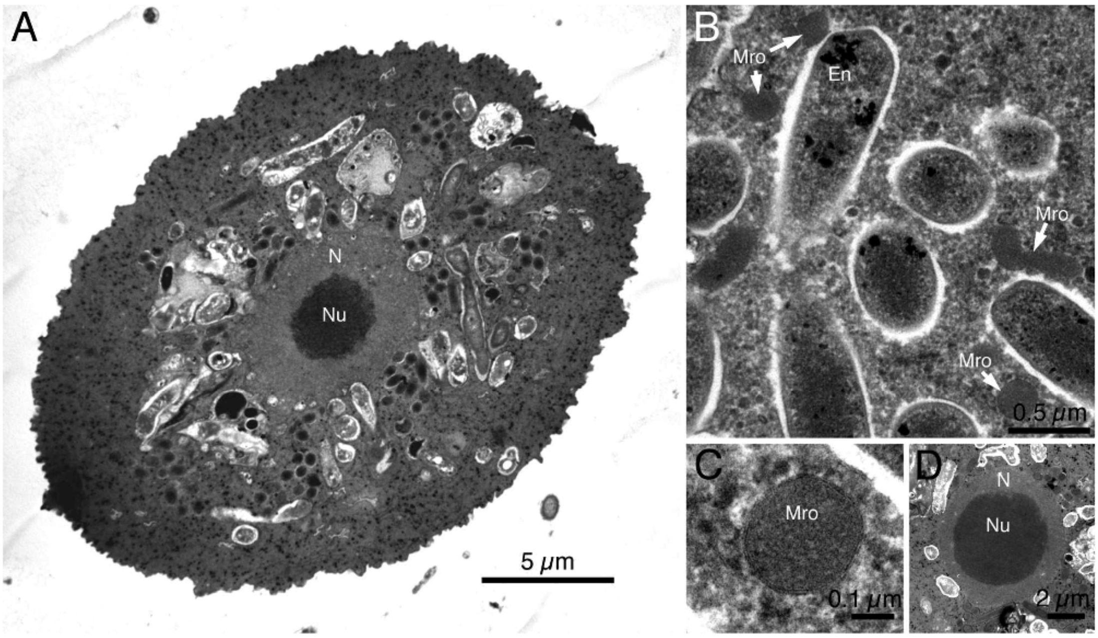

Figure 9. Ultrastructure of *Mastigella ineffigiata* sp. nov. strain OLB6AN. **A.** Longitudinal section of the cell, showing central nucleus with solid central nucleolus. **B** Section through the endosymbiotic prokaryotes and mitochondrion-related organelles. **C** Mitochondrion-related organelle. **D** Nucleus with large central nucleolus. En – endosymbionts; Mro – mitochondrion-related organelle; N – nucleus; Nu – nucleolus.

was surrounded by numerous prokaryotic elongated and oval-shaped endosymbionts and food vacuoles (Fig. 14G). In longitudinal sections, the cell was oval-shaped with two nuclei containing peripheral chromatin granules (Fig. 14I). The flagellum had a 9+n structure of microtubules and appeared to entirely lack dynein arms (Fig. 14H). The arrangement of the flagellar apparatus was not determined, but at least a broad, laterally-running flagellar root was present (Fig. 14K).

Pelomyxa schiedti Schaeffer, 1918 is distinguished by its small size (relative to many other *Pelomyxa* species), fast movement, and its highly characteristic nuclear structure with a concentric ring of chromatin around the periphery of the nucleus. Its canonical appearance is shown in Figures 10B, 11B, 12A, 13A.

Phylogenetic Analyses

The phylogenetic tree of Amoebozoa as inferred from actin gene sequences is shown in Figure 15. The relationships between most taxa were unresolved. Archamoebae appeared monophyletic, but the monophyly was not statistically supported. The internal topology of the Archamoebae was largely unresolved, and monophyletic *Mastigamoeba* was not recovered. The genus *Entamoeba* appeared robustly monophyletic. Importantly, a clade consisting of genera *Mastigella* and *Pelomyxa*, the Pelomyxidae (see below), was recovered with relatively strong support. The relationships within this clade remained unresolved, but monophyletic *Pelomyxa* was recovered and supported. The strain KIEL3 formed a sister branch of the sequence AAQ55803 deposited in GenBank under the name *Pelomyxa palustris*, but the relationship was not supported. Strains of *Mastigella* formed a paraphyletic grade at the base of *Pelomyxa* with *M. erinacea* sp. nov. being its closest relative, but this relationship was also not strongly supported.

In order to test the possible paraphyly of *Mastigella*, we determined SSU rDNA sequences from two potentially unrelated species (*M. eilhardi* and *M. erinacea* sp. nov.). Unfortunately, we were not able to determine SSU rDNA sequences from the other strains because they did not amplify at all. The results of phylogenetic analyses (Fig. 16) were consistent with previous studies (e.g. Fiore-Donno et al. 2010; Lahr et al. 2011; Ptáčková

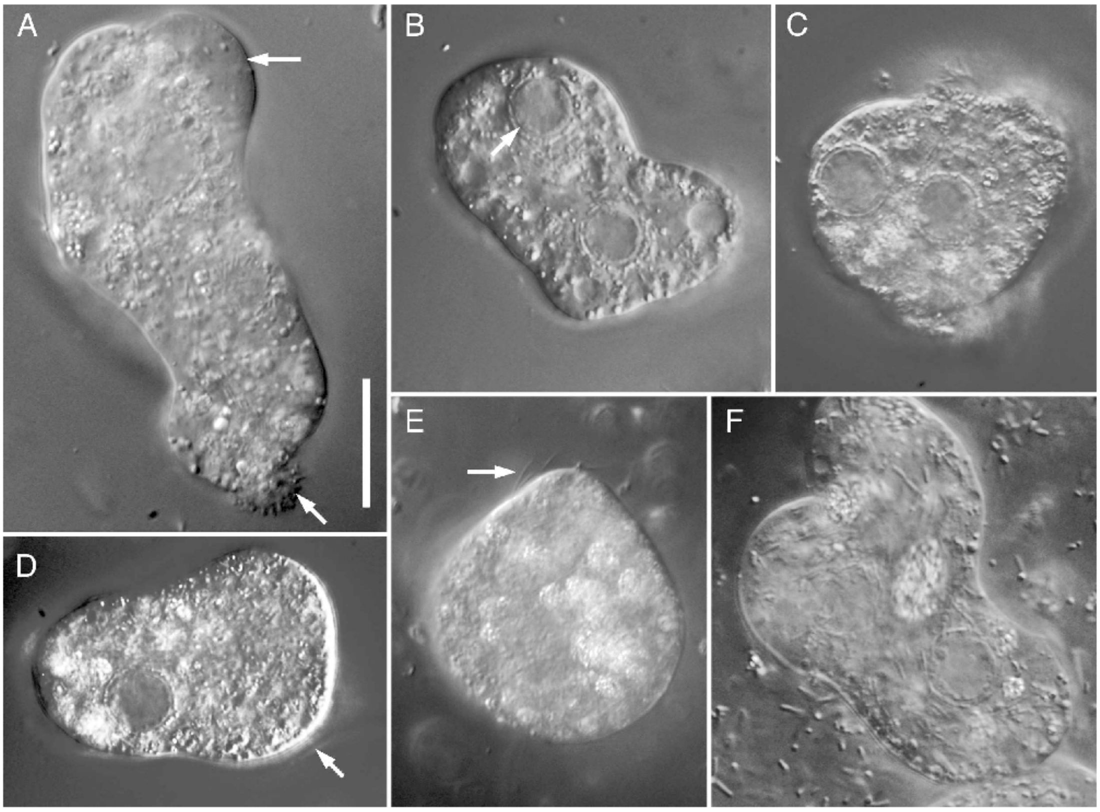

Figure 10. Light-microscopical morphology of *Pelomyxa schiedti* strain SKADARSKE, showing distinctive nuclear structure, posterior uroid-like area, and cells filled with granules and endosymbionts. **A – F** Locomotive amoebae. Scale bar in A = 20 μm. DIC (A – F). Arrows show eruptive anterior lobopodia and bulbous, villous uroid-like area in A; peripheral ring of chromatin granules in the nucleus in B; immotile, poorly visible flagella in D and E.

et al. 2013; Shadwick et al. 2009). The relatively well-supported clade of Archamoebae split into four lineages representing individual families: (1) Pelomyxidae comprising genera *Mastigella* and *Pelomyxa*, (2) Rhizomastixidae, represented by a single species *Rhizomastix libera*, (3) Entamoebidae comprising species of *Entamoeba*, (4) Mastigamoebidae including genera *Mastigamoeba*, *Endolimax*, and *Iodamoeba*. The clade of Pelomyxidae was relatively well supported. The genus *Mastigella* appeared paraphyletic with a good support, *M. erinacea* sp nov. being closely related to the robustly monophyletic genus *Pelomyxa*. *M. eilhardi* was sister to the clade of *Pelomyxa* + *M. erinacea* sp. nov.

To examine the strength of our taxonomic hypotheses, we used the likelihood-based AU test (Shimodaira 2002). Five alternative hypotheses were assessed for the actin gene dataset: (1) *Mastigella* is monophyletic. (2) *Mastigella* and *Entamoeba* form a clade. (3) *Mastigella* and *Rhizomastix* form a clade. (4) One or both *Mastigella* strains are sister to the rest of Archamoebae. (5) *Mastigella* and *Mastigamoeba* form a clade. All hypotheses but the first one (*Mastigella* is monophyletic) were rejected on the 5% significance level (p = 0.023, 0.032, 0.002, and 0.004, respectively). *Mastigella* was the sister taxon to *Pelomyxa* in (1).

The AU test was also performed on the SSU rRNA gene dataset. Six alternative hypotheses were evaluated: (1) – (5) as above; and (6) *Mastigella*, *Mastigamoeba*, *Endolimax*, and *Iodamoeba* form a clade (i.e. Mastigamoebidae sensu Ptáčková et al. 2013 is monophyletic). In

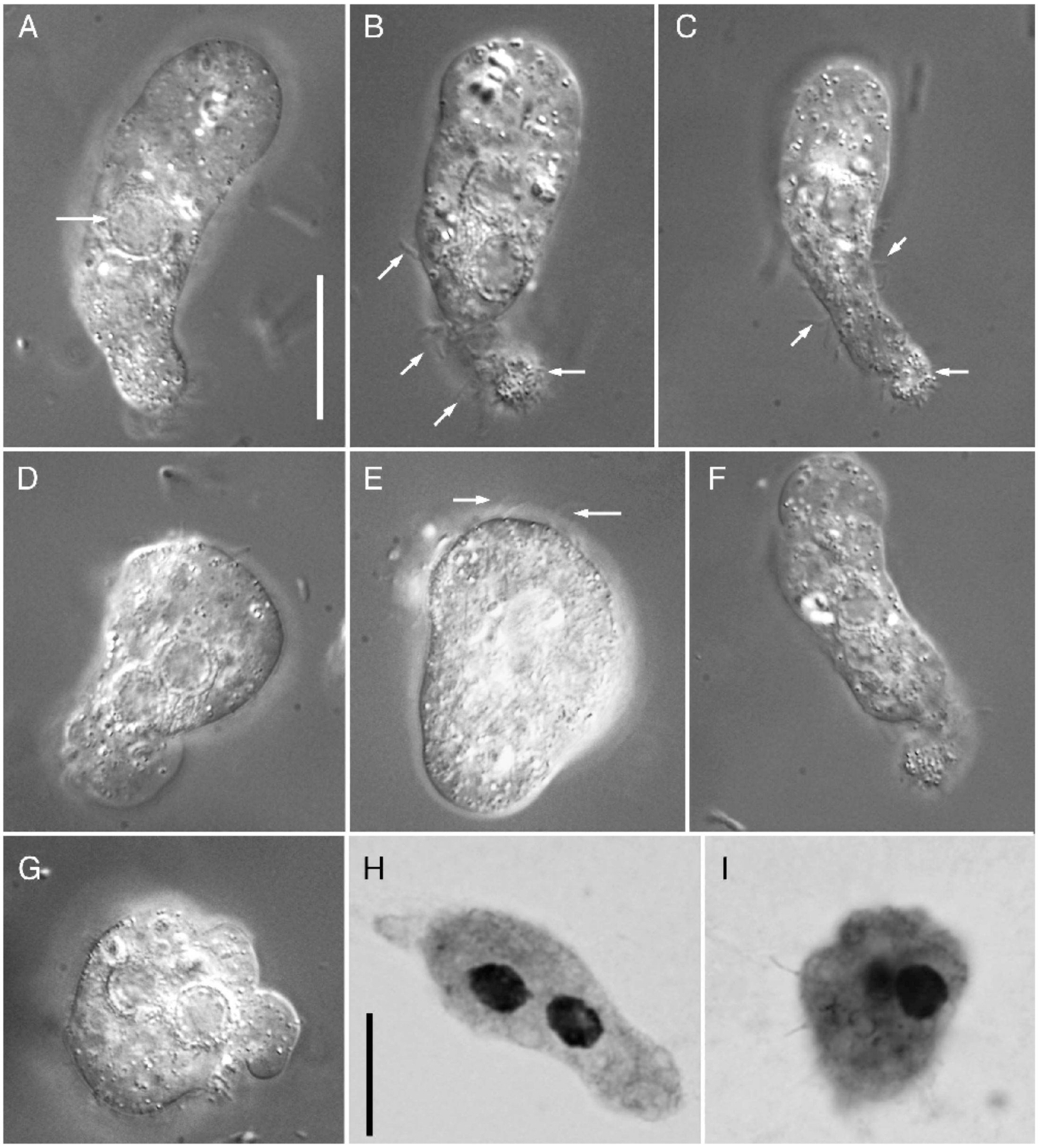

Figure 11. Light-microscopical morphology of *Pelomyxa schiedti* strain TIWI, showing distinctive nuclear structure, posterior uroid, and finger-shaped pseudopodia. **A – G** Locomotive amoebae. **H, I** Protargol-stained cells. Scale bar in A = 20 μm, in H = 10 μm. DIC (A – G) or bright field (H, I). Arrows show peripheral ring of chromatin granules in A; lateral irregular finger-shaped pseudopodia and uroid-like area in B and C; multiple immobile poorly visible flagella in E.

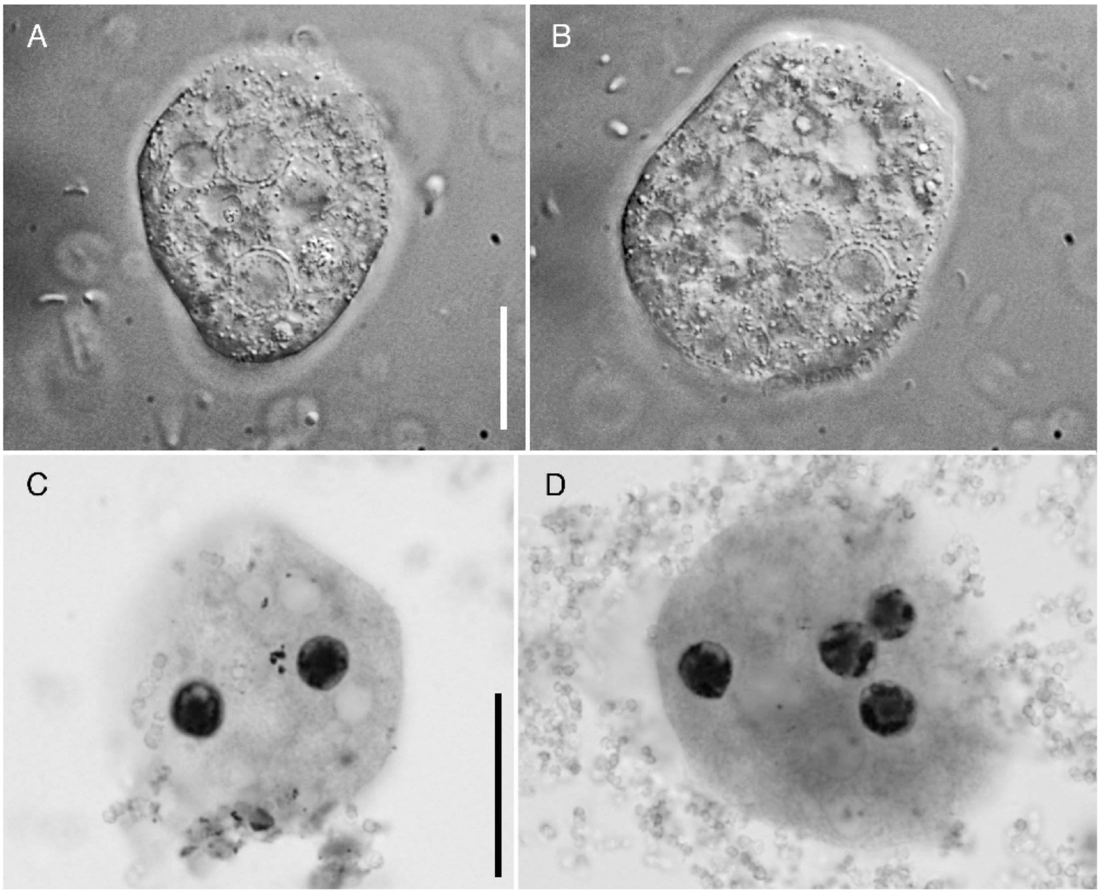

Figure 12. Light-microscopical morphology of *Pelomyxa schiedti* strain KIEL3 showing distinctive nuclear structure, and villous pseudopodia. **A, B** Locomotive amoebae. **C, D** Protargol-stained cells. Scale bar in A, C = 20 μm. DIC (A, B) or bright field (C, D). Arrow shows villous patch of pseudopodia.

this case, only topologies (5) and (6) were rejected (p = 0.002 and 0.047, respectively); the others could not be rejected.

Discussion

Species Identities of Strains

Following the accepted usage of *Mastigella* (Goldschmidt 1907 inter alia), strains with a single flagellum and the absence of a connection between the nucleus and flagellar base were assigned to this genus. The typical axonemal organization for the Archamoebae (9+2 microtubules with no outer dynein arms) was observed in *Mastigella rubiformis* sp. nov. The organisms described here fall into 4 species, 3 of which are new, on the basis of their morphology.

Mastigella erinacea sp. nov. is described as a new species here because it is typically binucleate, and it was isolated from brackish/saline sediments, which is not common in Archamoebae. To the best of our knowledge, the other archamoeba that has been found in brackish sediments, *Mastigamoeba simplex* (e.g. Bernard et al. 2000), is morphologically different from *Mastigella erinacea* sp. nov., and also from the truly marine archamoebae *Mastigamoeba schizophrenia* and *Pelomyxa marina* (Delphy 1938; Simpson et al. 1997). The presence of a villous area in *M. erinacea* sp. nov., from which the mostly immotile flagellum sometimes emerges

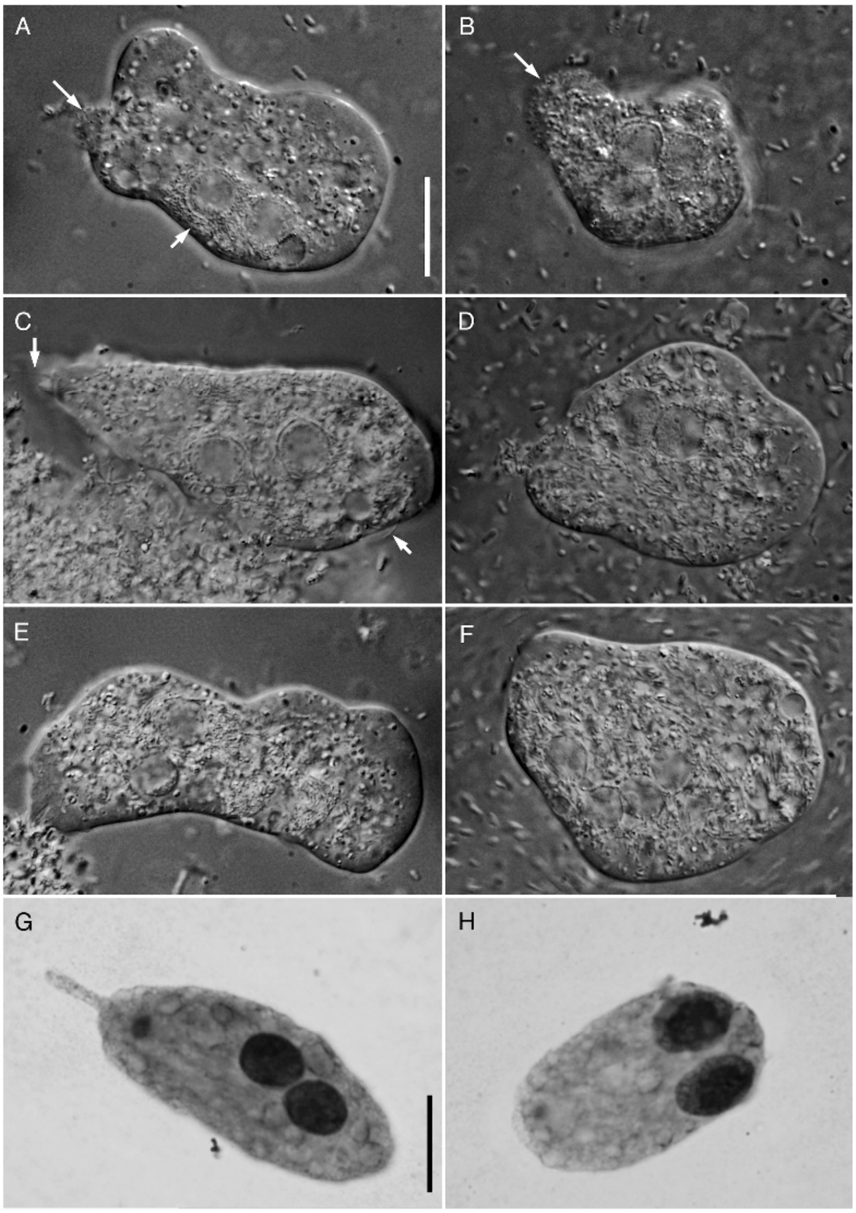

Figure 13. Light-microscopical morphology of *Pelomyxa schiedti* strain WACT07 showing distinctive nuclear structure, and villous pseudopodia and posterior uroid-like area. **A – F** Locomotive amoebae. **G, H** Protargol-stained cells. Scale bar in A = 20 μm, in G = 10 μm. DIC (A – F) or bright field (G, H). Arrow show uroid-like area in A, B and C; conspicuous endosymbionts in A.

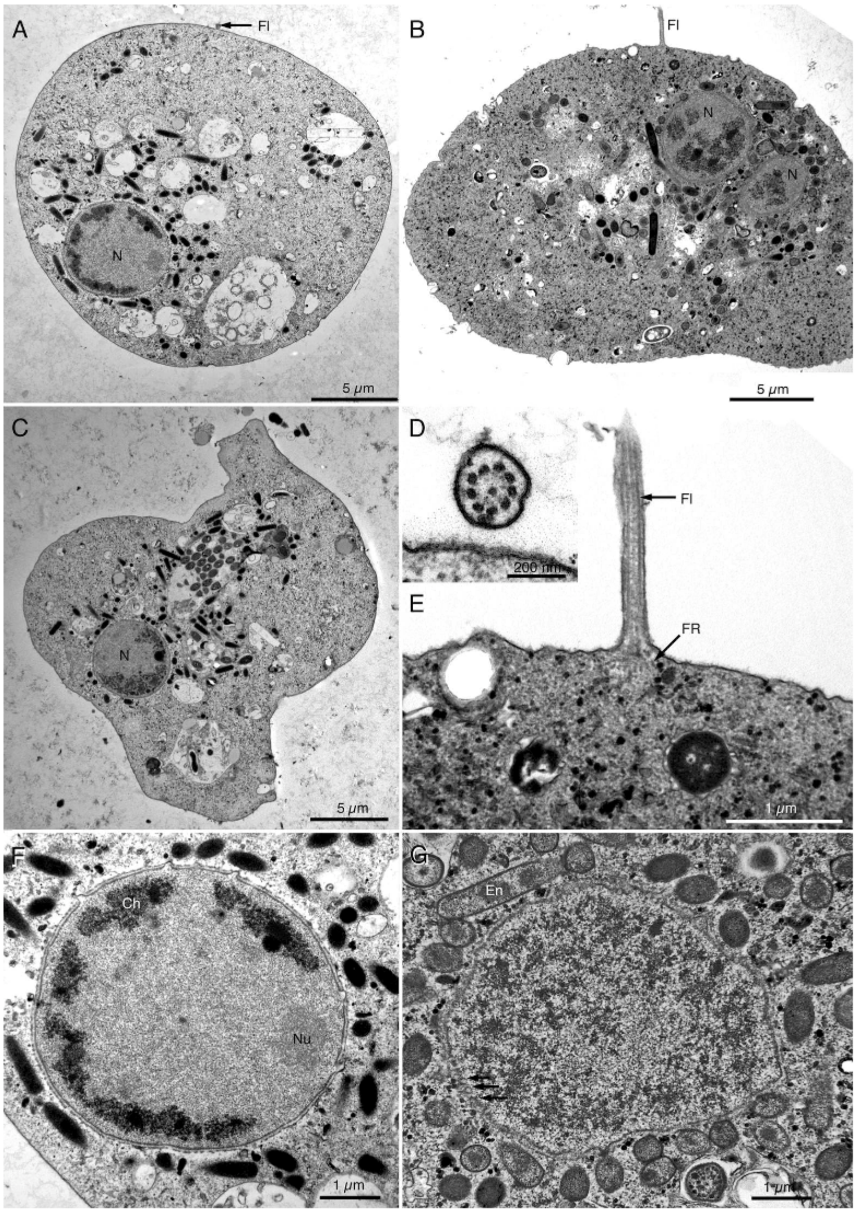

Figure 14. Ultrastructure of *Pelomyxa schiedti* strain WACT07. **A** Transverse section of the cell showing single nucleus, endosymbiotic prokaryotes, feeding vacuoles and flagellum. **B** Longitudinal section of the cell showing endosymbionts, flagellar apparatus and pair of nuclei. **C** Section through the cell showing the amoeboid body, single nucleus and endosymbionts. **D** Transversal section of the flagellum with aberrant arrangement of microtubules. **E** Longitudinal section of the flagellar apparatus. **F** Detail of the nucleus from the cell in A, showing peripheral chromatin and small nucleolus. **G** Section through the endosymbionts and nucleus. Ch – chromatin; En – endosymbionts; Fl – flagellum; FR – flagellar root; N – nucleus; Nu – nucleolus.

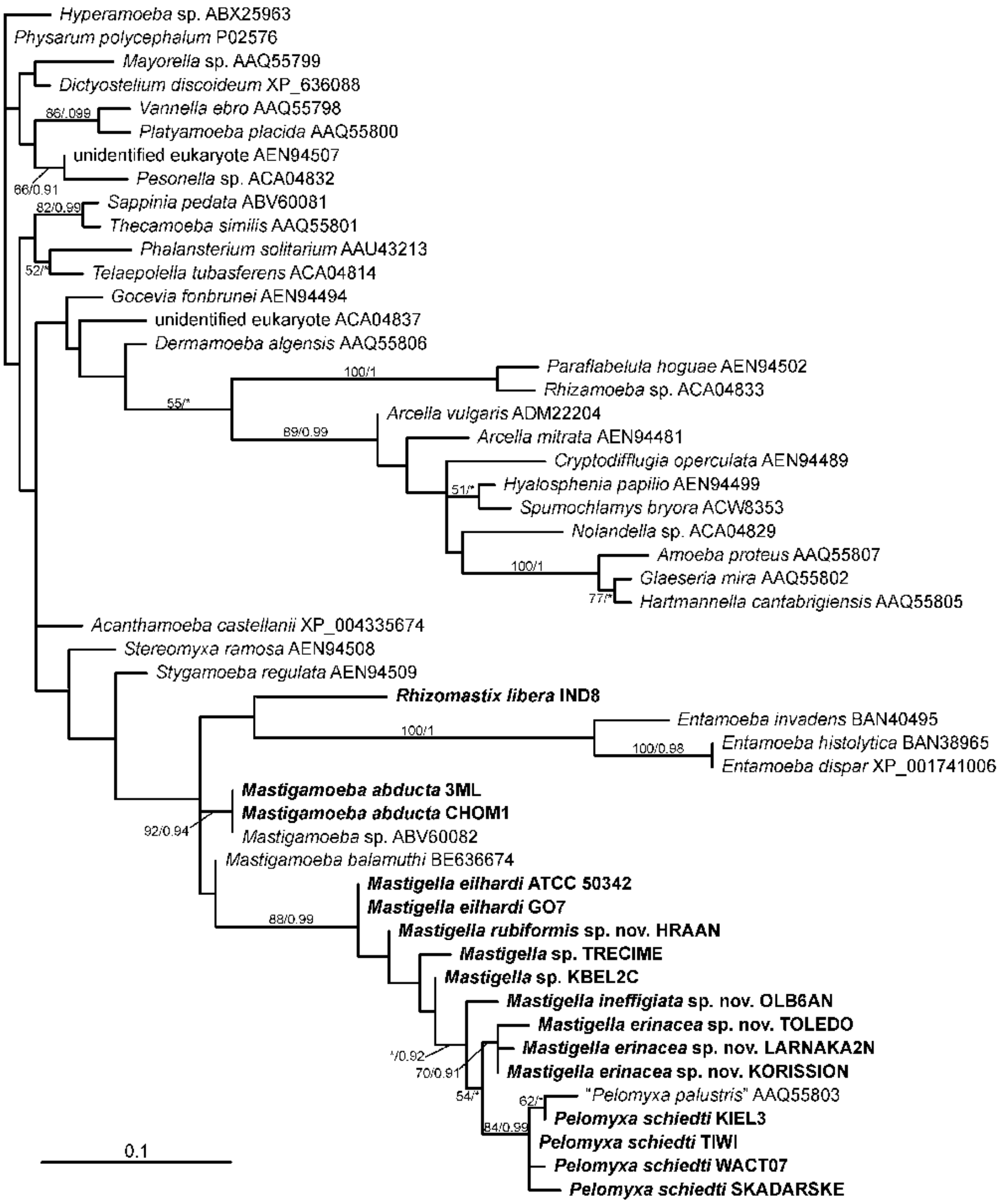

Figure 15. Phylogenetic tree of Amoebozoa based on actin gene sequences. The tree was constructed by the maximum likelihood method (PROTGAMMAILG model). The values at the nodes represent statistical support in maximum likelihood bootstrap values/Bayesian posterior probabilities. Support values below 50%/0.50 are not shown or are represented by an asterisk (*). New sequences are in bold.

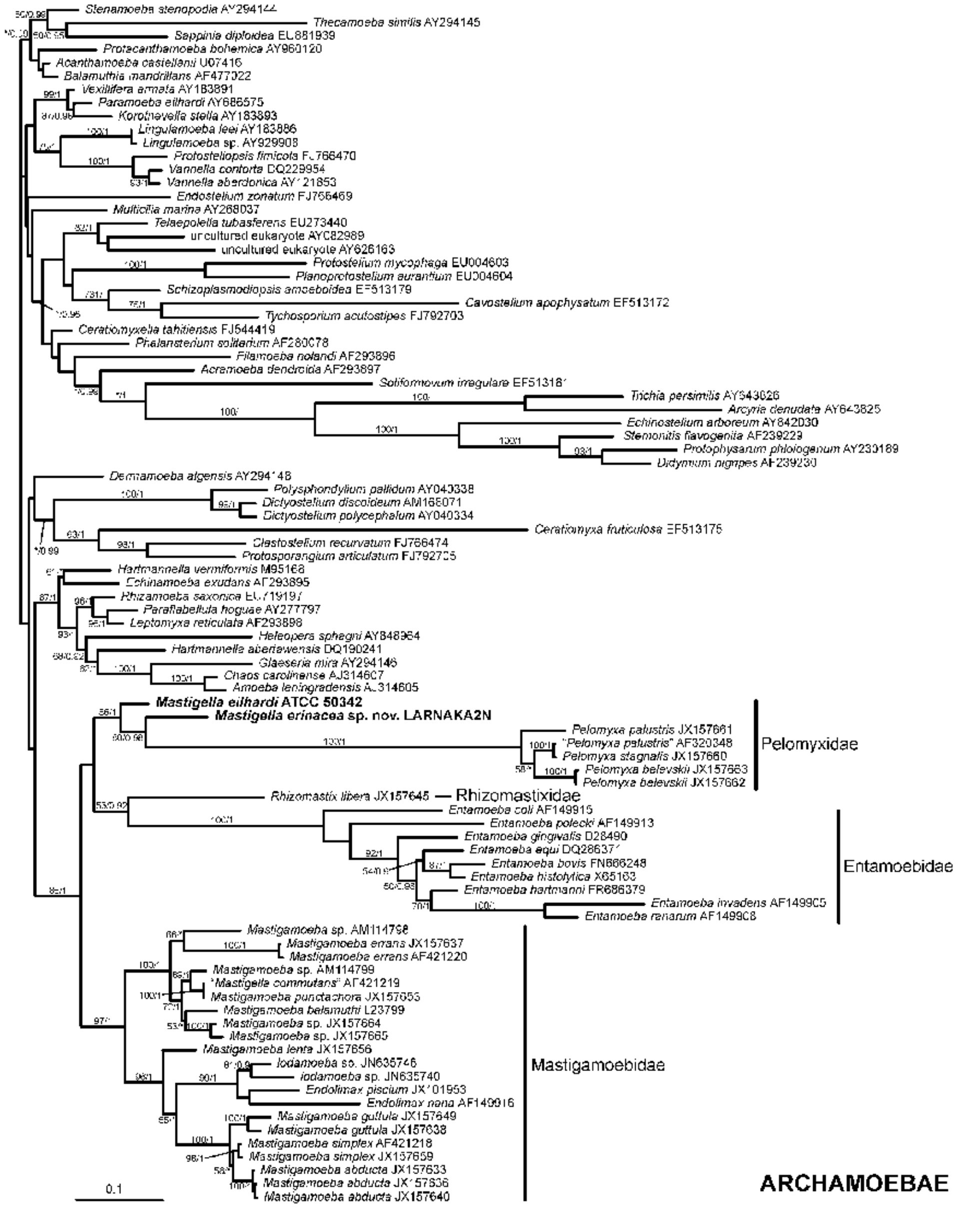

Figure 16. Phylogenetic tree of Amoebozoa based on SSU rDNA sequences. The tree was constructed by the maximum likelihood method (GTRGAMMAI model). The values at the nodes represent statistical support in maximum likelihood bootstrap values/Bayesian posterior probabilities. Support values below 50%/.50 are not showed or are represented by an asterisk (*). New sequences are in bold.

(not supported by a cytoplasmic “neck”), suggests a possible identity with *Mastigella unica* (Frenzel 1897), where the flagellum also emerges from a patch of villous pseudopodia; this species was originally placed in the genus *Limulina* and was transferred to *Mastigella* by Goldschmidt (1907). However *M. unica* was found in freshwater rather than saline sediments, its flagellum trails behind the cell and emerges from a cytoplasmic “neck”, cells are ca. 75 µm long, and possess 3 – 5 lateral, broad, finger-shaped hyaline pseudopodia on which the cells could “creep” (Frenzel 1897), none of which is seen in any of our strains. The nucleus or nuclei in *M. unica* were discussed as being not observed by Frenzel (1897, p. 42), so it is not possible to judge whether *M. unica* is uninucleate or binucleate.

Uninucleate individuals of *M. erinacea* sp. nov. can be differentiated from other mastigellas by being much smaller than *M. vitrea* (Goldschmidt 1907), being larger, less hyaline and having a much shorter flagellum than *M. commutans* (Meyer 1897; Walker et al. 2001); and being much larger than, and lacking the respective, specific pseudopodial characteristics of each of *M. penardi* (Lemmermann 1914), *M. caputmeduase* (Klug 1936) and *M. compacta* (Hamar 1979).

The nucleus observed in *Mastigella rubiformis* sp. nov. is unlike that seen in any previous descriptions of *Mastigella*. The hyaline cytoplasmic layer around the cell is similar to *Mastigella januarii* Frenzel, 1897, but the latter also has fingershaped pseudopodia and a morulate uroid in the swimming form.

Mastigella ineffigiata sp. nov. is distinguished by being much larger than the previously-described mastigellas that lack specific distinguishing features: *Mastigella polymastix* with average length 40 µm (Frenzel 1897); *Mastigella limax*, 38 – 42 µm long (Skuja 1964); and *Mastigella compacta*, 20 – 25 µm long (Hamar 1979).

On the basis of the morphological characterization described in Bürger (1905), we assign the strain ATCC 50342, deposited in the American Type Culture Collection under the name “Mastigella radicula (Moroff) Goldschmidt”, to the species *Mastigella eilhardi* Bürger, 1905. The original drawings and the description of *Mastigella radicula* by Moroff (1904) demonstrate this taxon to have been clearly a member of *Mastigamoeba* with an anterior nucleus clearly attached to the flagellar base, and it is unclear why it was transferred to *Mastigella* by Goldschmidt (1907); or why strain ATCC 50342, clearly a *Mastigella*, was previously identified as *Mastigella radicula*.

Species of *Pelomyxa* usually do not survive long in culture and tend to die after a few months. However, we cultivated several strains of *Pelomyxa schiedti* for one or two years, and strain SKADARSKE is still living. We assigned to *Pelomyxa* those strains that displayed monopodial locomotion and possessed cells with a posterior uroid and non-motile flagella. In the phylogenetic tree based on actin gene sequences, all four *Pelomyxa* strains clustered together with the sequence AAQ55803 deposited in GenBank under the name *Pelomyxa palustris* (Fahrni et al. 2003). *P. palustris* is usually reported as multinucleate amoeba that is 100 – 500 mm long (Whatley and Chapman-Andresen 1990). Since morphology of the sequenced strain was not examined in Fahrni et al. (2003), and distinguishing *Pelomyxa palustris* is quite complicated, it is possible that the sequence belongs, in fact, to other giant species of *Pelomyxa*. Our strains do not show similarities with *P. palustris*; but on the other hand, there are several features that correspond with original description of *Pelomyxa schiedti*: size, number and structure of nuclei, and fast movement (Schaeffer 1918). On the basis of these similarities, and because we do not know the complete life cycle of any our strains, we tentatively assigned them to *Pelomyxa schiedti*. However, there is the possibility that binucleate and tetranucleate cells of *Pelomyxa* strains may represent stages of the life cycles of diverse giant pelomyxas assigned to various nominal species, and may represent the only form able to survive in culture for a longer period than the lifespan of a typical microcosm. The ultrastructure of the flagellar apparatus of our *Pelomyxa* strains has not been fully determined. Expectedly, an aberrant arrangement of flagellar microtubules known from *Pelomyxa* species and *Tricholimax hylae* (Brugerolle 1982; Chistyakova and Frolov 2011; Frolov et al. 2005, 2006, 2007, 2011; Griffin 1988) is present in strain WACT07.

Mitochondrion-related Organelles and Endosymbionts

Double-membrane bound organelles that probably have mitochondrial ancestry have been previously reported from morphological descriptions of eight free-living species of Archamoebae (Constenla et al. 2013; Gill et al. 2007; Ptáčková et al. 2013; Seravin and Goodkov 1987; Simpson et al. 1997; Walker et al. 2001). Mitochondrion-related organelles have here been observed in the cells of *Mastigella rubiformis* sp. nov. and *M. ineffigiata* sp. nov. Although several species of *Pelomyxa* have been examined by means of electron microscopy, cytoplasmic double-membrane bound organelles, 300 – 500 nm long, have been detected only once in a single species, *Pelomyxa palustris* (Seravin and Goodkov 1987). Double membrane-bound organelles detected in *Mastigamoeba balamuthi*, which were characterized as mitosomes with extended biochemical functions, had similar dimensions to those of *Pelomyxa palustris* (Gill et al. 2007; Hampl and Simpson 2007; Nývltová et al. 2013). Electron-microscopical identification of prokaryotic endosymbionts in several species of *Pelomyxa* (Chistyakova and Frolov 2011; Chystjakova et al. 2014; Daniels and Pappas 1994; Frolov et al. 2005, 2006, 2007) may possibly be of mixed populations of endosymbionts and mitochondrial remnant organelles; further data are required.

Unidentified endosymbiotic prokaryotes have previously been observed by electron microscopy in *Mastigina trichophora*, *Mastigella nitens*, *Mastigamoeba aspera*, and *Rhizomastix libera* (Frolov 2011; Ptáčková et al. 2013). Three kinds of prokaryotes have been reported by light microscopy from *Mastigella* sp. and diverse *Pelomyxa* species: large rod-shaped bacteria with a longitudinal cleft, small rod-like bacteria and the methanogenic archaean *Methanobacterium formicicum* (Frolov et al. 2005, 2006, 2011; Goldschmidt 1907; Gould-Veley 1905; Griffin 1988; Gutiérrez 2012; Lauterborn 1916; Ptáčková et al. 2013; van Bruggen et al. 1983, 1985, 1988; Whatley and Chapman-Andresen 1990). *M. rubiformis* sp. nov., *M. ineffigiata* sp. nov., and *P. schiedti* possess rod-shaped bacteria in their cytoplasm, and *P. schiedti* probably contains two different types of endosymbionts. Since we have not yet enough information about these endosymbionts, we could not identify them more precisely, and their function is so far unknown. Recently, the genome sequence of *Methanobacterium formicicum* isolated from cells of *Pelomyxa palustris* was published (Gutiérrez 2012). The data suggest that this prokaryote represents a free-living organism rather than an endosymbiont or the prokaryotes might be a content of feeding vacuoles. No symbiotic prokaryotes have been observed in the cytoplasm of endobiotic genera *Entamoeba* and *Endolimax* (Constenla et al. 2013; Martínez-Palomo 1993), or in small *Mastigamoeba* species (Walker et al. 2001).

Phylogeny and Taxonomy of Pelomyxidae

Here we determined actin gene sequences of our strains of *Mastigella* and *Pelomyxa*. We also added new actin gene sequences from two strains of *Mastigamoeba abducta* and one from *Rhizomastix libera*, which means that now actin gene sequences from each of the main lineages of Archamoebae are available. Although we improved the taxon sampling, the monophyly of the Archamoebae remained unsupported, and its internal topology was unresolved. Nevertheless, genera *Mastigella* and *Pelomyxa* were closely related with relatively high statistical support. Internal branches in this lineage that comprised both genera were not resolved with the exception of relatively well-supported monophyly of *Pelomyxa*. Strains isolated from saline sediments and representing *Mastigella erinacea* sp. nov. cluster together although the group is weakly supported.

Since SSU rDNA is one of the most frequently used markers for reconstructing evolutionary history, SSU rDNA sequence of *Mastigella* might elucidate the phylogenetic position of this genus. We determined SSU rDNA sequences of *Mastigella eilhardi* (strain ATCC 50342) and *M. erinacea* sp. nov. (strain LAR2N). The Archamoebae appeared robustly monophyletic in SSU rDNA tree, accordingly with previous studies (Fiore-Donno et al. 2010; Nikolaev et al. 2006; Ptáčková et al. 2013). In accordance with the actin gene analysis, *Pelomyxa* clustered with *Mastigella* and formed an internal branch of *Mastigella*, and *M. erinacea* sp. nov. forms the sister branch of *Pelomyxa*. In this case, however, the paraphyly of *Mastigella* was statistically relatively well-supported. Unlike *Pelomyxa*, both *Mastigella* species form relatively short branches in the SSU rDNA tree; in fact, the branch of *M. eilhardi* is the shortest one among the Archamoebae.

AU testing of alternative hypotheses rejected a close relationship between *Mastigella* and *Mastigamoeba*, but provided no strong conclusions otherwise for the SSU rRNA gene dataset. For the actin dataset all alternatives were rejected other than monophyly of *Mastigella*, where the sister taxon in the constraint tree was *Pelomyxa*. This supports our interpretation of the taxonomic relationship between these two genera, though the statistical support for this from AU testing is low, as would be expected for single gene-tree phylogenies of archamoebae.

It has traditionally been held that *Mastigella* is specifically related to the genus *Mastigamoeba* (Adl et al. 2012; Cavalier-Smith et al. 2004; Chatton 1925; Goldschmidt 1907; Griffin 1988; Ptáčková et al. 2013). In contrast, the results of our phylogenetic analyses suggested that the genera *Mastigamoeba* and *Mastigella* are phylogenetically distant among the Archamoebae (at least the species from which sequence data are available). Our data support a close relationship between the genera *Mastigella* and *Pelomyxa*, which has previously been hypothesized based on the nuclear structure and presence of endosymbiotic prokaryotes in the cytoplasm (Cavalier-Smith 1991; Frolov 2011; van Bruggen et al. 1985; Walker et al. 2001). More specifically, our data favor the scenario suggested by Cavalier-Smith (1991), who postulated that *Pelomyxa* had evolved from within *Mastigella* by nuclear and flagellar multiplication. Interestingly, *M. erinacea* sp. nov. morphologically resembles *Pelomyxa* with respect to cell movement and presence of several nuclei per cell, and branches as a sister taxon to *Pelomyxa* in both gene trees.

The paraphyly of *Mastigella* and the close relationship between *M. erinacea* sp. nov. and *Pelomyxa* spp. seems to be further supported by several lines of morphological data. Cells of most *Mastigella* species, including *M. eilhardi*, *M. ineffigiata* sp. nov., and *M. rubiformis* sp. nov., are predominantly uninucleate. In contrast, the cells of *M. erinacea* sp. nov. mostly possess two nuclei, similarly to *Pelomyxa schiedti* and some other *Pelomyxa* species.

Trophozoites usually containing a single nucleus, with a large central nucleolus that is often visible under light microscope, are the most common stage occurring in Mastigamoebidae, Rhizomastixidae and in genera *Mastigella* and *Mastigina* (Constenla et al. 2013; Goldschmidt 1907; Ptáčková et al. 2013; Walker et al. 2001). *Mastigella eilhardi*, *M. erinacea* sp. nov., and *M. ineffigiata* sp. nov. possess a nucleus with a single large central nucleolus, a morphology that has been previously reported for example in *M. commutans* (Walker et al. 2001).

Peripheral chromatin granules in the nucleus are widely present across the archamoebae (Čepička 2011; Frolov et al. 2007; Martínez-Palomo 1993; Ptáčková et al. 2013). The greatest variability of nuclear ultrastructure occurs in species of *Pelomyxa*, where heterochromatin blocks are frequently dispersed in the nucleus without evident pattern; or a threadlike rounded body is occasionally present in the nucleolus (Chistyakova and Frolov 2011; Frolov et al. 2005, 2006, 2011; Griffin 1988; Ptáčková et al. 2013). The nuclei of *M. rubiformis* sp. nov. and *Pelomyxa schiedti* consist of a small nucleolus and peripheral chromatin. While nuclear chromatin appears to be useful in distinguishing individual species, its utility as a supra-specific character is still questionable in members of the Pelomyxidae.

Although the single flagellum of *M. erinacea* sp. nov. is motile, like that of other species of the genus and unlike the non-motile flagella of *Pelomyxa* spp. and *Tricholimax* (Brugerolle 1982; Frenzel 1897; Frolov et al. 2005, 2006, 2007, 2011; Griffin 1988; Chistyakova and Frolov 2011; this paper), its beating is comparatively slower than in other species of *Mastigella*.

It is possible that flagellar movement has been lost gradually in the lineage leading to *Pelomyxa*; the loss probably correlates with the degree of aberration of the axonemal structure. Because of the unavailability of sequence data from *Tricholimax*, another archamoeba with aberrant flagellar structure (Brugerolle 1982), a possible close relationship between *Tricholimax* and *Pelomyxa* suggested by Griffin (1988) remains unclear. Since the connection between the flagellum and nucleus occurs in *Tricholimax hylae* (Brugerolle 1982), but not in *Mastigella*, the absence of motility of the flagellum of *Tricholimax* could have evolved independently, as an adaptation to an endobiotic lifestyle. Slowing down of flagellar movement or its complete loss has also happened in other endobiotic species of the Archamoebae. While the beating of the flagellum in free-living *Rhizomastix libera* is quick (Ptáčková et al. 2013), that of the endobiotic species *R. biflagellata* moves slowly (own observation); and species of *Entamoeba*, *Endolimax* and *Iodamoeba* have completely lost their entire flagellar apparatus (see Martínez-Palomo 1993).

The genus *Mastigella* Frenzel, 1897 has traditionally been treated as belonging to the family Mastigamoebidae Chatton, 1925, while *Pelomyxa* Greeff, 1874 has been regarded as the sole member of the family Pelomyxidae Schulze, 1877 (Adl et al. 2012; Griffin 1988; Larsen and Patterson 1990; Ptáčková et al. 2013). In order to emphasize morphological differences between genera *Mastigamoeba* and *Mastigella*, Cavalier-Smith (1991) removed *Mastigella* from Mastigamoebidae and created family Mastigellidae. However, Mastigellidae Cavalier-Smith, 1991 has not been adopted by the other authors. We propose a new taxonomic concept of *Mastigella* by transferring it to the family Pelomyxidae. The family Pelomyxidae thus now contains two genera, *Pelomyxa* and *Mastigella*. Although we are convinced we have improved the understanding of the systematics of the Archamoebae, the taxonomy of *Mastigella* and Pelomyxidae is still far from being settled due to the possible paraphyly of *Mastigella* with respect to *Pelomyxa*. The situation is further complicated by two issues: (1) Sequence data from the type species *M. polymastix* Frenzel, 1897 are unavailable. Thus, it is unclear to which of the two lineages of *Mastigella* revealed by our SSU rDNA analysis, it belongs. (2) We were able to determine only SSU rRNA gene sequences of *Mastigella ineffigiata* sp. nov. and *M. rubiformis* sp. nov. We cannot rule out the possibility that one or both species represent a separate evolutionary lineage from the rest of *Mastigella*. It seems likely that *Mastigella* will be split into at least two genera in the future. It is currently unclear which lineage would retain the name *Mastigella* after the splitting. Therefore, we retain paraphyletic *Mastigella* here, similarly to the current situation in the genus *Mastigamoeba* (Ptáčková et al. 2013).

Taxonomic Summary

The type material of newly described species is deposited in the collection of the Department of Parasitology, Charles University in Prague, Czech Republic.

Eukaryota: Amoebozoa: Archamoebae

Family Pelomyxidae Schulze, 1877. Diagnosis: Anaerobic or microaerophilic flagellated amoebae with slow-beating monokinetid or immobile polykinetids. Type genus: *Pelomyxa* Greeff, 1874. Included genera: *Pelomyxa* Greeff, 1874; *Mastigella* Frenzel, 1897.

***Mastigella* Frenzel, 1897**. Diagnosis: Amoeboid cells with flagellated basal body and microtubular cone not associated with the nucleus. Type species: *Mastigella polymastix* Frenzel, 1897.

***Mastigella erinacea* sp. nov.** Zoobank registration: urn:lsid:zoobank.org:act:101FB8D9-48A9-4146-9894-4604028009BF. Description: see Results. Type locality: Larnaka, Cyprus. 34°51′N, 33°37′E. Habitat: Shallow anoxic brackish sediments, salt marsh. Holotype: Protargol-stained cell of the strain LAR2N depicted in Figure 4E. The preparation is deposited with the catalogue number 11/25. Etymology: L. fem. adj. *erinacea* – like a hedgehog. Referring to the spiny pseudopodia of this species.

***Mastigella ineffigiata* sp. nov.** Zoobank registration: urn:lsid:zoobank.org:act:ED574EC2-7DB5-4D9C-B09B-80BA105698FD. Description: See results. Type locality: Olbasee lake, Germany. 51°16′N, 14°35′E. Habitat: Anoxic freshwater sediment. Syntype: Protargol preparations of the strain OLB6AN with *M. ineffigiata* sp. nov. and *Rhizomastix* sp., catalogue numbers 11/27 – 11/34. Figure 8H, I are images from the syntype. Etymology: L. fem. adj. *ineffigiata* – shapeless. Referring to the amorphous appearance of the cell.

***Mastigella rubiformis* sp. nov.** Zoobank registration: urn:lsid:zoobank.org:act:6DF37D99-51B7-4763-BD03-68502E36E46F. Description: see results. Type locality: Hradiště peak, Czech Republic. 50°27′N, 13°20′E. Habitat: Anoxic freshwater sediment. Syntype: Protargol preparations of the strain HRAAN with *M. rubiformis* sp. nov., *Mastigamoeba errans* and unidentified ciliates, catalogue numbers 6/38 – 6/40. Figure 6K, L are images from the syntype. Etymology: L. fem. adj. *rubiformis* – like a raspberry. Referring to the swimming cell appearance reminiscent of a raspberry.

Methods

Organisms: Most new strains were isolated from fresh-water anoxic/microoxic sediments. The strains KORISSION and LAR2N were isolated from brackish sediments; the strain TOLEDO was obtained from salt-marsh sediments. The strain ATCC 50342 was obtained from the American Type Culture Collection. The strains 3ML, CHOM1, and IND8 were isolated by Ptáčková et al. (2013). Fresh-water samples were inoculated into 15 ml Falcon tubes containing 9 ml of Sonneborn's *Paramecium* medium (ATCC medium 802: http://www.lgcstandards-atcc.org/~/media/91F4C9697D734A1F89DE0E2474F43743.ashx) made using Ward's cereal grass (Ward's Science). The strains KORISSION and LAR2N were cultivated in seawater 802 medium (ATCC medium 1525: http://www.atcc.org/~/media/D88E997F5B8B4B9F9DA267829BD8E27B.ashx). For TOLEDO a 1:1 mixture of Sonneborn's and fresh-water medium was used. Approximately 2 ml of each sample was initially inoculated into the medium and were maintained in a xenic culture at room temperature with transfers of 1 ml to new medium occurring once weekly. Unidentified bacteria and eukaryotes (e.g. ciliates, diplomonads, *Rhizomastix* sp., *Mastigamoeba* sp.) were present in the cultures besides the *Mastigella* and *Pelomyxa*. Strains GO7, OLB6AN, and SKADARSKE are deposited in the culture collection of the Department of Parasitology of Charles University in Prague, Czech Republic; the other strains have been lost.

Light microscopy: The morphology of living and protargol-stained cells was examined under a light microscope (Olympus BX51). DIC was used to observe living cells. Protargol-stained preparations were prepared as follows: the strains were centrifuged at 1000 *g* for 10 minutes. The pelleted cultures were spread on cover slips forming moist films as described in Pánek et al. (2014). The films were fixed in Bouin-Hollande's fluid for 10 hours, washed with 70% ethanol, and stained with 1% protargol (Bayer, I. G. Farbenindustrie) following Nie's (1950) protocol.

DNA extraction, amplification, cloning and sequencing: Genomic DNA was isolated from cultures using the DNeasy Blood and Tissue Kit (Qiagen). Universal eukaryotic primers for amplification of actin gene sequences actFY (AACTGGGAYGAYATGGARAAGAT) and actRY (ATCCACATYTGYTGGAANGT) (Yoon et al. 2008) were used. These primers preferentially amplified sequences of Archamoebae, and were inefficient at amplifying other groups present in the cultures. SSU rDNA sequnces of strains ATCC 50342 and LAR2N were amplified using universal eukaryotic primers EK42F (CTCAARGAYTAAGCCATGCA) and EK1498R (CACCTACGGAAACCTTGTTA) (Marande et al. 2009). PCR fragments were purified from agarose gels using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research) and cloned using the pGEM® T-Easy Vector System (Promega). In cases of mixed culture, PCR fragments were separated using gel electrophoresis, and were then cloned individually. The new sequences are available in GenBank database under accession numbers KJ879559 – KJ879587.

Phylogenetic analyses: Two datasets were created, respectively containing sequences of actin and SSU rRNA genes. The actin dataset contained inferred amino acid sequences, including 16 newly-determined sequences from *Mastigella*, *Pelomyxa*, *Mastigamoeba*, and *Rhizomastix*, 6 sequences from *Mastigamoeba*, *Pelomyxa* and *Entamoeba* obtained from GenBank, and 29 sequences of non-archamoebae Amoebozoa. The sequences were aligned using MAFFT (Katoh et al. 2002) with the help of the MAFFT 7 server http://mafft.cbrc.jp/alignment/server/ with G-INS-i algorithm at default settings. The resulting alignment was manually edited in BioEdit 7.0.9.0 (Hall 1999). The final dataset contained 264 amino acid positions. A maximum likelihood phylogenetic tree was constructed in RAxML 7.2.3 (Stamatakis 2006), using the PROTGAMMAILG model. Bootstrap values were estimated from 1000 permutations. Bayesian analysis was performed in PhyloBayes 3.3f (Lartillot and Philippe 2004) using the CAT POI model. Two independent chains were run until their maximum observed discrepancy was lower than 0.1, and the effective sample size of all model characteristics was at least 100. The first 25% of trees were removed as burn-in. Consensus was calculated every 10 trees.

The SSU rRNA gene dataset contained two newly-determined sequences of *Mastigella eilhardi* and *M. erinacea* sp. nov., respectively, 36 sequences of Archamoebae obtained from GenBank, and 52 sequences of non-archamoebae Amoebozoa. The sequences were aligned, and the alignment was edited as for the actin gene. The final dataset contained 1160 nucleotide positions. A maximum likelihood phylogenetic tree was constructed in RAxML using the GTRGAMMAI model of sequence evolution; *b*ootstrap values were estimated from 1000 permutations. Bayesian analysis was performed in MrBayes 3.2.2 (Ronquist et al. 2012) using the GTR + I + Γ + covarion model. Four MCMC chains were run for 3.10^6 generations, until the mean standard deviation of split frequencies based on last 75% of generations was lower than 0.01. The trees were sampled every 500^{th} generation. The first 25% of trees were removed as burn-in.

Various alternative phylogenetic positions of *Mastigella* strains were tested using AU tests implemented in consel 0.1i (Shimodaira and Hasegawa 2001). The null hypothesis was that there was no difference between trees. The alternative topologies were inferred using RAxML with a prior phylogenetic hypothesis set as a constraint. Site likelihoods were calculated using RAxML.

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7. 3. Zadrobílková et al. 2015b

Zadrobílková E, Smejkalová P, Walker G, Čepička I (2015b) Morphological and molecular diversity of the neglected genus *Rhizomastix* Alexeieff, 1911 (Amoebozoa: Archamoebae) with description of five new species. J Euk Microbiol, *in press* doi:10.1111/jeu.12266

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ORIGINAL ARTICLE

Morphological and Molecular Diversity of the Neglected Genus Rhizomastix Alexeieff, 1911 (Amoebozoa: Archamoebae) with Description of Five New Species

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ABSTRACT

The genus *Rhizomastix* is a poorly known group of amoeboid heterotrophic flagellates living as intestinal commensals of insects, amphibians or reptiles, and as inhabitants of organic freshwater sediments. Eleven *Rhizomastix* species have been described so far, but DNA sequences from only a single species have been published. Recently, phylogenetic analyses confirmed a previous hypothesis that the genus belongs to the Archamoebae; however, its exact position therein remains unclear. In this study we cultured nine strains of *Rhizomastix*, both endobiotic and free-living. According to their light-microscopic morphology and SSU rRNA and actin gene analyses, the strains represent five species, of which four are newly described here: *R. bicoronata* sp. nov., *R. elongata* sp. nov., *R. vacuolata* sp. nov. and *R. varia* sp. nov. In addition, *R. tipulae* sp. nov., living in the intestine of crane flies, is separated from the type species, *R. gracilis*. We also examined the ultrastructure of *R. elongata* sp. nov., which revealed that it is more complicated than the previously described *R. libera*. Our data show that either the endobiotic lifestyle of some *Rhizomastix* species has arisen independently from other endobiotic archamoebae, or the free-living members of this genus represent a secondary switch from the endobiotic lifestyle.

ARCHAMOEBAE is a small but phylogenetically interesting group of Amoebozoa, which comprises approximately 250 species of amoeboid flagellates and amoebae (Ptáčková et al. 2013). Members of Archamoebae are obligately anaerobic or microaerophilic, and can be either free-living or endobiotic. They were originally thought to lack mitochondria, and were thus thought to be basal eukaryotes, placed in the Archezoa. However, mitochondrial homologues were later discovered in some species (Gill et al. 2007; Tovar et al. 1999); and molecular phylogenetics has placed Archamoebae in the Amoebozoa (Arisue et al. 2002; Milyutina et al. 2001), distant from other taxa with degenerate mitochondria. Cells of Archamoebae have a simple cytoskeleton that consists of a single basal body giving rise to a single flagellum, microtubular cone and lateral root (Brugerolle 1991).

Archamoebae have traditionally been divided into pelobionts, which encompass the flagellated, mostly free-living genera *Mastigamoeba*, *Mastigella*, *Pelomyxa*, *Tricholimax*, and *Mastigina*; and entamoebae, containing aflagellated and predominantly endobiotic genera *Entamoeba*, *Endamoeba*, *Iodamoeba*, and *Endolimax*. Subsequent phylogenetic analyses have shown that neither of these two groups is monophyletic within the Archamoebae (Cavalier-Smith et al. 2004; Edgcomb et al. 2002; Milyutina et al. 2001; Nikolaev et al. 2006). Archamoebae is currently divided into four families: mostly endobiotic Entamoebidae and Rhizomastixidae, and predominantly free-living Mastigamoebidae and Pelomyxidae (Ptáčková et al. 2013; Zadrobílková et al. 2015). The best-known and most extensively studied member of the group is the human parasite *Entamoeba histolytica*, which causes amoebic dysentery

(Martínez-Palomo 1993). Free-living archamoebae, on the other hand, have been studied much less often, and mostly from a taxonomic point of view (Brugerolle 1982, 1991; Chistyakova et al. 2014; Chystyakova and Frolov 2011; Chystyakova et al. 2012; Frolov 2011; Frolov et al. 2004, 2005a,b, 2006, 2011; Ptáčková et al. 2013; Simpson et al. 1997; Walker et al. 2001; Zadrobílková et al. 2015). The endobiotic lifestyle has arisen at least two times independently within Archamoebae: in Entamoebidae, genus *Entamoeba*, and in Mastigamoebidae, genera *Iodamoeba* and *Endolimax* (Ptáčková et al. 2013; Stensvold et al. 2012; Zadrobílková et al. 2015); but important endobiotic taxa remain from which molecular data are still missing (*Endamoeba*, *Tricholimax*, *Rhizomastix* spp.).

The genus *Rhizomastix*, the only member of the family Rhizomastixidae, comprises 11 species. Most of them are intestinal symbionts of insects (Bhaskar Rao 1963, 1970; Ludwig 1946; Mackinnon 1913; Sultana 1976), amphibians (Alexeieff 1911; Cepicka 2011; Krishnamurthy 1969) and reptiles (Cavalier-Smith and Scoble 2013). One species was also found in human faeces (Yakimoff and Kolpakoff 1921). Two *Rhizomastix* species were isolated from freshwater sediments or polluted water, and are considered free-living (Ptáčková et al. 2013; Zhang and Yang 1990). The most characteristic feature of the genus *Rhizomastix* is the rhizostyle, a long cytoskeletal fibre that arises from the basal body of the flagellum and extends posteriorly into the cytoplasm (Alexeieff 1911). It has subsequently been shown that the rhizostyle of the free-living species *Rhizomastix libera* is composed of a bundle of microtubules, and it has been hypothesized that it is a homologue of the microtubular cone of other archamoebae (Ptáčková et al. 2013). Other important characters of *Rhizomastix* include binucleated cysts; and morphology of the nucleus, which often contains a large central nucleolus connected with peripheral chromatin granules (Bhaskar Rao 1970; Cepicka 2011; Ludwig 1946; Mackinnon 1913). The cells of *Rhizomastix* usually have a single flagellum, though aflagellated cells have been observed in some species; and approximately half of the population of *R. biflagellata* consists of biflagellated cells (Cepicka 2011).

Although the genus *Rhizomastix* was discovered just over 100 yr ago (Alexeieff 1911), and most nominal species were described more than 30 yr ago (Alexeieff 1911; Bhaskar Rao 1963, 1970; Krishnamurthy 1969; Sultana 1976; Yakimoff and Kolpakoff 1921), it has been largely ignored in recent decades: molecular data, confirming the affinity of *Rhizomastix* with the Archamoebae, were published only recently (Ptáčková et al. 2013). Phylogenetic analysis of the SSU rRNA gene of the free-living species *R. libera* showed that it forms a deep branch in the Archamoebae and indicated that *Rhizomastix* might be closely related to Entamoebidae or Pelomyxidae (Ptáčková et al. 2013). Sequence data from other species have not hitherto been obtained. It is thus unclear whether the genus is monophyletic, and whether its endobiotic species represent an independent origin of parasitism.

In order to examine the diversity of the genus *Rhizomastix*, we cultured seven strains and examined their light-microscopic morphology and phylogenetic position, using SSU rRNA and actin gene sequences. Additionally, we examined the ultrastructure of a single strain. Our data show that *Rhizomastix* is diverse, as the strains represent four new species. Our data show either that parasitism has arisen at least three times independently within Archamoebae; or that, if parasitism has only arisen twice, then all members of the genus *Rhizomastix* are descendants of endobiotic organisms, and thus the free-living members are secondarily free-living.

MATERIALS AND METHODS

Sampling and culture conditions

Information on the origin of *Rhizomastix* strains included in the study is summarized in Table 1. Strain VELKA1 of *R. bicoronata* sp. nov. was obtained from the lower intestine of a millipede. Strains GOL1 and GOL18 (*R. vacuolata* sp. nov.) were isolated from the lower intestine of beetle larvae. After the hosts were dissected, the intestinal contents were inoculated into Dobell and Laidlaw's (1926) biphasic medium; this medium was used also for subsequent cultivation. Strain VAVRH of *Rhizomastix elongata* sp. nov. was isolated from the contents of a cesspool. The cesspool was a concrete pool, approximately 3-m long by 2.5-m wide, filled by ground-water to a depth of approximately 70 cm, and with a ca. 30-cm-thick layer of black organic sediment. It had not been used for some years, prior to sampling. Larvae of hoverflies (*Eristalis* sp.) were observed occasionally, and larvae of mosquitoes (*Culex* sp.) were observed frequently, inside the cesspool. Of the sediment, 2 ml was inoculated to two different media: Dobell and Laidlaw's biphasic medium and Sonneborn's *Paramecium* medium (ATCC medium 802; http://www.lgcstandards-atcc.org/~/media/91F4C9697D734A1F89DE0E2474F43743.ashx). *Rhizomastix elongata* sp. nov. survived in both media for a week; Dobell and Laidlaw's biphasic medium was then used for routine cultivation. Strains BOTANKA and IPSALA of *R. libera* and strain FBAN of *Rhizomastix varia* sp. nov. were obtained from freshwater sediments. The samples were inoculated into Sonneborn's *Paramecium* medium; this medium was used for routine cultivation. Cultures IND8MA, OLB6AN and SKADARSKE containing *R. libera* were maintained as described in Ptáčková et al. (2013) and Zadrobílková et al. (2015).

The strains were maintained in a xenic culture at room temperature with transfers occurring once per week. Besides *Rhizomastix*, most cultures contained unidentified bacteria as well as several unrelated species of protists, usually trichomonads, retortamonads, oxymonads, stramenopiles or kinetoplastids, which were morphologically and phylogenetically easily distinguished from *Rhizomastix* spp. Strain OLB6AN of *R. libera* grew in culture with *Mastigella ineffigiata*; strain SKADARSKE was co-cultured with *Pelomyxa schiedti* (Zadrobílková et al. 2015). The strains, except for BOTANKA, GOL18 and IPSALA, are deposited in the culture collection of the Department of Parasitology of Charles University in Prague, Czech Republic.

Table 1. List of the strains included in the study

Species	Strain	Locality/host	Habitat	Coordinates
Rhizomastix bicoronata sp. nov.	VELKA1	Millipede	Intestine	N.A.
Rhizomastix elongata sp. nov.	VAVRH	Vejvanov-Pajzov, Czech Republic	Abandoned cesspit	49°51′N 13°39′E
Rhizomastix libera	BOTANKA	Prague, Czech Republic	Freshwater sediment	50°04′N 14°24′E
Ptáčková *et al.*, 2013	IND8[a]	Bhangarh, India	Freshwater sediment	27°05′N 76°17′E
	IPSALA	Ipsala, Turkey	Freshwater sediment	40°56′N 26°19′E
	OLB6AN	Olbasee lake, Germany	Freshwater sediment	51°16′N 14°35′E
	SKADARSKE	lake Skadar, Albania	Freshwater sediments	42°3′N 19°29′E
Rhizomastix vacuolata sp. nov.	GOL1	Larva of Goliath beetle	Intestine	N.A.
	GOL18	Larva of Goliath beetle	Intestine	N.A.
Rhizonastix varia sp. nov.	FBAN	Ribeiro Frio, Madeira, Portugal	Freshwater sediment	32°44′N 16°53′E

N.A. = not available.

[a]Strain isolated by Ptáčková et al. (2013).

Light microscopy

The morphology of living and protargol-stained cells was examined under a light microscope (Olympus BX51, Tokyo, Japan). DIC was used to observe living cells.

Protargol-stained preparations were prepared as follows: 1 ml of culture was centrifuged at 1,000 *g* for 10 min. The pelleted cultures were spread on coverslips forming moist films. The films were fixed in Bouin–Hollande's fluid for 10 h, washed with 70% ethanol and stained with 1% protargol (Bayer, I. G. Farbenindustrie, Frankfurt am Main, Germany; defunct since 1952) following Nie's (1950) protocol.

Transmission electron microscopy

A cell suspension of strain VAVRH (*R. elongata* sp. nov.) was prepared by centrifugation of the culture for 10 min at 1,000 *g*. The sample was high-pressure frozen using a Leica EM PACT2 (Leica Microsystems, Wetzlar, Germany), and cryosubstituted in a Leica EM AFS2, using acetone with 2% OsO_4 at −90 °C for 96 h. Embedding was done at room temperature, using Epon resin (Poly/Bed 812/Araldite; Polysciences, Warrington, PA), having been infiltrated in an ascending series of concentrations changed every hour. Samples were sectioned at 60 nm thickness using a diamond knife on an Ultracut E ultramicrotome (Reichert, Vienna, Austria) and collected on copper mesh grids coated with formvar film. Ultrathin sections were stained with lead citrate and uranyl acetate (2–3%) and examined using a TEM JEOL 1011 (Jeol, Tokyo, Japan) transmission electron microscope.

DNA extraction, amplification, cloning and sequencing

Genomic DNA was isolated from cultures using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Two types of universal eukaryotic primers were used to amplify SSU rRNA genes: (1) MA (CTGGTTGATCCTGC CAG) and MB (TGATCCTTCTGCAGGTTCACCTAC) (Medlin et al. 1988) for strains VAVRH and VELKA1, (2) EK42F (CTCAARGAYTAAGCCATGCA) and EK1498R (CACCTACG GAAACCTTGTTA) (Marande et al. 2009) for strains GOL1, GOL18, BOTANKA, and OLB6AN. Actin gene sequences were amplified using universal eukaryotic primers actFY (AACTGGGAYGAYATGGARAAGAT) and actRY (ATCCACA TYTGYTGGAANGT) (Yoon et al. 2008). PCR fragments were purified from agarose gels using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, CA) and cloned using the pGEM® T-Easy Vector System (Promega, Fitchburg, WI). In cases of mixed culture, PCR fragments were separated using gel electrophoresis and were then cloned individually. The new sequences are available in GenBank under accession numbers KP343610–KP343638.

Phylogenetic analyses

Two data sets were created, respectively, containing sequences of SSU rRNA and actin genes. The data set of SSU rRNA gene contained six newly determined sequences of *Rhizomastix*, 37 sequences of Archamoebae obtained from GenBank, and 56 sequences of non-archamoebae Amoebozoa. The sequences were aligned with MAFFT (Katoh et al. 2002), using the G-INS-I algorithm with default settings, on the MAFFT 7 server (http://mafft.cbrc.jp/alignment/server/). The resulting alignment was manually edited in BioEdit 7.0.9.0 (Hall 1999) to remove ambiguously aligned sites. The final data set contained 1,263 nucleotide positions. A maximum-likelihood phylogenetic tree was constructed in RAxML 7.2.3 (Stamatakis 2006) using the GTRGAMMAI model of sequence evolution; bootstrap values were estimated from 1000 permutations. Bayesian analysis was performed in MrBayes 3.2.2 (Ronquist et al. 2012) using the GTR + I + Γ + covarion model. Four MCMC chains were run for 3×10^6 generations, until the mean standard deviation of split frequencies based on the previous 75% of generations was lower than 0.01. Trees were sampled every 500th generation. The first 25% of trees were discarded as burn-in.

The data set of the actin gene consisted of inferred amino acid sequences and contained 14 newly determined sequences of genus *Rhizomastix*, 22 sequences of archamoebae obtained from GenBank, and 28 sequences of non-archamoebae Amoebozoa. The sequences were aligned using MAFFT with the G-INS-i algorithm; the resulting alignment was manually edited in BioEdit to remove ambiguously aligned sites. The final data set contained 264 amino acid positions. A maximum-likelihood phylogenetic tree was constructed in RAxML using the PROTGAMMAILG model of sequence evolution. Bootstrap values were estimated from 1,000 permutations. Bayesian analysis was performed in PhyloBayes 3.3f (Lartillot et al. 2009) using the CAT POI model. Two independent chains were run until their maximum observed discrepancy was lower than 0.1, and the effective sample size of all model characteristics was at least 100. The first 25% of trees were discarded as burn-in. Consensus was calculated every 10 trees.

RESULTS

Light microscopy

Rhizomastix bicoronata sp. nov.

The strain VELKA1 consisted almost exclusively of flagellated cells (Fig. 1). Living cells were 16.9 ± 3.0 (14.1–25.0) µm long and 5.2 ± 1.1 (3.8–7.8) µm wide, with length/width ratio ca. 3.4 ± 0.8 (*n* = 11). The flagellum was 25.6 ± 7.4 (15.6–39.1) µm long (*n* = 8), with the ratio of flagellar length/cell length being ca. 1.7 ± 0.5. The single flagellum did not beat as fast as in *R. elongata* sp. nov. (see below). The movement of actively swimming cells was relatively fast, and a bulbous uroid was occasionally formed. Thorn-like pseudopodia were produced in the anterior and the posterior hyaline end of the cell during swimming (Fig. 1B, C, F). Trophozoites possessed a single, rounded nucleus, which was situated in the centre of elongated cells. Some crawling cells, with the flagellum emerging from the anterior hyaloplasm, were also present (Fig. 1A). Occasionally, cells produced fine pseudopodia (not shown). The rhizostyle was sometimes visible in the hyaline, anterior end of the cell (Fig. 1A). A few aflagellated cells were observed in the culture (Fig. 1D). A layer of hyaline, lobate pseudopodia was produced around the outside of the cell during amoeboid movement. Cysts were rarely present in the culture (Fig. 1E). They were rounded and possessed two nuclei. The rhizostyle was not observed in cysts.

Protargol-stained cells were smaller than living cells, usually oval and rounded, rarely elongated (Fig. 1G–I). They were 9.3 ± 3.1 (6.1–18.3) µm long and 6.3 ± 1.0 (4.4–8.5) µm wide, with length/width ratio 0.7 ± 0.2 (*n* = 30). The flagellum was 15.6 ± 7.1 (5.9–27.1) µm long (*n* = 30), and the ratio between the length of the flagellum and cell was 1.7 ± 0.8. They sometimes showed the anterior or posterior crown-like pseudopodia (Fig. 1I). In the protargol-stained cells, the rhizostyle was sometimes visible (Fig. 1G, I), as well as a large central nucleolus and peripheral chromatin in the nucleus (Fig. 1G, H). The rhizostyle arose from the flagellar base and ran posteriorly through the cell, beyond the nucleus (Fig. 1G).

Rhizomastix vacuolata sp. nov.

Living cells of strain GOL1 were amoeboid, sometimes rounded or elongated (Fig. 2A–G) with highly vacuolated cytoplasm (Fig. 2A–D, F, G). The cells were 14.7 ± 2.8 (10.6–21.2) µm long and 7.4 ± 2.3 (4.1–14.0) µm wide, with length/width ratio 2.2 ± 0.9 (*n* = 30), and usually equipped with a single flagellum, which was 19.5 ± 5.4 (7.7–31.1) µm long (*n* = 30). The ratio between the length of the flagellum and cell was 2.2 ± 0.9. Aflagellated and biflagellated cells were also rarely observed (not shown). Crawling cells that formed small pseudopodia around the whole body predominated in the culture (Fig. 2B, C, G). A fasciculate uroid (see Smirnov and Brown 2004) was occasionally formed during slow amoeboid locomotion (Fig. 2B). The direction of movement was not easily determined because the cells were often highly amoeboid. Swimming cells sometimes appeared in large numbers (Fig. 2F). They moved very quickly, using the flagellum. A bulbous uroid was sometimes visible (Fig. 2F). A single, rounded nucleus with a clearly recognizable nucleolus was most often situated in the central or slightly anterior part of the elongated cell (Fig. 2A, F), or was very rarely located posteriorly (Fig. 2D). Cysts with two nuclei were rarely observed in the culture (Fig. 2E). The rhizostyle was neither visible in living cells nor in cysts.

Protargol-stained cells were rounded or oval, sometimes with conserved amoeboid shape (Fig. 2H–J). They were 6.7 ± 1.4 (2.5–8.7) µm long and 5.6 ± 1.2 (2.7–8.2) µm wide, with length/width ratio 1.2 ± 0.4 (*n* = 30). The flagellum was 14.2 ± 5.0 (6.5–24.8) µm long (*n* = 30), and the ratio between the length of the flagellum and cell was 1.2 ± 0.4. The rhizostyle was not visible in stained cells. The single nucleus contained a large nucleolus, and what appeared to be heavily stained peripheral chromatin. The conspicuous vacuoles seen in living cells were also visible in many stained cells (Fig. 2I, J).

Strain GOL18 was lost before its morphology could be examined in detail, but it was similar to GOL1.

Rhizomastix elongata sp. nov.

The strain VAVRH consisted almost exclusively of flagellated cells, which were 27.6 ± 4.9 (19.7–37.8) µm long and 3.2 ± 0.5 (2.2–4.0) µm wide, with length/width ratio 8.7 ± 1.5 (*n* = 30), and with a single flagellum 15.1 ± 2.8 (10.1–19.5) µm long (*n* = 30). The ratio between the length of the flagellum and cell was 0.9 ± 0.1. The flagellar beat was of low amplitude, moving the cell quickly and jerkily. Cells with more than one flagellum were not observed. Swimming cells were long, very thin, and usually curved (Fig. 3A). The single nucleus was typically situated behind the hyaline anterior of the cell, in the middle or slightly in the posterior of the cell body (Fig. 3A, B, D). The rhizostyle was sometimes visible in the hyaline anterior of the living cell (Fig. 3A, D). A bulbous uroid (Fig. 3A), villous-bulbous uroid (Fig. 3D) or uroidal filament (Fig. 3B) was frequently present. As cells started to crawl, they were only slightly elongated, rather than being extremely long and thin like swimming cells. These crawling cells and fully spread cells often produced fine pseudopodia

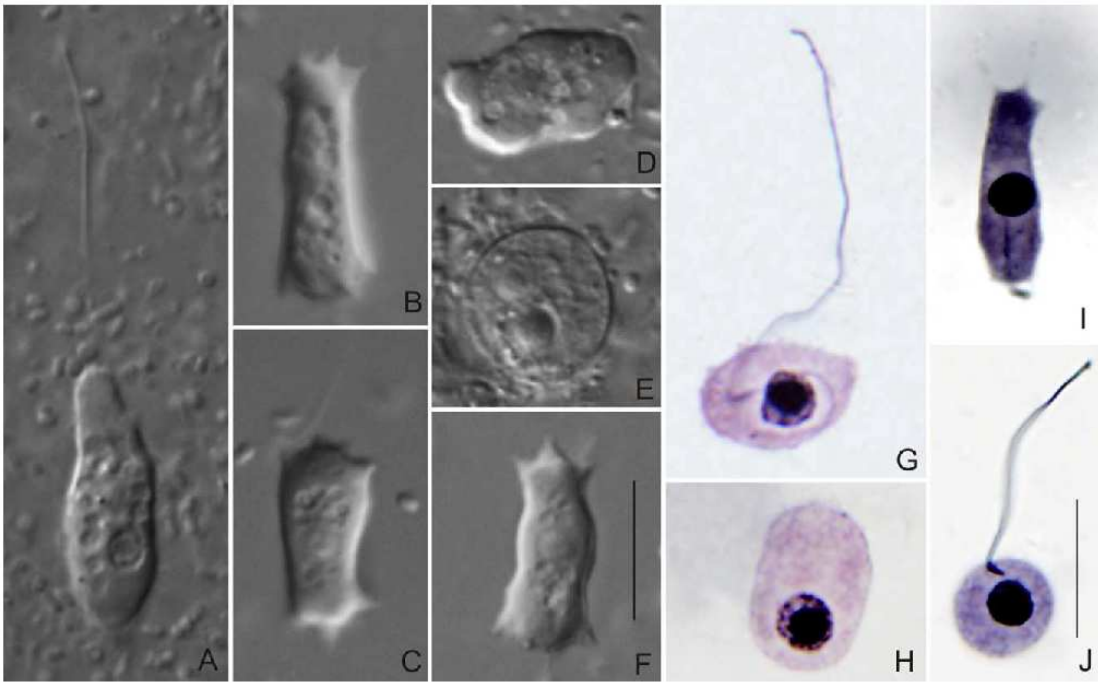

Figure 1 Light-microscopical morphology of *Rhizomastix bicoronata* sp. nov. strain VELKA1. **A.** Gliding cell. **B, C, F.** Elongated swimming cells. **D.** Aflagellated crawling cell. **E.** Binucleated cyst. **G–J.** Protargol-stained cells. Scale bar in (F, J) = 10 μm. DIC (A–F) or bright field (G–J).

(Fig. 3E). Binucleated cysts without a visible rhizostyle were rarely observed in culture (Fig. 3C).

Protargol-stained cells were rounded or elongated, and were 9.9 ± 3.7 (4.4–18.0) μm long and 3.2 ± 1.0 (1.2–6.5) μm wide, with length/width ratio 4.7 ± 2.8 ($n = 30$). The flagellum was 15.8 ± 3.7 (9.6–24.3) μm long ($n = 30$), and the ratio between the length of the flagellum and cell was 1.9 ± 0.4. There was a visible rhizostyle that often ended approximately in the anterior or central part of the cell (Fig. 3G–J) or, sometimes, continued behind the nucleus to the posterior end of the cell (Fig. 1F). The cells possessed a heavily stained nucleus, meaning that peripheral chromatin could not be seen (Fig. 3F–J). A semi-circular structure that attached the nucleus was present in some cells. Other presumably microtubular structures rarely occurred around the nucleus (Fig. 3J).

Rhizomastix libera Ptáčková et al., 2013

Morphology of two strains, IND8MA (the type strain of this species; Ptáčková et al. 2013) and OLB6AN, was examined. Living cells of strain OLB6AN were elongated or amoeboid, sometimes with tiny pseudopodia in the anterior part (Fig. 4F, H, I, K, L). They were 11.0 ± 3.0 (5.7–19.8) μm long and 5.6 ± 1.3 (3.1–8.4) μm wide, with length/width ratio 2.2 ± 1.2 ($n = 30$), and usually had a single flagellum, which was 15.7 ± 5.3 (8.8–30.5 μm long ($n = 30$). The ratio between the length of the flagellum and cell was 1.5 ± 0.6. Flagellar movement was relatively slow. Cells with two flagella were occasionally observed. A villous-bulbous uroid was occasionally formed in the posterior part of the cell (Fig. 4I, L). During amoeboid movement, which was faster than in *R. varia* sp. nov., filose pseudopodia were formed (Fig. 4A–C). The nucleus was situated in the central or anterior part of the cell, behind the anterior hyaloplasm (Fig. 4A–D). Protargol-stained cells were mostly rounded, 4.6 ± 1.6 (3.0–10.5) μm long and 3.1 ± 0.6 (1.8–4.2) μm wide, with length/width ratio 1.6 ± 1.0 ($n = 30$); tiny pseudopodia (Fig. 4M) were sometimes present. The flagellum sometimes had fine projections on its surface (Fig. 4N), and was 13.1 ± 5.8 (5.4–37.7) μm long ($n = 30$), with the ratio between the length of the flagellum and cell 2.9 ± 0.8. The rhizostyle was short and inconspicuous (Fig. 4O). The nucleus was rounded without visible internal structure (Fig. 4F–H). Cysts were not observed.

Living cells of strain IND8MA were amoeboid or elongated, with a fasciculate uroid (Fig. 5A, B) or uroidal filaments (Fig. 5C, D) in the posterior part. Swimming cells were occasionally observed (Fig. 5A, B), but their movement was not as fast and jerky as in the cells described previously (Ptáčková et al. 2013). Amoeboid and crawling cells predominated over swimming cells. A single flagellum was present; the nucleus was usually situated in the central part of the cell, behind the anterior hyaline zone

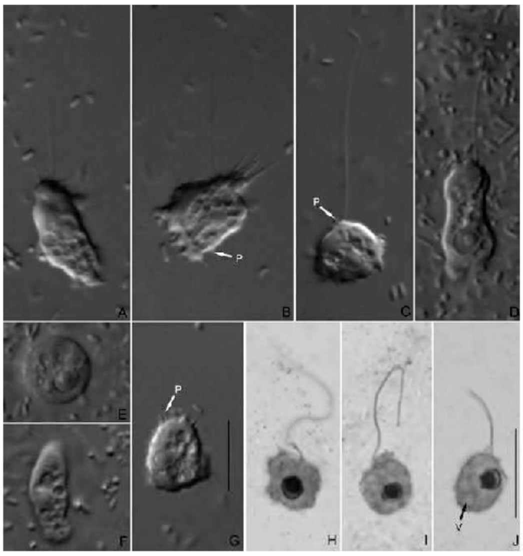

Figure 2 Light-microscopical morphology of *Rhizomastix vacuolata* sp. nov. strain GOL1. **A, C, D.** Gliding cells. **B, G.** Crawling cells. **E.** Binucleated cyst. **F.** Swimming cell. **H–J.** Protargol-stained cells. Scale bar in (G, J) = 10 μm. DIC (A–G) or bright field (H–J). P – small pseudopodia; U – fasciculate uroid; V – vacuole.

(not shown). The rhizostyle was sometimes visible in living cells (Fig. 5B). Filose pseudopodia were often formed in the anterior part of elongated cells (Fig. 5C) or around the whole surface in amoeboid cells (Fig. 5E–H). Protargol-stained preparations were not examined.

Rhizomastix varia sp. nov.

Crawling cells of the strain FBAN were often amoeboid, and rarely elongated. They were 11.4 ± 3.5 (7.0–19.3) μm long and 5.2 ± 1.3 (3.0–8.9) μm wide, with length/width ratio 2.4 ± 1.2 (*n* = 30), and usually had a single flagellum, which was 12.9 ± 3.1 (5.1–21.4) μm long (*n* = 30). The ratio between flagellar and cell length was 1.2 ± 0.4. Cells moved very slowly, often using anterior pseudopodia (Fig. 6A–G, I–L). Many spiny filopodia were formed over the surface of the cell during amoeboid movement (Fig. 6A–E, G, J, L). Movement appeared undirected, and the single flagellum was not anchored at a fixed point but moved fluently around the cell. Amoeboid cells attached to the substrate by well-developed ramified pseudopodia (Fig. 6K). The flagellum arose from a hyaline neck; it was inconspicuous, short and thick, without distinctive movement; it did not beat but flopped (Fig. 6D, G, J, L). The rhizostyle was not visible. The cells possessed a single nucleus, oriented anteriorly, or in the central part of the cell behind a relatively indistinct hyaline zone. Actively swimming cells were relatively common in fresh cultures (Fig. 6H), but were very rare after the strain had been in culture for several years. They were elongated, without any conspicuous pseudopodia, and showed the typical quick and jerky movement of *Rhizomastix*. Cysts were not observed.

Protargol-stained cells were elongated, sometimes with fine pseudopodia around the anterior end of the cell (Fig. 6N). The cells were 4.6 ± 1.7 (2.8–8.8) μm long and 2.7 ± 0.5 (1.4–3.6) μm wide, with length/width ratio 1.8 ± 0.9 (*n* = 30). The flagellum was 10.1 ± 3.0 (5.5–17.7) μm long (*n* = 30), and the ratio between flagellar and cell length was 2.4 ± 1.0. The rhizostyle was clearly visible and extended from the base of the flagellum to the posterior end of the cell (Fig. 6M, N). The flagellum was thick and appeared to be covered by short, fine projections (Fig. 6M–O), which may be consistent with the paraflagellar vanes seen in *R. libera* (Ptáčková et al. 2013). The internal structure of the nucleus was not discernible (Fig. 6M–O). Cysts were not observed under light microscope.

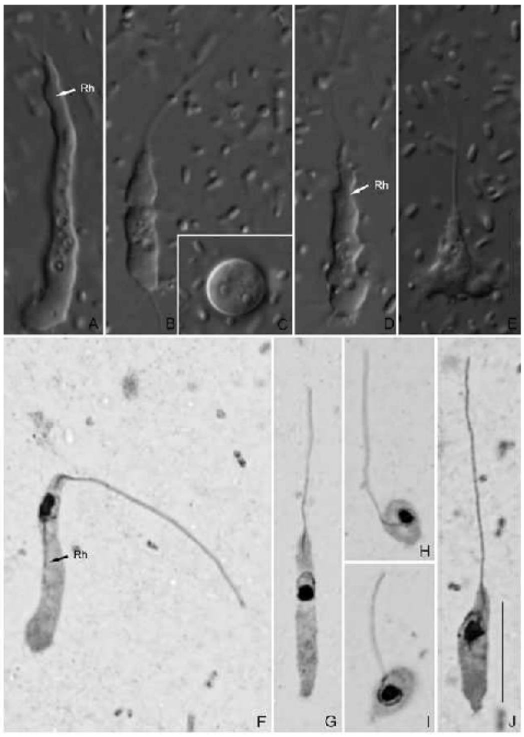

Figure 3 Light-microscopical morphology of *Rhizomastix elongata* sp. nov. strain VAVRH. **A.** Extremely long and thin swimming cell. **B.** Elongated gliding cell. **C.** Binucleated cyst. **D.** Elongated swimming cell. **E.** Crawling cell. **F–J.** Protargol-stained cells. Scale bar in (E, J) = 10 μm. DIC (A–E) or bright field (F–J). Rh – rhizostyle.

Transmission electron microscopy of *R. elongata* sp. nov.

Transmission electron microscopy showed elongated cells with a flagellum, microtubular flagellar apparatus composed of a rhizostyle and two microtubular roots, a

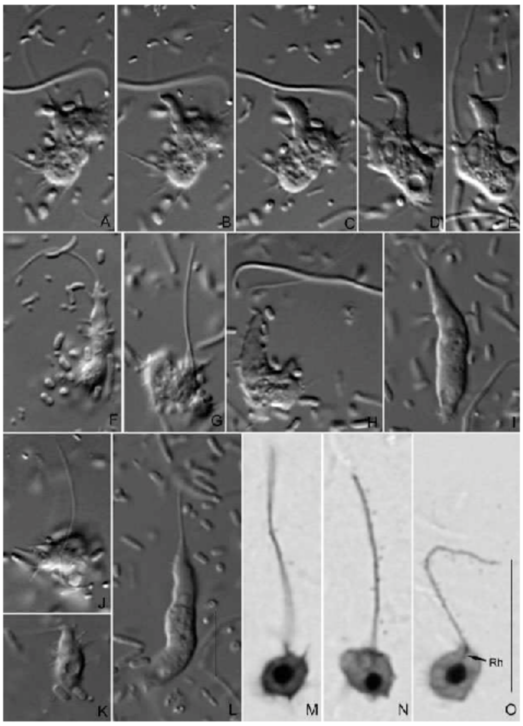

Figure 4 Light-microscopical morphology of *Rhizomastix libera* strain OLB6AN. **A–E, G, H, J.** Crawling cells. **F, I, K, L.** Gliding cells. **M–O.** Protargol-stained cells. Scale bar in (L, O) = 10 μm. DIC (A–L) or bright field (M–O). Rh – rhizostyle.

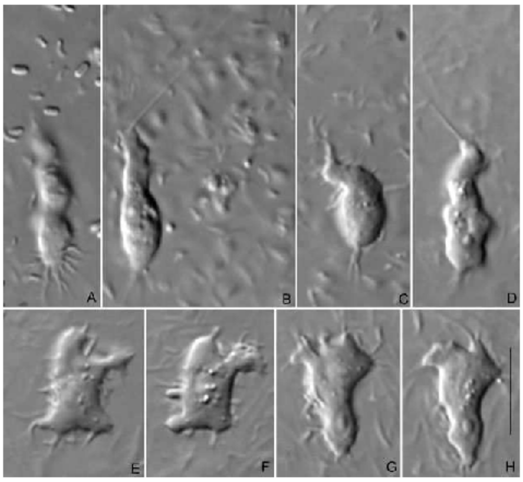

Figure 5 Light-microscopical morphology of *Rhizomastix libera* strain IND8. **A, B.** Swimming cells. **C, D.** Gliding cells. **E–H.** Crawling cells. Scale bar = 10 μm. DIC (A–H).

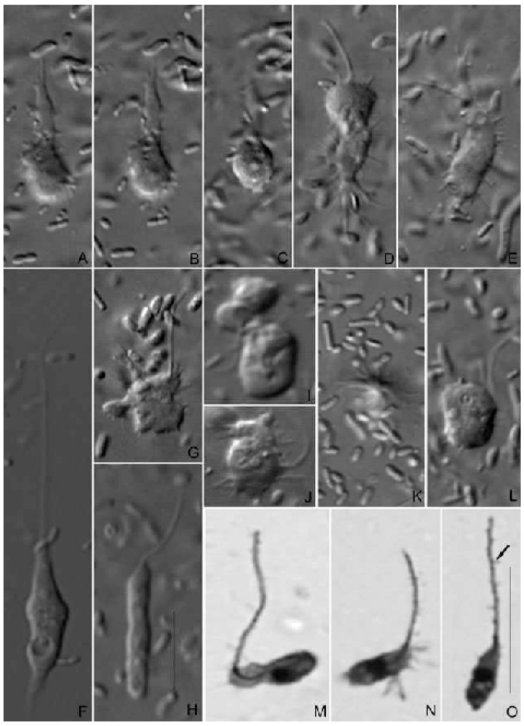

Figure 6 Light-microscopical morphology of *Rhizomastix varia* sp. nov. strain FBAN. **A–C, F.** Gliding cells. **D, E, G, I–L.** Crawling cells. **H.** Elongated swimming cell. **M–O.** Protargol-stained cells. Scale bar in (H, O) = 10 μm. DIC (A–L) or bright field (M–O). Arrow shows fine projections on the surface of the flagellum.

nucleus with a highly characteristic membrane structure, food vacuoles and endoplasmic reticulum. The longitudinal axis of the cell is defined as running from the apical flagellar apparatus through the nucleus; and the large flagellar root is defined as running laterally to the right.

In axial longitudinal sections (Fig. 7A), the body was elongated but amoeboid, with no theca or cytoskeletal structures supporting the cell membrane. There was a posterior or central nucleus, which had electron-dense chromatin distributed through it, and a central nucleolus (Fig. 7B, C). The nuclear envelope showed a characteristic pocket or loop structure, anteriorly, which was present in all cells examined (Fig. 7B, C; 8B–E); the nuclear envelope was closely surrounded by a single layer of endoplasmic reticulum, which also surrounded both sides of the loop structure. Stacked endoplasmic reticulum was present posteriorly in the cell (Fig. 7C, F). The cytoplasm contained food vacuoles (Fig. 7A, C); and small, cylindrical, electron-dense, double-membrane-bound organelles (Fig. 7E; 8A, E), about 250 nm long and 50 nm in diameter, characteristic of mitochondrial remnant organelles seen in other Archamoebae. Cysts were present (Fig. 7D) and contained remnants of the flagellar apparatus.

The flagellar apparatus (Fig. 8A–K) consisted of a flagellum, a single basal body, a flagellar root of nine microtubules, a doublet flagellar root of two microtubules, and a rhizostyle of 11 microtubules extending proximally into the cell. The fine structure of the flagellar axoneme, transition zone and basal body were not determined in detail. The basal body was single in every cell observed, and appeared to have a normal triplet structure (Fig. 8H). In the transition zone, a transitional spiral similar to that seen in *Mastigamoeba* sp. by Brugerolle (1991), and a transitional cylinder, similar to those seen by Walker et al. (2001), were both visible (Fig. 9).

The rhizostyle was a semi-circular root of eleven microtubules, which initiated on either the bottom corner or slightly below the bottom edge of the basal body (Fig. 8H); it extended proximally from the base of the basal body (Fig. 8A–F) surrounding electron-dense material for the first ca. 150 nm (Fig. 8I–K), and then for ca. 1 μm enclosed vesicles that were ultrastructurally similar to acidocalcisomes (Fig. 8A, D, F). The rhizostyle extended posteriorly into the cell, tapering sharply at its endpoint (Fig. 8A), with five microtubules being the fewest seen (Fig. 8G). In each of the rhizostyle and the flagellar root, the microtubules appeared to be interconnected by fine fibrils (Fig. 8H–K). Similar fine fibrils joined the microtubules of the rhizostyle to the basal body (Fig. 8H), and the microtubules of both the rhizostyle and the flagellar root to the electron-dense material below the basal body (Fig. 8I). Further serial sectioning would be required to show how far along the rhizostyle or root the fibrils extend.

A root of nine microtubules emerged laterally from the side of the basal body and extended posteriorly into the cell, initially close to the rhizostyle but posteriorly more separate from it (Fig. 8A–F, I–K). A second root of two microtubules (the "doublet root") emerged at the base of the basal body (Fig. 8J, K) and extended posteriorly into

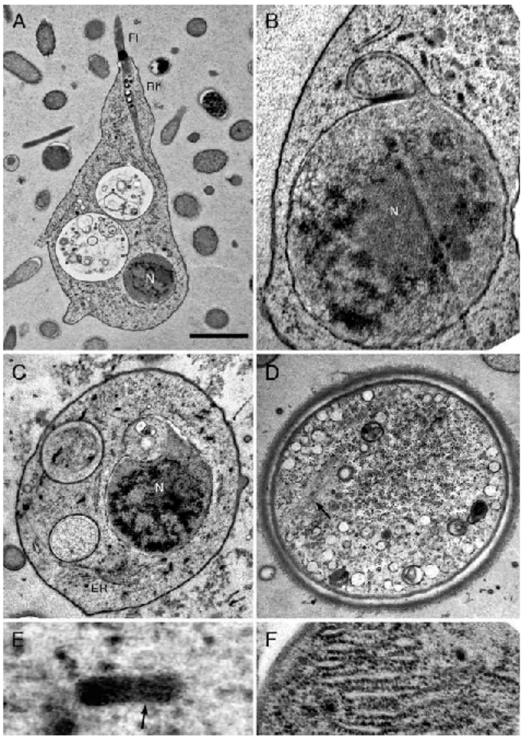

Figure 7 Ultrastructure of *Rhizomastix elongata* sp. nov. strain VAVRH. **A.** Longitudinal section of the cell, showing single nucleus, vacuoles, and flagellar apparatus with rhizostyle. **B.** Nucleus with nuclear pocket. **C.** Section through cell, showing the nucleus with the central nucleolus and nuclear loop. **D.** Cyst with the cyst wall. Arrow shows remnant of the flagellar apparatus. **E.** Mitochondrion-related organelle. **F.** Detail of the endoplasmic reticulum. ER = endoplasmic reticulum; Fl = flagellum; N = nucleus; Rh = rhizostyle. Scale bar 2 μm for (A), 500 nm for (B), 1 μm for (C, E), 1.25 μm for (D), 250 nm for (F).

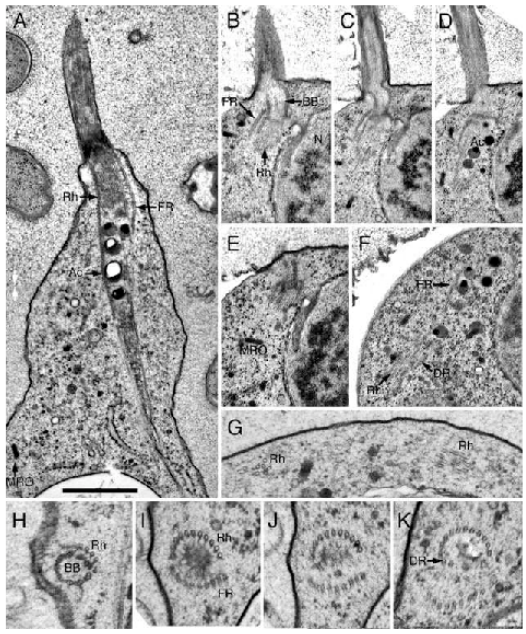

Figure 8 Ultrastructure of *Rhizomastix elongata* sp. nov. strain VAVRH. **A.** Compound of two pictures showing flagellar apparatus with the flagellum, rhizostyle and flagellar root. The join between the two pictures is shown with white arrows. **B–F.** Serial longitudinal sectioning through the flagellar apparatus, showing mutual position of the flagellum, basal body, rhizostyle and two flagellar roots. **G.** Section through the rhizostyle along the cell. **H–K.** Serial transversal sectioning through the flagellar apparatus, showing mutual position of the basal body, rhizostyle and two flagellar roots. Ac = acidocalcisome-like body; BB = basal body; DR = doublet root; FR = flagellar root; MRO = mitochondrion-related organelle; N = nucleus; Rh = rhizostyle. Scale bar 1 μm for (A–G), 500 nm for (H–K).

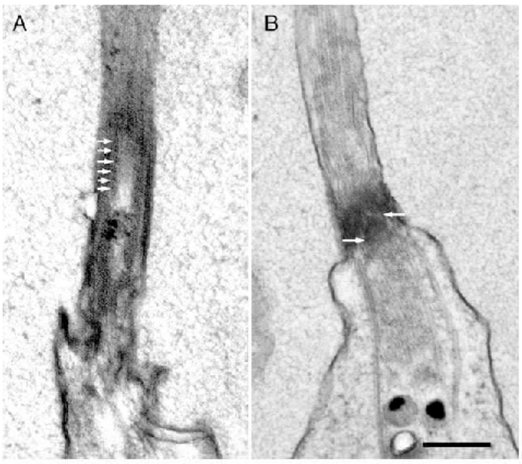

Figure 9 Transition zone structures in *Rhizomastix elongata*. **A.** Transition zone spiral (arrowheads) within the nine microtubular doublets of the axoneme, similar to that seen in *Mastigamoeba* sp. (Brugerolle 1991). **B.** Transition zone cylinder (upper and lower edges shown with arrowheads), similar to that seen previously in *Mastigamoeba* and *Mastigella* (Walker et al. 2001). Scale bar 200 nm for (A), 300 nm for (B).

the cell, enclosed by the rhizostyle, sometimes appearing as a "central pair" as would be seen in a hypothetically inverted flagellum. Its endpoint was not determined.

Phylogenetic analyses

We determined the SSU rRNA gene sequences of six strains belonging to four *Rhizomastix* species. The phylogenetic tree of Amoebozoa as inferred from SSU rRNA gene sequences is shown in Fig. 10. Archamoebae were robustly monophyletic. Four main Archamoebae lineages, Mastigamoebidae, Pelomyxidae, Rhizomastixidae and Entamoebidae, which had been identified in previous studies (Ptáčková et al. 2013; Zadrobílková et al. 2015), were recovered and statistically supported (99/1; 92/1; 91/1; 100/1). Rhizomastixidae formed a sister branch of Entamoebidae with medium support (79/1). Mastigamoebidae and Pelomyxidae appeared closely related, but the relationship was unsupported. *Rhizomastix*, the only genus of Rhizomastixidae, split into two robust clades. The first one comprised three strains of *R. libera* and an uncultured eukaryote (GenBank sequence GU921236). Strains BOTANKA and OLB6AN were closely related; the type strain of *R. libera*, IND8MA, was closely related to the uncultured eukaryote indicating that the latter belongs to this species as well. The second lineage of *Rhizomastix* consisted of *R. elongata* sp. nov., *R. bicoronata* sp. nov., and *R. vacuolata* sp. nov. Two strains of *R. vacuolata* sp. nov. formed a clade, which was closely related to *R. bicoronata* sp. nov.

We also determined the actin gene sequences of six strains belonging to four *Rhizomastix* species. The phylogenetic tree of Amoebozoa as inferred from actin gene sequences is shown in Fig. 11. Although Archamoebae formed a clade, its monophyly was unsupported, similarly to our previous study (Zadrobílková et al. 2015). Pelomyxidae (*Pelomyxa* + *Mastigella*) formed a robust clade. Mastigamoebidae A (*Mastigamoeba balamuthi*) and B (*M. abducta*, *Mastigamoeba* sp.) did not form a common clade. Instead, the latter was closely related to *Entamoeba*, though without any statistical support. Genus *Rhizomastix* appeared monophyletic, but without support. Two sequences of strain FBAN *Rhizomastix varia* sp. nov. formed an unsupported clade that was sister to the rest of *Rhizomastix*. Four strains of *R. libera* formed a clade that was statistically supported in the maximum-likelihood analysis, but not in the Bayesian analysis. *Rhizomastix bicoronata* sp. nov. was closely related to *R. elongata* sp. nov. Despite some clones of the actin gene of *R. elongata* sp. nov. forming relatively long branches, all sequences belonging to this species formed a well-supported clade.

DISCUSSION

Species diversity in the genus *Rhizomastix*

Prior to this study, eleven *Rhizomastix* species, both free-living and endobiotic, had been described. The endobiotic species were isolated from insects, namely crane flies, cockroaches and mole crickets (*R. dastagiri*, *R. gracilis*, *R. gryllotalpae*, *R. murthii*, *R. periplanetae*) (Bhaskar Rao 1963, 1970; Ludwig 1946; Mackinnon 1913; Mali et al. 2002; Sultana 1976), amphibians (*R. biflagellata*, *R. gracilis*, *R. ranae*) (Alexeieff 1911; Cepicka 2011; Jiménez et al. 2001; Krishnamurthy 1969) and reptiles (*R. scincorum*) (Bovee and Telford 1962; Cavalier-Smith and Scoble 2013). *Rhizomastix hominis* was isolated from human faeces (Yakimoff and Kolpakoff 1921). Two species, *R. borealis* and *R. libera*, were obtained from freshwater environments and are considered free-living (Ptáčková et al. 2013; Zhang and Yang 1990). Most species descriptions were based almost exclusively on the morphology of stained cells, and the morphology of living organisms was neglected. The exceptions are the two cultured species, *R. biflagellata* and *R. libera*, whose living cells were observed for a long period (Cepicka 2011; Ptáčková et al. 2013). Moreover, all species except for *R. gracilis* were isolated only once and have not been reported again after the original description. *Rhizomastix* species differ morphologically from each other in cell shape and size, length of the flagellum, and thickness and length of the rhizostyle.

Rhizomastix gracilis was originally described by Alexeieff (1911) from an axolotl. Later, the species was reported from larvae of crane flies (Ludwig 1946; Mackinnon 1913). *Rhizomastix gracilis* thus has the broadest host range within the genus. However, Alexeieff's and Mackinnon's descriptions of trophozoites of *R. gracilis* were rather brief, and it is difficult to compare them with other studies. Nevertheless, based on our observations of *Rhizomastix*, we believe that the organism observed by

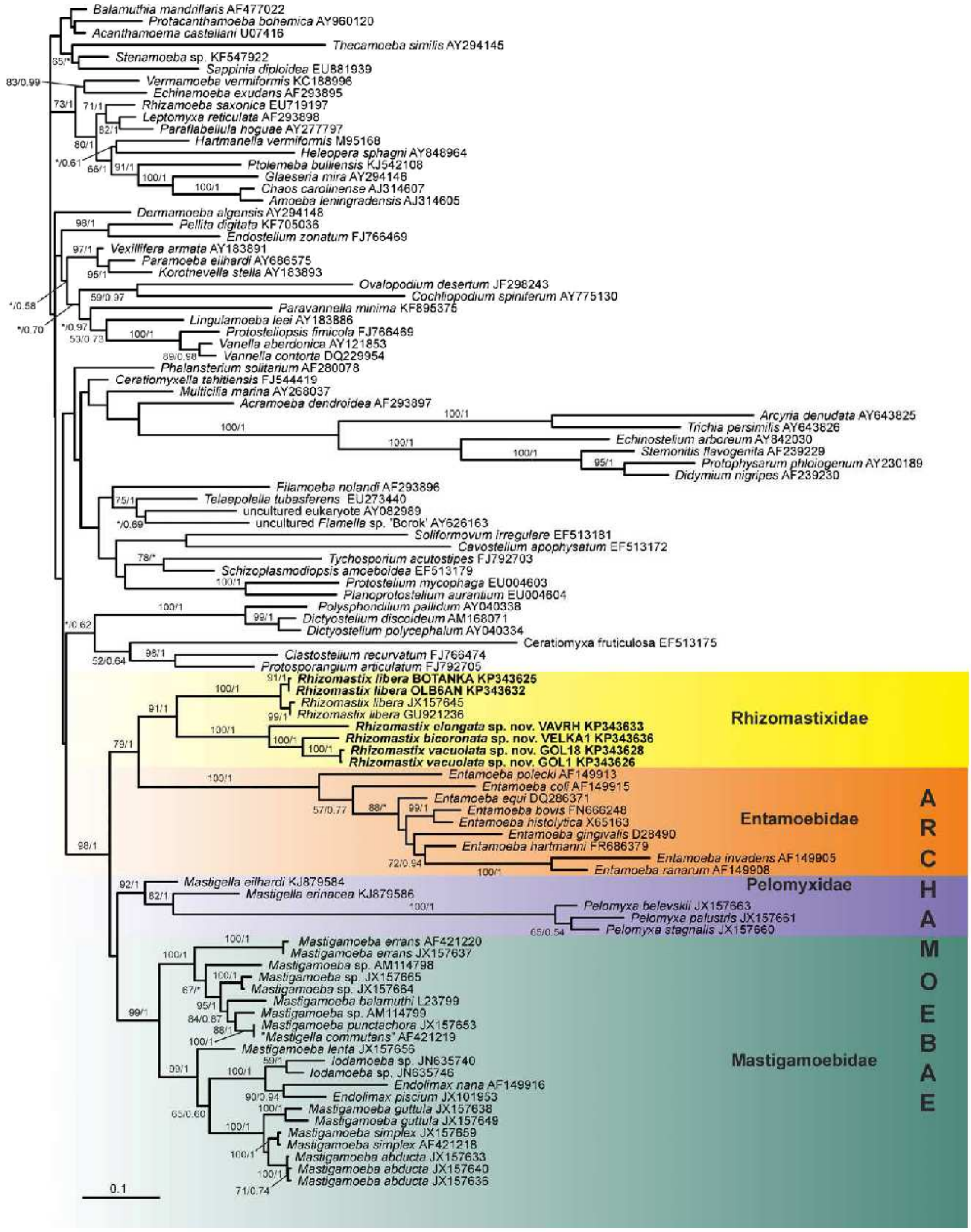

Figure 10 Phylogenetic tree of Amoebozoa based on SSU rRNA gene sequences. The tree was constructed by the maximum-likelihood method (GTRGAMMAI model). The values at the nodes represent statistical support in maximum-likelihood bootstrap values/Bayesian posterior probabilities. Support values below 50%/0.50 are not showed or are represented by an '*'. New sequences are in bold.

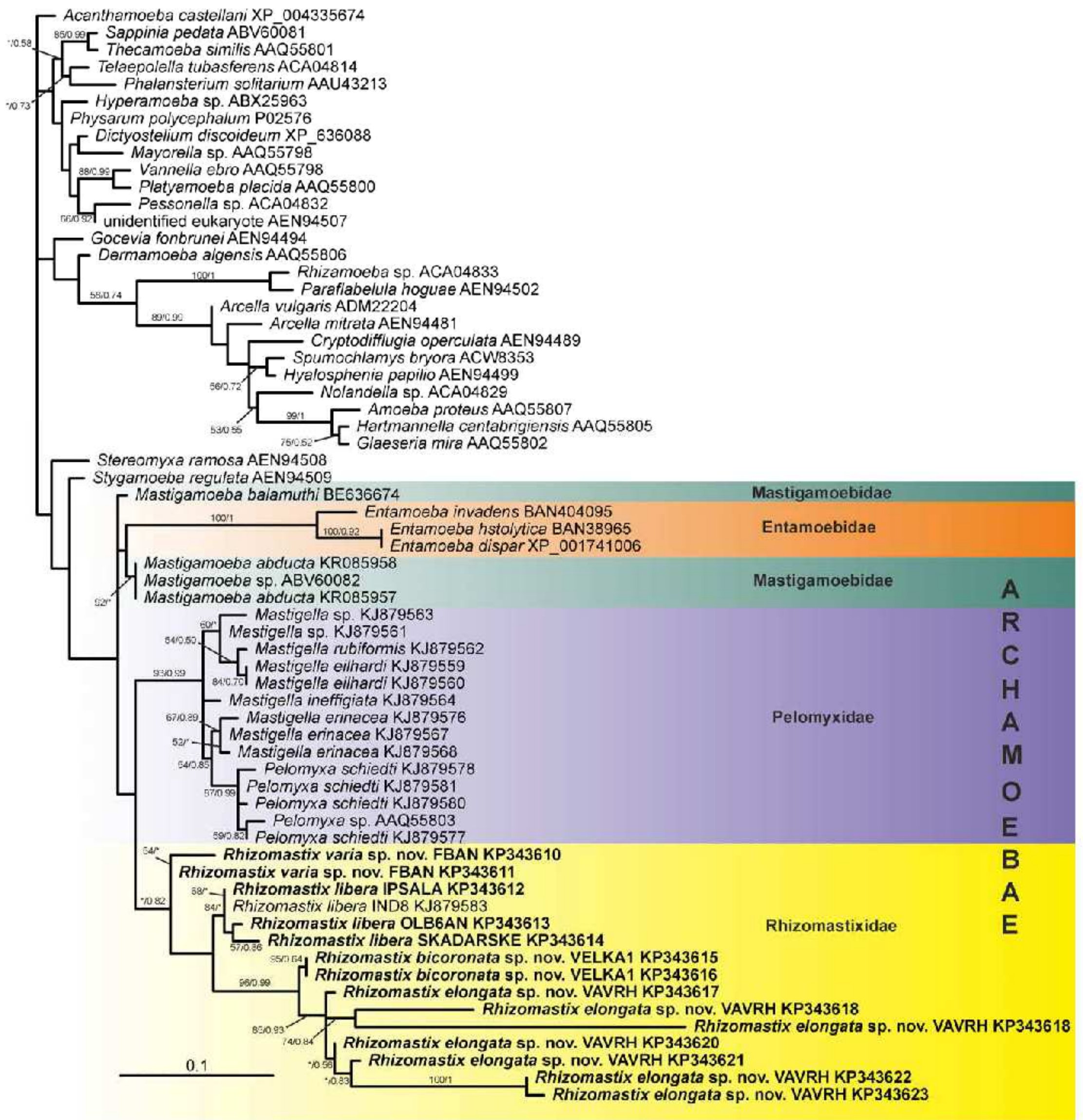

Figure 11 Phylogenetic tree of Amoebozoa based on actin gene sequences. The tree was constructed by the maximum-likelihood method (PROTGAMMAILG model). The values at the nodes represent statistical support in maximum-likelihood bootstrap values/Bayesian posterior probabilities. Support values below 50%/0.50 are not shown or are represented by an '*'. New sequences are in bold.

Ludwig (1946) is different from *R. gracilis*—it was much more elongated and the flagellum and rhizostyle were shorter relative to the cell body—and consider it a separate species, here described as *Rhizomastix tipulae* sp. nov. The species identity of the organism observed by Mackinnon (1913) remains uncertain, and it is thus impossible to assess the true host range of *R. gracilis*.

In this study, we have cultured nine new strains of *Rhizomastix*. Three of them were endobiotic, being isolated from beetle larvae and a millipede. Five strains were isolated from freshwater sediments, and a single strain was isolated from a cesspit. Based on the presence of rhizostyle, typical jerky movement and phylogenetic position the strains were assigned to the genus *Rhizomastix*. After careful examination of the morphology, we conclude that the majority of them represent novel, morphologically distinct species.

The distinguishing feature of *Rhizomastix bicoronata* sp. nov. are the conical pseudopodia present at both ends of the cell body of swimming cells. The presence of pseudopodia was briefly mentioned in descriptions of some species (Bhaskar Rao 1963; Bovee and Telford 1962; Cepicka 2011; Krishnamurthy 1969; Ptáčková et al. 2013), but they were usually present only on crawling cells and have never been reported to be thick and conical. In addition, the pseudopodia of *R. bicoronata* sp. nov. are uniquely arranged in crown-like patterns, a feature not reported in any *Rhizomastix* species.

Rhizomastix vacuolata sp. nov. from the larvae of a Goliath beetle is morphologically most similar to *R. murthii* isolated from a cockroach *Periplaneta americana* by Mali et al. (2002). Both species are ovoid with the flagellum being approximately twice as long as the cell body. However, the cells of *R. vacuolata* sp. nov. are considerably smaller than that of *R. murthii* (3–9 μm long vs. 7–16 μm), and the rhizostyle is almost invisible in stained cells of the new species, whereas it was reported to be delicate but clearly visible in *R. murthii*. The cells of *R. vacuolata* sp. nov. also appear much more vacuolated than the cells of the other *Rhizomastix* species.

The only available strain of *R. elongata* sp. nov. was isolated from organic sediment of an abandoned cesspit. Its phylogenetic affinity supports the possibility that it is, in fact, endobiotic (with unknown host) or that it has reverted recently from an endobiotic to a coprozoic/free-living lifestyle. As *R. elongata* sp. nov. thrives in culture media for endobiotic protists, and it is closely related to the clade of endobiotic species of *Rhizomastix*, the former alternative seems to be more probable. As larvae of hoverflies and numerous mosquitoes did have contact with the cesspit, but no vertebrate hosts had access into it, it is possible, that the natural habitat of this species may be the intestine of an insect. *Rhizomastix elongata* sp. nov. differs morphologically from *R. hominis* isolated from human faeces by Yakimoff and Kolpakoff (1921). Its cells are larger (20–38 μm vs. 12–15 μm in living conditions) and much more elongated. Also, the movement of the new species is much faster. *Rhizomastix elongata* sp. nov. is morphologically most similar to *R. tipulae* sp. nov. (see above); the cells of both species are markedly elongated. However, the rhizostyle of *R. elongata* extends beyond the nucleus, whereas in *R. tipulae* sp. nov. it is often limited to the prenuclear part of the cell. The cells of *R. elongata* sp. nov. are considerably longer than the cells of *R. tipulae* sp. nov.—the elongated ones are almost always longer than 10 μm when stained, whereas Ludwig (1946) reported 3.7–10 μm for the latter species (the stated sizes of cells, however, do not correspond with the bar in Plate I in Ludwig 1946). The nucleus of *R. elongata* sp. nov. is situated more anteriorly (usually in the anterior half of the cell) than that in *R. tipulae* sp. nov. (which is in the middle of the cell). *Rhizomastix elongata* sp. nov. differs markedly from both free-living species, *R. libera* and *R. borealis*, in both cell shape and diameter.

Strain OLB6AN was assigned to *R. libera*, although it is much more amoeboid than the type strain (IND8MA) as reported by Ptáčková et al. (2013). In order to compare the morphology of the two strains in detail, we examined strain IND8MA again and realized that its morphology has changed slightly since its original description, which is characteristic of cells kept in continuous culture for some years. Currently, amoeboid cells predominate in the culture of IND8MA, similarly to OLB6AN. *Rhizomastix libera* is now represented by five cultured strains isolated from India (Ptáčková et al. 2013) and various European countries (this paper), and a single environmental sequence GU921236 obtained from activated sludge. It seems that this species is widespread and common in freshwater environments.

Rhizomastix varia sp. nov. was isolated from an environment similar to *R. libera*, and their overall morphology is similar, but the two species differ in some aspects. The cells of *R. varia* sp. nov. produce multiple pseudopodia all over the surface, and the flagellar base does not occupy a stable position and moves freely around the cell body. In addition, the flagellum does not beat as frequently as in *R. libera*, only flopping very slowly. The existence of *R. varia* sp. nov. as a species separate from *R. libera* is further supported by our actin gene analysis. *Rhizomastix varia* sp. nov. also differs in its morphology from another *Rhizomastix* species isolated from freshwater sediment, *Rhizomastix borealis*. This species does not display any of the features mentioned above.

Phylogeny of *Rhizomastix* and origin of parasitism within Archamoebae

The phylogenetic position of the genus *Rhizomastix* has long been uncertain, and several incompatible hypotheses were formulated on the basis of light-microscopic morphology in the first half of the 20th century (see Cepicka 2011). Considering the fact that members of the genus *Rhizomastix* are intestinal symbionts of various animals, the first sequence data of this genus became available surprisingly recently (Ptáčková et al. 2013). SSU rRNA gene analysis of a single species, *R. libera*, clearly showed that *Rhizomastix* is an archamoeba. A similar result was obtained by subsequent analysis of the actin gene, including the same *Rhizomastix* species, though relationships within the archamoebae as well as its monophyly were generally unsupported (Zadrobílková et al. 2015). Importantly, Ptáčková et al. (2013) showed that *Rhizomastix* might be a close relative of the parasitic genus *Entamoeba*, though the statistical support for this interpretation was low. Another interesting aspect of *Rhizomastix* is that it comprises both endobiotic and free-living species. As data have only been available from a free-living species prior to this study, it could not hitherto be excluded that free-living *Rhizomastix* species are not specifically related to the endobiotic ones, i.e. that the genus is nonmonophyletic. Here, we have added four additional *Rhizomastix* species into the phylogenetic analysis. The results of SSU rRNA gene analysis strongly suggest that *Rhizomastix* indeed is monophyletic. Our data also support a close relationship between *Rhizomastix* and *Entamoeba*. The sister relationship seems to be supported by similar nuclear ultrastructure. In both genera, the cells often have nuclei possessing a central nucleolus and peripheral chromatin granules. However, it is worth noting that a similar arrangement of heterochromatin has also been observed in *Mastigella rubiformis* and *P. schiedti* (Zadrobílková et al. 2015). It is almost certain that phylogenetic relationships between the main lineages of archamoebae cannot be satisfactorily elucidated by a single-gene analysis.

The genus *Rhizomastix* splits into two lineages in SSU rRNA gene analysis. The first one is comprised of endobiotic (*R. bicoronata* sp. nov., *R. vacuolata* sp. nov.) or possibly endobiotic (*R. elongata* sp. nov.) species, while the other one contains the free-living species *R. libera*. The actin gene tree, although generally unresolved, strongly supports the existence of these two lineages. Unfortunately, the phylogenetic position of *R. varia* sp. nov., another very likely free-living species, was not elucidated by the actin gene analysis, and we were unable to amplify its SSU rRNA gene. Nevertheless, the actin gene analysis supports the distinctiveness of *R. libera* and *R. varia* sp. nov. as suggested by light-microscopical morphology (see above).

Based on molecular phylogenetic results, the endobiotic lifestyle has arisen at least two times independently within the Archamoebae: (1) In the last common ancestor of *Entamoeba* + *Rhizomastix*. (2) In the last common ancestor of *Endolimax* and *Iodamoeba*. However, further clarification is needed on several points before the number of endobiotic taxa/origins of parasitism in the Archamoebae can be stated with confidence: (i) whether *Rhizomastix* really is the closest relative of *Entamoeba*, (ii) whether *Tricholimax hylae*, *Endamoeba* spp., and *Mastigella bovis*, whose sequence data are currently unavailable, are either closely related to one of the two endobiotic lineages or are not archamoebae at all, and (iii) whether at least *R. libera* and maybe *R. varia* sp. nov. as well are secondarily free-living, similarly to *Entamoeba moshkovskii*. If any of (i–iii) is invalid, it would mean that the endobiotic lifestyle of archamoebae arose more than two times independently. Further data are required before a robust hypothesis can be tested.

Peculiarities in ultrastructure of *R. elongata* sp. nov

So far, only a single *Rhizomastix* species (the free-living *R. libera*) has been studied by means of transmission electron microscopy (Ptáčková et al. 2013). It was shown that *R. libera* has a unique ultrastructure among flagellated archamoebae, because the microtubular cone seen in other archamoebae has been modified into a rhizostyle, a relatively thin bundle of microtubules that winds around the nucleus and extends posteriorly to it (Ptáčková et al. 2013). A lateral microtubular root arising from the edge of the basal body and running laterally into the cell was also observed in *R. libera* (Ptáčková et al. 2013). A diagrammatic summary of current knowledge of the ultrastructure of *R. libera* is presented in Fig. 12A, and of *R. elongata* in Fig. 12B.

The flagellar cytoskeleton of *R. elongata* sp. nov. presented here is very similar to, but slightly different from that described previously in *R. libera*. The rhizostyle of *R. elongata* sp. nov. does not appear to wind around the nucleus; and it is composed of eleven rather than 13–15 microtubules as seen in *R. libera*. Both species have electron-dense material in the centre of the rhizostyle close to the basal body, but acidocalcisome-like structures have only been seen in *R. elongata* sp. nov. Both species show the rhizostyle initially being arranged as a semicircle as it arises from the basal body, and more proximally being a circle, though this is more pronounced in *R. libera*.

In *R. elongata* sp. nov., the large root of nine microtubules arising from the proximal edge of the basal body, and extending proximally, is likely to be homologous to the laterally emerging and extending flagellar root of eight microtubules seen in *R. libera* (Ptáčková et al. 2013) and in other archamoebae (Walker et al. 2001; inter alia). The doublet root has not previously been described in *R. libera* or other archamoebae. A microtubular doublet was observed within the rhizostyle of *R. libera* and this may be homologous to the doublet root of *R. elongata* sp. nov.: more detailed description of the mastigont system is required in both species. The hypothesis that rhizostyle could fix the position of the nucleus in the central part of the cell was raised by Cepicka (2011). Subsequently, the possibility that the rhizostyle functions as an anchor of the basal body was discussed (Ptáčková et al. 2013). As the cells of *R. elongata* sp. nov. are very thin and

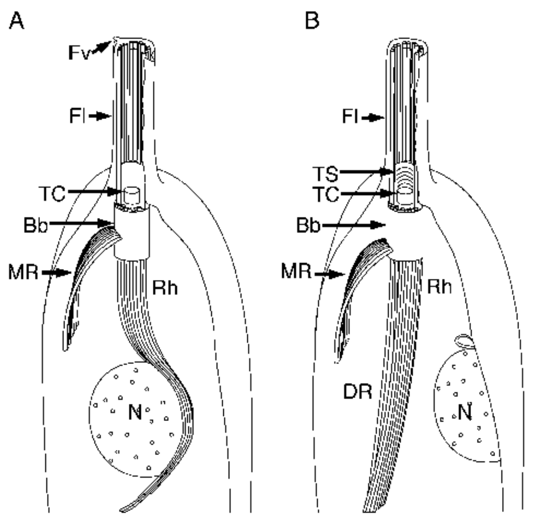

Figure 12 Diagrammatic interpretations of ultrastructure in (**A**) *Rhizomastix libera* and (**B**) *Rhizomastix elongata*. Bb = basal body; DR = doublet microtubular root; Fl = flagellum; FV = flagellar vane; MR = lateral microtubular root; N = nucleus; Rh = rhizostyle; TC = transition zone cylinder; TS = transition zone spiral.

extremely long in comparison with previously reported species (see table 1 in Mali et al. 2002) and, in addition, *R. elongata* sp. nov. moves very quickly and jerkily, these two roots with the rhizostyle may anchor both the flagellum and the nucleus, using the anterior part of the cell as a fulcrum during cell motion.

Vesicles that closely resemble acidocalcisomes are present mostly near the flagellar base, enclosed by the rhizostyle. Acidocalcisomes were originally defined in trypanosomes, but have subsequently been found in diverse organisms (e.g. bacteria, parasitic protists) where they function in storage of pyrophosphates, polyphosphates and cations, and potentially have a role in pH homoeostasis and osmoregulation (Docampo and Moreno 2011; Moreno and Docampo 2009; Seufferheld et al. 2003, 2004; Vercesi et al. 1994). Similar unspecified organelles have previously been observed in the mastigont of *Mastigamoeba simplex* (Walker et al. 2001) but were not detected in *R. libera* (Ptáčková et al. 2013). In contrast to the vesicles in *M. simplex* and *R. elongata* sp. nov. the acidocalcisomes of other eukaryotic organisms are distributed randomly through the whole cell (Docampo and Moreno 2011; Moreno and Docampo 2009). The location of vesicles near the flagellar apparatus might indicate their possible function in flagellar movement or flagellar apparatus formation, as an energy source for replacing ATP.

Nuclear pocket or loop structures were observed in all cells where the nucleus was fully sectioned (Fig. 7B, C; 8B–E). To the best of our knowledge, similar nuclear projections have been frequently observed in lymphocytic diseases in man and diverse mammals (Ghadially 1988), but they have not been observed in protists, particularly not in *R. libera*. The invagination of nuclear membrane can occur in protists during nuclear division (Sun and Bowen 1972; Wustman et al. 2004), however, the structures in *R. elongata* sp. nov. do not appear to have any temporal connection with cell division. Moreover they are morphologically different forming a fold or curled cleft, often with a fused margin. The nuclear pockets are bounded by bands of chromatin, and always enclose cytoplasmic material. In an experimental study on yeast it was shown that depletion of nucleoporin Nup170p plays a key role in the formation of nuclear pore complexes and causes some damage to the nucleus (Makio et al. 2009). This phenotype somehow resembles the nuclear pockets in *R. elongata* sp. nov., but they do not look as similar as the projections in cells affected by lymphocytic diseases (Ghadially 1988; Makio et al. 2009).

Cylindrical or rounded electron-dense structures that might be mitochondrion-related organelles were present in cytoplasm of *R. elongata* sp. nov. Double membranes surrounding the organelles were sometimes visible. Although their shape is not extremely similar to that of the mitosome-like organelles found in *R. libera*, their size is similar (Ptáčková et al. 2013). It has previously been shown that energetic metabolism of mitochondrion-related organelles is diverse in diverse species of Archamoebae, and the organelles thus have been characterized as mitosomes or hydrogenosome-like organelles (Chan et al. 2005; Clark and Roger 1995; Ghosh et al. 2000; Gill et al. 2007; León-Avila and Tovar 2004; Mai et al. 1999; Mi-ichi et al. 2011; Nývltová et al. 2013; Tovar et al. 1999). Presumable mitochondrial derivatives discovered in *Rhizomastix* species have more similar dimensions to those of parasitic *E. histolytica* than to those of free-living Archamoebae: therefore we tentatively assume that these organelles are mitosomes.

TAXONOMIC SUMMARY

Amoebozoa: Archamoebae: Rhizomastixidae Ptáčková, Kostygov, Chistyakova, Falteisek, Frolov, Patterson, Walker & Cepicka, 2013: *Rhizomastix* Alexeieff, 1911

Rhizomastix bicoronata sp. nov

ZooBank registration: urn:lsid:zoobank.org:act:B8C88FBD-CB98-4C56-9998-C8BBD0762125

Diagnosis. Trophozoites uninucleated, with a single flagellum or aflagellated. Rhizostyle runs beyond the nucleus. Swimming cells typically with conical pseudopodia at both ends arranged in a crown-like pattern. Living trophozoites ca. 16.9 (14.1–25) μm long and 5.2 (3.8–7.8) μm wide, with the flagellum ca. 25.6 (15.6–39.1) μm long. Protargol-stained trophozoites 9.3 (6.1–18.3) μm long and 6.3 (4.4–8.5) μm wide with the flagellum 15.6 (5.9–27.1) μm long.

Type locality. Members of the millipede order Spirostreptida naturally occur in Africa, Asia, Australia, and America. The type host specimen was kept in Prague, Czech Republic.

Type host. Unidentified member of the order Spirostreptida (Diplopoda: Juliformia).

Habitat. Lower intestine.

Holotype. Protargol-stained cell of the strain VELKA1 depicted in Fig. 1I. The preparation containing the cell is deposited in the collection at the Department of Parasitology, Charles University in Prague, catalogue number 6/83.

Etymology. L. fem. adj. *bicoronata*—with two crowns.

Rhizomastix vacuolata sp. nov

ZooBank registration: urn:lsid:zoobank.org:act:AC48F854-600C-497F-B314-C297F5B20F1F

Diagnosis. Trophozoites uninucleated, with a single flagellum or aflagellated. Rhizostyle not visible. Crawling cells amoeboid with multiple small pseudopodia around the body. Swimming cells rare. Cytoplasm highly vacuolated. Living trophozoites 14.7 (10.6–21.2) μm long and 7.4 (4.1–14.0) μm wide with the flagellum 19.5 (7.7–31.1) μm long. Protargol-stained cells 6.7 (2.5–8.7) μm long and 5.6 (2.7–8.2) μm wide with the flagellum 14.2 (6.5–27.1) μm long.

Type locality. Members of genus *Goliathus goliatus* naturally occurs in equatorial Africa. The type host specimen was kept in Prague, Czech Republic.

Type host. larva of *Goliathus goliatus* (Coleoptera: Scarabaeidae: Cetoniinae)

Habitat. Lower intestine.
Hapantotype. Protargol preparations of the strain GOL1, deposited in the collection at the Department of Parasitology, Charles University in Prague, catalogue numbers 10/15–10/17, 10/84–10/87, 12/24, and 12/25.
Etymology. L. fem. adj. *vacuolata*—vacuolated.

Rhizomastix tipulae sp. nov

ZooBank registration: urn:lsid:zoobank.org:act:CBE4EE02-D323-40CE-B9C8-45F951EAED4B
Diagnosis. Trophozoites uninucleated, with a single flagellum. Cells elongated, rounded or amoeboid. Cytoplasm finely granular. Rhizostyle extends posteriorly beyond the nucleus. Cells stained with Heidenhain's haematoxylin 7.5 (3.7–10.0) μm long and 2.5 (2.0–4.0) μm wide with the flagellum at least twice the length of the body.
Type locality. Chester, Delaware, and Montgomery counties, Pennsylvania, USA.
Type host. larva of *Tipula abdominalis* (Diptera: Tipulidae).
Habitat. Lower intestine.
Holotype. Cell depicted in fig. 5 in Ludwig (1946).
Etymology. L. fem. adj. *tipulae*—from a crane fly.

Rhizomastix elongata sp. nov

ZooBank registration: urn:lsid:zoobank.org:act:459B3020-73FC-4774-AB0F-1525AB26B44B
Diagnosis. Trophozoites uninucleated, with a single flagellum or aflagellated. Rhizostyle ends approximately in the anterior or central part of the cell or runs behind the nucleus. Movement fast and jerky. Swimming cells extremely elongated and very thin, 27.6 (19.7–37.8) μm long and 3.2 (2.2–4.0) μm wide with flagellum 15.1 (10.1–19.5) μm long. Protargol-stained trophozoites 9.9 (4.4–18.0) μm long and 3.2 (1.2–6.5) μm wide with flagellum 15.8 (9.6–24.3) μm long.
Type locality. Vejvanov-Pajzov, Czech Republic. 49°51′N, 13°39′E.
Habitat. Organic-rich sediment of a disused cesspit.
Hapantotype. Protargol preparations of the strain VAVRH, deposited in the collection at the Department of Parasitology, Charles University in Prague, catalogue numbers 10/20–10/22 and 10/49–10/52.
Etymology. L. fem. adj. *elongata*—elongated.

Rhizomastix varia sp. nov

ZooBank registration: urn:lsid:zoobank.org:act:3A4E917D-EED1-4C59-814F-D12EA7B9C852
Diagnosis. Trophozoites uninucleated, with a single flagellum or aflagellated. Rhizostyle extends to the posterior end of the cell. Movement slow. Crawling cells amoeboid with multiple spiny pseudopodia. Swimming cells rarely observed. Flagellum thick. Flagellar base moves fluently around the cell. Living trophozoits 11.2 (5.7–19.8) μm long and 5.4 (3.0–8.9) μm wide with the flagellum 14.3 (5.1–30.5) μm long. Protargol-stained cells 4.6 (2.8–10.5) μm long and 2.9 (1.4–4.2) μm wide with the flagellum 11.6 (5.4–37.7) μm long.
Type locality. Ribeiro Frio, Madeira, Portugal. 32°44′N, 16°53′E.
Habitat. Freshwater sediment.
Hapantotype. Protargol preparations of the strain FBAN, deposited in the collection at the Department of Parasitology, Charles University in Prague, catalogue numbers 6/67 and 6/68.
Etymology. L. fem. adj. *varia*—diverse, various, different.

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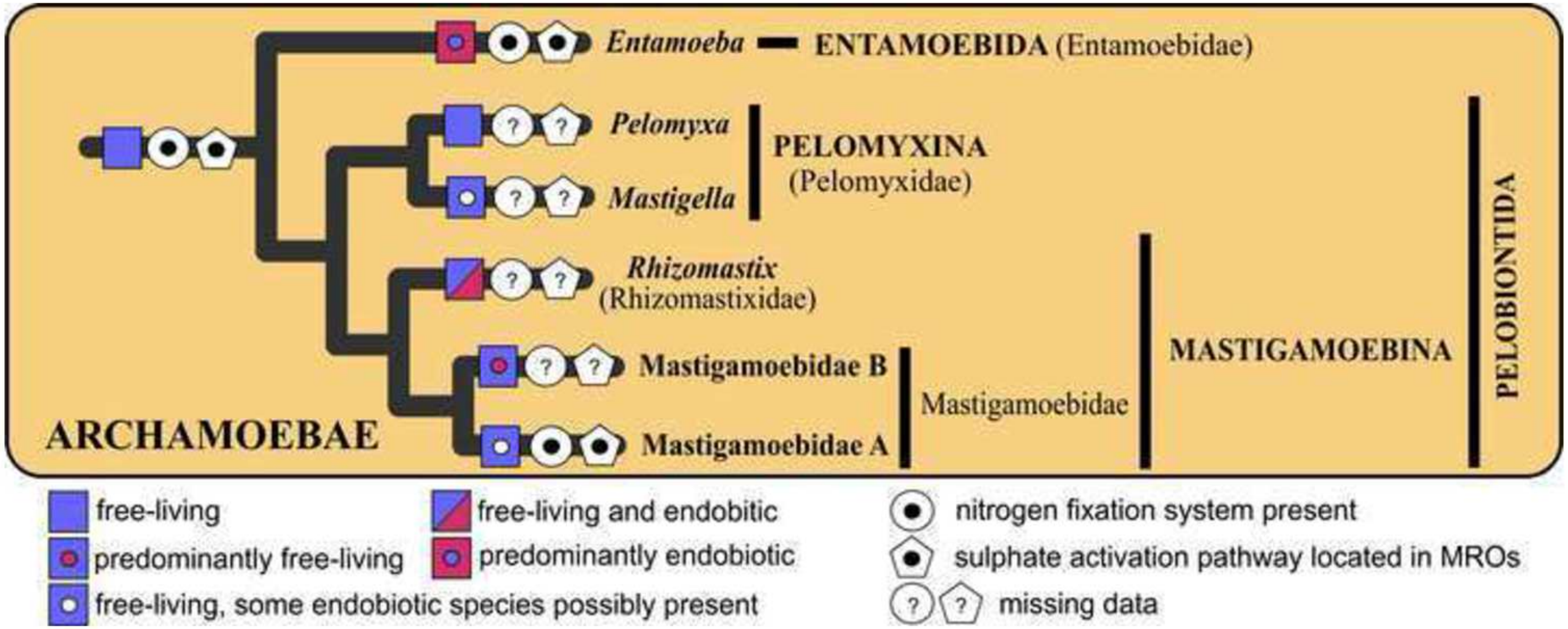
7. 4 Pánek et al., *in press*

Pánek T, Zadrobílková E, Walker G, Brown MW, Gentekaki E, Hroudová M, Kang S, Roger A, Tice AK, Vlček Č, Čepička I. First multigene analysis of Archamoebae (Amoebozoa: Conosa) robustly reveals its phylogeny and shows that Entamoebidae represents a deep lineage of the group. Mol Phylogenet Evol, *in press*.

Graphical Abstract (RGB)



*Highlights

HIGHLIGHTS

- 7-gene phylogenetic analysis clearly resolves relationships within Archamoebae
- The endobiotic lifestyle appeared at least three times during the evolution of the group
- The bacterial nitrogen fixation system was present in the last common ancestor of Archamoebae (LCAA)
- Mitochondrial derivatives of the LCAA contained a sulfate activation pathway
- Comparative ultrastructural analysis of Mastigamoebidae „A“ and „B“ clades is presented.

First multigene analysis of Archamoebae (Amoebozoa: Conosa) robustly reveals its phylogeny and shows that Entamoebidae represents a deep lineage of the group

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ABSTRACT

Archamoebae is an understudied group of anaerobic free-living or endobiotic protists that constitutes the major anaerobic lineage of the supergroup Amoebozoa. Hitherto, the phylogeny of Archamoebae was based solely on SSU rRNA and actin genes, which did not resolve relationships among the main lineages of the group. Because of this uncertainty, several different scenarios had been proposed for the phylogeny of the Archamoebae. In this study, we present the first multigene phylogenetic analysis that includes members of Pelomyxidae, and Rhizomastixidae. The analysis clearly shows that Mastigamoebidae, Pelomyxidae and Rhizomastixidae form a clade of mostly free-living, amoeboid flagellates, here called Pelobiontida. The predominantly endobiotic and aflagellated Entamoebidae represents a separate, deep-branching lineage, Entamoebida. Therefore, two unique evolutionary events, horizontal transfer of the nitrogen fixation system from bacteria and transfer of the sulfate activation pathway to mitochondrial derivatives, predate the radiation of recent lineages of Archamoebae. The endobiotic lifestyle has arisen at least three times independently during the evolution of the group. We also present new ultrastructural data that clarifies the primary divergence among the family Mastigamoebidae which had previously been inferred from phylogenetic analyses based on SSU rDNA.

KEYWORDS

Conosa, Pelobiontida, evolution of parasitism, nitrogen fixation system, flagellar apparatus, classification

1. INTRODUCTION

Archamoebae is a group of anaerobic amoeboid flagellates and amoebae. It was originally created to unite two groups of previously-known, presumptively primarily-amitochondriate, amoeboid protists – pelobionts and Entamoebae (Cavalier-Smith, 1983). Subsequently, remnants of mitochondria were reported from several of its species (e.g. Clark and Roger, 1995; Tovar *et al.*, 1999; Walker *et al.*, 2001; Gill *et al.*, 2007; Ptáčková *et al.*, 2013, Zadrobílková *et al*. 2015a). Using ultrastructural and molecular data, a close relationship between Archamoebae and mycetozoan slime moulds (grouping together with some other taxa as Conosa) has been hypothesised and discussed (Cavalier-Smith, 1998, 2013; Walker *et al.*, 2001; Cavalier-Smith *et* *al*., 2004); and the relationship has been strongly supported in recent multigene analyses (Cavalier-Smith *et al.*, 2015). Very few other possibly anaerobic species (*Vannella peregrinia*, *Flamella citrensis*) have been described within the supergroup Amoebozoa so far (Bovee, 1956; Smirnov and Fenchel, 1996), so Archamoebae constitutes the major anaerobic lineage of Amoebozoa.

Currently, Archamoebae comprises approximately 450 nominal species, distributed among five families – Entamoebidae, Pelomyxidae, Mastigamoebidae, Tricholimacidae, and Rhizomastixidae. Most described species are free-living, but the group also contains numerous endobionts (more than 100 nominal species) including the prevalent and significant human parasite *Entamoeba histolytica,* and other protists infecting humans (Clark *et al.*, 2006; Stensvold *et al.*, 2012).

In analyses of SSU rDNA, each of the four families containing more than a single species appears robustly monophyletic; Tricholimacidae is monotypic and no molecular data exists for *Tricholimax hylae* (Ptáčková *et al.*, 2013; Zadrobílková *et al.*, 2015; Zadrobílková *et al.*, in press). Monophyly of Pelomyxidae (*Mastigella* + *Pelomyxa*) is further supported by actin gene phylogeny (Zadrobílková *et al.*, 2015). In SSU rDNA trees, family Mastigamoebidae splits into two diverse, statistically well-supported clades, provisionally called Mastigamoebidae A and B (Ptáčková *et al.*, 2013). The latter clade also contains the endobiotic and aflagellate genera *Iodamoeba* and *Endolimax* (Stensvold *et al.*, 2012; Ptáčková *et al.*, 2013).

Nevertheless, relationships within the Archamoebae are currently unclear, because neither actin nor SSU rDNA trees are able to resolve relationships between families (Cavalier-Smith *et al.*, 2004; Ptáčková *et al.*, 2013; Stensvold *et al.*, 2012; Zadrobílková *et al.*, 2015; Zadrobílková *et al.*, in press). Both *Pelomyxa* and *Entamoeba* form very long branches in SSU rDNA trees, and their phylogenetic positions are probably affected by long-branch attraction (Ptáčková *et al.*, 2013). Sequence data for use in multigene phylogenetic analyses was hitherto available just for two lineages – *Mastigamoeba balamuthi* (Mastigamoebidae A) and *Entamoeba* spp. (Entamoebidae). Morphology is also ambiguous as to relationships between families and even genera, in the absence of heuristic arguments as to which characters are genuinely taxonomically informative (discussed in Walker *et al*. 2001; *c.f.* the revised interpretation of the placement of *Mastigella* in Ptáčková *et al.*, 2015).

Anaerobic mitochondrial derivatives (MROs) of two species, *Entamoeba histolytica* and *Mastigamoeba balamuthi*, have been biochemically characterized. It was shown that MROs of each species have a sulfate activation pathway, which is not present in any other known mitochondria, and whose key enzyme (ATP sulfurylase) has been acquired laterally from bacteria (Mi-Ichi *et al.*, 2011; Nývltová *et al.*, 2015). Moreover, both species possess an ε-proteobacterial nitrogen fixation system (NIF system), the only eukaryotes to do so. This system has replaced the ancestral mitochondrial iron-sulfur cluster machinery (ISC machinery). It is thought that the ISC machinery exports a sulfur-containing moiety from the mitochondrial matrix to the cytoplasm, for use in cytoplasmic FeS protein biogenesis (CIA pathway). It has also been shown that FeS cluster biogenesis is the only known function of yeast mitochondria that is indispensable to cellular viability (see Lill, 2009). In both, *M. balamuthi* and *E. histolytica*, this ancestral mitochondrial pathway has been lost and replaced by the NIF system, which is active both in MROs and the cytosol of *Mastigamoeba balamuthi*, and in the cytosol of *E. histolytica* (Nývltová *et al.*, 2013). As compared to MROs of *E. histolytica*, MROs of *M. balamuthi* additionally contain a set of proteins typically involved in hydrogenosomal metabolism that allows anaerobic acetyl CoA-dependent synthesis of ATP (Nývltová *et al.*, 2015).

Here, we present the first multigene phylogenetic analysis that is based on seven protein-coding genes and includes members of four families in the Archamoebae. Our results clearly show that the predominantly endobiotic and parasitic family Entamoebidae represents a deep lineage of Archamoebae (Entamoebida Cavalier-Smith, 1993), and the other three families form the second clade of the group, the order Pelobiontida Page, 1976. Based on the results, plesiomorphic features and convergent evolution within the Archamoebae is discussed. Using transmission electron microscopy, we define the morphological characteristics of the two clades that currently fall within Mastigamoebidae, "Mastigamoebidae A" and "B".

2. MATERIAL AND METHODS

2.1. Organisms and RNA extraction

Four examined strains (*Mastigella eilhardi* ATCC 50342, *Rhizomastix libera* IND8, *R. elongata* VAVRH, and *Mastigamoeba abducta* CHOM1) were grown in monoeukaryotic cultures with various unidentified bacteria, following previously published protocols (Ptáčková *et al.*, 2013; Zadrobílková *et al.*, 2015; Zadrobílková *et al.*, in press). *Pelomyxa* sp. was isolated directly from anaerobic sediment collected from a small freshwater farm pond outside of Fayetteville, AR, USA in September, 2014.

Total RNA samples were extracted from 28 – 50 ml of the culture (2 ml of cell suspension lying at the bottom of 10 ml of culture medium, in 15 ml Falcon tubes). Cells of *Mastigella eilhardi* strain ATCC 50342 were filtered through a membrane filter with 5µm pores (Whatman, GE Healthcare Bio-Sciences, USA) to remove bacteria and centrifuged at 1,200 *g* for 10 minutes. Cells of other strains were centrifuged at 1,200 *g* for 10 minutes without the filtration step. Total RNA was extracted from harvested cells using TriReagent Solution (Ambion, USA) according the manufacturer´s instructions.

A single *Pelomyxa* sp. cell was transferred from a ~0.5ml drop of the anoxic suspension to a fresh ~0.5ml drop of filter-sterilized natural spring water. Immediately the cell was processed as follows. To remove any other contaminating eukaryotic cells, the *Pelomyxa* cell was successively washed in five fresh aliquots of sterile spring water. Once free of any other potential contaminating eukaryotes, the cell was picked up with a loop made from 32-gauge platinum (https://youtu.be/nSZuTOZ0QyY) and transferred to a 200µL PCR tube containing cell lysis buffer. Total RNA was extracted from *Pelomyxa* sp. using a modified version of Smart-seq2 (Picelli *et al*., 2014) that includes an additional six rounds of a freeze thaw cycle to aid in cell lysis in -80°C isopropanol and ~25°C H_2O respectively.

2.2. **cDNA libraries construction, sequencing, cluster assembly**

mRNAs from all four examined strains were isolated by selection with Dynabeads Oligo$(dT)_{25}$ (Invitrogen). Illumina sequencing libraries were prepared from mRNA (double polyA selection in a case of IND8 and triple polyA selection in a case of 50342, CHOM1 and VAVRH strains) using BIOO Scientific developed protocol NEXTflex RNA-Seq Kit and sequenced on appropriate platform (MiSeq 150bp paired end for IND8 or HiSeq2000 100bp paired end for 50342, CHOM1 and VAVRH strains). Illumina sequence read data were filtered based on quality scores with the fastq_quality_filter program of FASTXTOOLS (http://hannonlab.cshl.edu/fastx_toolkit/), using a cut-off filter (a minimum 70% of bases must have quality of 20 or greater). Filtered sequences were then assembled into clusters using the INCHWORM assembler of the TRINITY package (Grabherr *et al.*, 2011).

The resultant *Pelomyxa* sp. single cell cDNA generated from the SmartSeq-2 method was fragmented using sonification in a Covaris S220 (Woburn, MA, USA) (set at Duty %: 10, Intensity: 5, Burst Cycle: 200, Time: 30s, Mode: Frequency Sweeping). The fragmented cDNA was used as the starting material for a DNA Illumina library generated using the NEBnext Ultra DNA library construction kit (New England Biolabs, Boston, USA) following the manufacturer's protocol. The resultant Illumina library was pooled with four other libraries from non-related protists from a different experiment (5-plex pool). The library pool was sequenced using the MiSeq platform (300bp paired end reads). Reads were demultiplexed by the MiSeq. Reads were trimmed to remove low-quality sequences using Trimmomatic v. 0.30 with a sliding window of 10 nucleotides and a PHRED33 quality threshold of 25 (Bolger et al. 2014). The quality trimmed reads were assembled using Trinity 2.0.4 (Grabherr et al. 2011). Open-reading frames (ORFs) were predicted using TransDecoder (from the Trinity package) and translated to protein sequences.

2.3.**Protein Data sets Construction and Gene Searching in ESTs**:

Single-gene protein datasets were based on datasets published by Pánek *et al.* (2015) and Cavalier-Smith *et al.* (2014, 2015). In addition, we included also sequences of *Phalansterium solitarium* (GenBank) and screened available ESTs of 11 amoebozoans deposited in MMETSP (Keeling *et al.*, 2014) classified as *Mayorella* sp. ATCC 50980, *Sapocribrum chincoteaguense* ATCC 50979, *Filamoeba nolandi* ATCC 50430, *Neoparamoeba aestuarina* SoJaBio B1-5/56/2, *Paramoeba atlantica* CCAP 1560/9, *Pessonella* sp. PRA-29, *Stereomyxa ramosa* ATCC 50982, *Trichosphaerium* sp. ATCC 40318, *Vannella robusta* DIVA3 518/3/11/1/6, *Vannella* sp. DIVA3 517/6/12, and *Vexillifera* sp. DIVA3 564/2. Those ESTs were screened for previously published gene orthologues of actin, α-tubulin, β-tubulin, cytosolic HSP70, cytosolic HSP90, EF-2, and EF-1α genes using local BLAST (tBLASTN) in BioEdit 7.0.4.1. The tBLASTN hits were then translated to amino acid residues. Those sequences as well as sequences from five archamoebae obtained during this study were then added to the single-gene datasets and aligned with the help of the MAFFT 7.221 server (http://mafft.cbrc.jp/alignment/software/) at default settings, checked and trimmed manually. No stop codons were observed in the coding regions. To test for undetected paralogs or contaminants, we performed phylogenetic analyses of the single-gene alignments (Supplementary Material S1). Newly obtained sequences are deposited as Supplementary Material S2.

Final single-gene alignments were concatenated into the multi-protein data set, which contained 3585 aligned characters (amino acid residues) of seven genes, and is provided as Supplementary Material S3. We did not include alpha-tubulin and beta-tubulin gene sequences of *Entamoeba invadens* and *E. histolytica* in the final multi-protein dataset, since *Entamoeba* tubulins formed extremely long branches in the single gene trees. This trimmed dataset contained more than 3560 amino acid residues for all species of pelobionts excepting *Pelomyxa*, which was represented by 1360 residues. *Entamoeba* spp. was represented by 2615 and 2716 residues, respectively.

2.4.**Phylogenetic Analyses**

The single-gene Maximum Likelihood trees (ML trees) were constructed in RAxML 8.0.19 (Stamatakis and Ott, 2008) using PROTGAMMAILG or PROTGAMMAILGF model of the sequence evolution with 10 ML tree searches and 500 non-parametric bootstrap replicates. The best partitioning scheme for the multi-protein data set was estimated in PartitionFinder Protein 1.1.0 (Lanfear *et al*., 2012) under the Bayesian Information Criterion with greedy searching. The Best-scoring ML tree was found using 50 independent heuristic searches. Branch support was estimated from 1000 non-parametric bootstrap replicates. Because RAxML is not equipped with C-series models, we also ran IQ-TREE v. 1.3.3 (Nguyen *et al*. 2015) on the multigene dataset. The best-fitting model available under ML analyses as determined under the Akaike Information Criterion was LG+G4+C60+F with class weights optimized from the dataset (using the exchangeabilities from the LG Q-Matrix (LG+G4+FMIX{empirical,C60pi1...C60pi60}) (Wang, *et al*. 2014). Branch support was estimated from 1000 ultrafast bootstrap replicates in IQ-Tree. Besides two ML approaches, we also used Bayesian Monte Carlo Markov Chain software, PhyloBayes v. 3.3 (Lartillot and Philippe, 2004), which is able to use non-parametric methods for modeling heterogeneous site specific features of sequence evolution. PhyloBayes was run on the multi-protein data set using the CAT-Poisson model. Two independent chains were run until they converged (*i.e.* their maximum observed discrepancy was lower than 0.3 and the effective sample size of all model characteristics was at least 80). Topologies were congruent between chains. Consensus trees and posterior probabilities were calculated using the bpcomp program with the first 25 % of the generations as burn-in, sampling every 50 trees.

Light Microscopy and Transmission Electron Microscopy

As a representative of Mastigamoebidae A, *Mastigamoeba balamuthi* was obtained from the CCAP (Strain 1557/1) in 1999. This strain has subsequently been lost from the CCAP, but is the same strain as ATCC 30984. *M. balamuthi* was cultured in Jones' Horse Serum medium (http://www.ccap.ac.uk/media/documents/HSM.pdf), and was observed under the light microscope and prepared for Transmission Electron Microscopy as described previously for *Mastigella commutans* (Walker et al. 2001).

As a representative of Mastigamoebidae B, *Mastigamoeba guttula* was cultured and observed under the light microscope as described previously for the two strains HRADANAN and LU2HNS4 (Ptáčková *et al.,* 2013). A cell suspension of strain HRADANAN was prepared by centrifugation of a 15 ml tube containing 12 ml culture, for ten minutes at 1,000 × *g*. The sample was high-pressure frozen using a Leica EM PACT2 (Leica Camera, Wetzlar, Germany), and cryosubstituted in a Leica EM AFS2 using 100% acetone with 2% OsO_4 as follows: −90 °C for 96 hours, 5 °C for 14 hours; −20 °C for 24 hours; 3 °C for 8 hours; 4 °C for 18 hours. Samples were then washed three times in 100 % acetone. Embedding was done at room temperature, using Epon resin (Poly/Bed 812/Araldite, Polysciences, Warrington, USA), having been infiltrated in an ascending series of concentrations changed after an hour (1:2, 1:1, 2:1). Samples were sectioned at 60 nm thickness using a diamond knife on an Ultracut E ultramicrotome (Reichert) and collected on copper mesh grids coated with formvar film. Ultrathin sections were stained with lead citrate and uranyl acetate (2–3 %) and examined using a TEM JEOL 1011 (Jeol, Tokyo, Japan) transmission electron microscope.

3. RESULTS AND DISCUSSION

3.1. Phylogenetic analyses

In the present study, we obtained EST data from five species, meaning that the broad diversity of Archamoebae could be represented in multigene analyses for the first time: *Rhizomastix libera* and *R. elongata* (Rhizomastixidae), *Mastigella eilhardi* and *Pelomyxa* sp. (Pelomyxidae), and *Mastigamoeba abducta* (Mastigamoebidae B). The latter species represents a lineage that also includes endobiotic genera *Endolimax* and *Iodamoeba* (Ptáčková *et al.*, 2013).

Monophyly of the family Rhizomastixidae and Entamoebidae was highly supported across phylogenies of all seven used molecular markers (genes for actin, alpha-tubulin, beta-tubulin, elongation factor 1-alpha, elongation factor 2, cytosolic HSP70, and HSP90). On the other hand, the resolution of individual gene trees was not sufficient either to clearly show relationships between other lineages of Archamoebae, or to show monophyly of Archamoebae itself (see Fig. 1 for RAxML bootstrap supports or Supplementary Material S1 for RaxML gene trees). No robustly or moderately-supported conflicting nodes (bootstrap support > 70) were observed among the gene trees inferred from individual molecular markers.

To resolve the internal phylogeny of Archamoebae more robustly, we concatenated sequences from these markers in the final multi-protein phylogenetic analysis. The Bayesian and Maximum Likelihood (ML) analyses yielded a highly congruent topology of Archamoebae. Maximum likelihood analyses (RAxML and IQ-Tree) as well as Bayesian approach (PhyloBayes) showed that Archamoebae form a clade with absolute statistical support. Similarly, relationships within Archamoebae have been recovered with absolute statistical support using Bayesian and IQ-tree ML analyses and at least 93 % using RAxML. These results (see Fig. 2) allowed us to reconstruct the phylogeny of Archamoebae, make inferences about its evolution and revise its taxonomy.

Three competing hypotheses about the phylogeny of Archamoebae have been proposed so far: **(1)** Pelomyxidae and Entamoebidae form a clade, sister to the rest of Archamoebae (Cavalier-Smith *et al.*, 2004; Cavalier-Smith, 2013); **(2)** Rhizomastixidae and Entamoebidae constitute a clade (Ptáčková *et al.*, 2013); **(3)** Entamoebidae forms a separate lineage from the rest of Archamoebae (Cavalier-Smith, 1991). Our analysis based on concatenated dataset clearly showed Entamoebidae as the sister clade to the rest of the Archamoebae. This result conclusively supports the third proposed hypothesis about the deep phylogeny of Archamoebae. The phylogenetic position of Rhizomastixidae is surprising since previous actin and SSU rDNA analyses have suggested possible close relationship between Rhizomastixidae and Entamoebidae.

Our Bayesian analysis of multiprotein dataset further recovered Amoebozoa as a clade, although its statistical support is very low. As currently shown by Cavalier-Smith *et al.* (2015), Amoebozoa splits into two clades, Conosa and Lobosa. Archamoebae, besides Macromycetozoa and Variosea *sensu* Berney *et al.* (2015), is one of the three conosean lineages. Our analysis demonstrated monophyly of each of them (see Fig. 2). On the other hand, it was unable to resolve relationships between these lineages and to recover Conosa as monophyletic, since the putative loboseans *Sapocribrum* and *Pessonella* (Amoebozoa: Lobosa) branched sister to Variosea with high statistical support in Bayesian and IQ-Tree ML analyses (1 and 96, respectively).

3.2. **Transmission Electron Microscopy**

Molecular markers (SSU rDNA trees) strongly support division of Mastigamoebidae into two clades, Mastigamoebidae A and B (see Ptáčková *et al*., 2013). On the other hand, no morphological differences between the clades have been reported so far. Therefore, we decided to thoroughly examine the cell morphology of representatives of both clades (see Table 1 and Figs 3 and 4). Representatives of Mastigamoebidae A (e.g. *Mastigamoeba balamuthi, M. aspera,* or *M. punctachora*) generally show more morphological variation and are larger than representatives of Mastigamoebidae B (e.g. *Mastigamoeba guttula*, *M. simplex*).

Full details of the flagellar apparatus characteristics known from each group are summarised in Table 1 and Figure 4, but the main differences are presented briefly here. The cone of microtubules arising from the basal body originates differently in each group: in members of Mastigamoebidae A, microtubules of the cone arise laterally, from along the sides of the basal body, and in some cases arise from the base of the basal body as well; whereas those in Mastigamoebidae B arise longitudinally, close to the base of the basal body, in a single layer. There may be an MTOC present immediately below the basal body, in some taxa of Mastigamoebidae A, but not B. In Mastigamoebidae A, the flagellar transition zone is long and, in some taxa, contains either a dense column or a spiral (potential homologies of the dense column are discussed in Walker et al. 2001); whereas the transition zone is short and no extra elements have been seen hitherto in members of Mastigamoebidae B. These results provide synapomorphies for the yet-unclassified groups Mastigamoebidae A and B, which were originally identified by molecular phylogenetics of the SSU rRNA gene, and have subsequently been confirmed by the detailed analyses presented here. Formal classification of these two groups is not given below, as it would require a complete revision of all nominal species of *Mastigamoeba*, *Iodameba* and *Endolimax*, which is beyond the scope of the current paper. However, our comparative analysis (see Table 1) indicates that *Mastigamoeba aspera*, the type species of the genus *Mastigamoeba*, belongs to the Mastigamoebidae “A” clade. Some of the present authors are currently working on a detailed taxonomic revision of Archamoebae which will reflect these findings and will deal with classification of species and genera within Mastigamoebidae.

3.3. **Classification of Archamoebae**

Currently, the class Archamoebae Cavalier-Smith, 1983 contains five families: Pelomyxidae Schulze, 1877; Mastigamoebidae Goldschmidt, 1907; Rhizomastixidae Ptáčková *et al.*, 2013; Tricholimacidae Cavalier-Smith, 2013; Entamoebidae Chatton, 1925. Based on our results, we distinguish two orders, Pelobiontida Page, 1976 and Entamoebida Cavalier-Smith 1993. Furthermore, we have divided Pelobiontida Page, 1976 into two suborders - Pelomyxina Starobogatov, 1980 (stat. nov.) and Mastigamoebina Frenzel, 1897 (stat. nov.). We have defined taxa using node-based and branch-based phylogenetic definitions. We have classified the genera *Endamoeba* and *Mastigina*, and the family Tricholimacidae as Archamoebae *incertae sedis*.

Order Pelobiontida Page, 1976

(Eukaryota: Amoebozoa: Conosa: Archamoebae)

Definition: The clade consisting of *Mastigella eilhardi* Bürger, 1905 and all organisms or species that share a more recent common ancestor with *Mastigella eilhardi* Bürger, 1905 than with *Entamoeba histolytica* Schaudinn, 1903. This is a branch-based definition; qualifying clause – the name does not apply if *Protosporangium articulatum* Olive & Stoianovich, 1972, *Dictyostelium discoideum* Raper, 1935, or *Filamoeba nolandi* Page, 1967 fall within the specified clade.

Remarks: The term Pelobiontida originally included only the genus *Pelomyxa* Greef, 1874. The term has since been emended by Griffin (1988) specifically to include *Pelomyxa* and mastigamoebids on the grounds both have flagella; it has subsequently been used to include different lineages of Archamoebae (see Ptáčková *et al*., 2013). Using the definition presented here, Pelobiontida is composed of three families (Pelomyxidae, Rhizomastixidae, Mastigamoebidae), of which Mastigamoebidae includes aflagellated mastigamoebids—genera *Endolimax* and *Iodamoeba*—that have in the past been treated as entamoebids. We choose to use this name for the whole order because it indicates the typical life style of most species (they live in freshwater sediments; greek word “pelos” means mud), and because Pelobiontida or pelobionts has been used to refer to the whole group in numerous publications from the past two decades.

Suborder Mastigamoebina Frenzel, 1897 (stat. nov.)

(Eukaryota: Amoebozoa: Conosa: Archamoebae: Pelobiontida)

Definition: The least inclusive clade containing *Rhizomastix libera* Ptáčková *et al.*, 2013, *Mastigamoeba balamuthi* (Chávez *et al.*, 1986), and *Mastigamoeba abducta* Ptáčková *et al.*

2013. This is a node-based definition: it is intended to apply to a crown clade; qualifying clause – the name does not apply if *Entamoeba histolytica* Schaudinn, 1903, *Mastigella eilhardi* Bürger, 1905, *Pelomyxa palustris* Greeff, 1874, or *Dictyostelium discoideum* Raper, 1935 fall within the specified clade.

Remarks: Mastigamoebina encompasses two families, Mastigamoebidae Goldschmidt , 1907 and Rhizomastixidae Ptáčková *et al.* 2013. We do not list the family Endolimacidae here since it has been shown that both its genera, *Endolimax* and *Iodamoeba*, branch within Mastigamoebidae (Stensvold *et al*. 2012) and the genus Endolimax was transfered to the family Mastigamoebidae by Ptáčková et al. 2013. Mastigamoebidae encompasses two clades, currently named Mastigamoebidae "A" (e.g. *Mastigamoeba balamuthi*, *M. punctachora*, *M. schizophrenia*) and Mastigamoebidae "B" (e.g. *Mastigmoeba simplex*, *M. guttula*, *Endolimax* spp., *Iodamoeba butschlii*) (Ptáčková *et al.* 2013), which should be given the rank of subfamily upon formal revision of the nominal species and genera contained within them (synapomorphies are defined in section 3.2).

Suborder Pelomyxina Starobogatov, 1980 (stat. nov.)

(Eukaryota: Amoebozoa: Conosa: Archamoebae: Pelobiontida)

Definition: The clade consisting of *Pelomyxa palustris* Greeff, 1874 and all organisms or species that share a more recent common ancestor with *Pelomyxa palustris* Greeff, 1874 than with *Mastigamoeba balamuthi* (Chávez *et al.*, 1986). This is a branch-based definition; qualifying clause – the name does not apply if *Rhizomastix libera* Ptáčková *et al.*, 2013, *Entamoeba histolytica* Schaudinn, 1903, *Dictyostelium discoideum* Raper, 1935, or *Mastigamoeba guttula* Ptáčková *et al.*, 2013 fall within the specified clade.

Remarks: Pelomyxina encompasses a single family, Pelomyxidae. We formally transfer *Mastigamoeba bovis* Liebetanz, 1910 to the genus *Mastigella* as ***Mastigella bovis*** **comb. nov.**, because it shows no connection between the nucleus and the flagellum.

Order Entamoebida Cavalier-Smith, 1993

(Eukaryota: Amoebozoa: Conosa: Archamoebae)

Definition: The clade consisting of *Entamoeba histolytica* Schaudinn, 1903 and all organisms or species that share a more recent common ancestor with *Entamoeba histolytica* Schaudinn, 1903 than with *Mastigella eilhardi* Bürger, 1905. This is a branch-based definition; qualifying clause –

the name does not apply if *Dictyostelium discoideum* Raper, 1935, *Pelomyxa palustris* Greeff, 1874, or *Mastigamoeba balamuthi* (Chávez *et al.*, 1986) fall within the specified clade.

Remarks: Entamoebida encompasses a single family, Entamoebidae. Cavalier-Smith (1993) did not specify genera included in the family Entamoebidae. Because genera *Endolimax* and *Iodamoeba* have been transferred to the different family several years later (Cavalier-Smith *et al*., 2004), it is clear that originally, Entamoebida was composed of genera *Entamoeba*, *Endamoeba*, *Endolimax* and *Iodamoeba*. We consider genera *Endolimax* and *Iodamoeba* as members of Mastigamoebidae.

Archamoebae *incertae sedis*

The phylogenetic position of some taxa within Archamoebae remains unclear and needs to be resolved using molecular methods. Currently, no molecular data from these organisms are available and the morphology of the taxa does not suggest obvious synonymy with any of the taxa defined above. 1. Family Tricholimacidae Cavalier-Smith, 2013 with sole genus and species *Tricholimax hylae*. 2. Genus *Endamoeba*. 3. Genus *Mastigina*.

3.4. Ancestral features and evolutionary trends in Archamoebae

The last common ancestor of all Archamoebae was an anaerobic amoeboid flagellate. Subsequently, several groups within Archamoebae partially lost flagellum-mediated movement, and some have even lost the entire flagellar apparatus, as seen in Entamoebidae and some mastigamoebids (*Endolimax*, *Iodamoeba*). *Mastigamoeba balamuthi* (Mastigamoebidae A) and *Entamoeba histolytica* (Entamoebidae) differ significantly in the complexity of their mitochondrial metabolism, with *E. histolytica* possessing an extremely reduced mitochondrial derivative, the mitosome, while *M. balamuthi* possessing a hydrogenosome (Tovar *et al.*, 1999; Mi-Ichi *et al.*, 2011; Nývltová *et al.*, 2015). Based on our phylogenetic analysis, we can conclude that both of the laterally transferred biochemical pathways that have been found in those species, *i.e.* the ε-proteobacterial NIF system for FeS cluster assembly and the mitochondrial-targeted sulphur activation pathway (see above), were present in the last common ancestor of Archamoebae.

Now, we can also conclude that the ancestors of Archamoebae, Pelobiontida, Pelomyxina, and Mastigamoebina were free-living protists. The endobiotic life style appeared at least three times during the evolution of Archamoebae: in the ancestor of Entamoebidae; in the ancestor of the '*Endolimax* + *Iodamoeba*' clade (in Mastigamoebidae B), and within the genus *Rhizomastix*. Although we cannot exclude the hypothesis that the last common ancestor of Mastigamoebina, or even of the whole Archamoebae, was endobiotic, we consider such scenarios much less plausible because other conoseans and most Archamoebae species are free-living. Almost all members of Pelomyxidae and Mastigamoebidae are free-living, with *Tricholimax hylae, Mastigella bovis* and *Mastigamoeba* sp. ('Mastigamoebidae A') found in vacuoles in *Pelomyxa belevskii* as potential exceptions (Ptáčková *et al.*, 2013, see below). In SSU rRNA gene trees the closely related genera of endobiotic mastigamoebids, *Endolimax* and *Iodamoeba*, form a internal branch of otherwise free-living Mastigamoebidae (Stensvold *et al.*, 2012; Ptáčková *et al.*, 2013). The deepest split in the genus *Rhizomastix* is between free-living *Rhizomastix libera* and other *Rhizomastix* spp. Some of these species are endobiotic, while *R. elongata* was isolated from abandoned cesspit and was suspected to be free-living as well (Zadrobílková *et al.*, in press). Thus an "early-endobiotic" scenario would require multiple reversions of the endobiotic lifestyle within Pelobiontida. Endobiotic-to-free-living transitions are rare in the nature and thus less probable than *vice versa*. Nevertheless, Archamoebae is one of very few protistan lineages that are suspected to contain secondarily free-living organisms that evolved from endobiotic ancestors. The most studied example is *Entamoeba moshkovskii* (Clark *et al.*, 2006; Clark and Diamond, 1997), which has repeatedly been isolated from animal or human stool as well as water sediments (see Heredia *et al.*, 2014) and is probably amphizoic (*i.e.*, both free-living and endobiotic). Recently, *Entamoeba marina,* that is closely related to E. moshkovskii, has been isolated from tidal flat sediment (Shiratori and Ishida, in press). It indicates that Entamoebida is still an undersampled group of protists.

There remain three endobiotic lineages of Archamoebae whose phylogenetic position is uncertain, and for which no sequence data are currently available: *Tricholimax hylae*, *Endamoeba* spp., and *Mastigella bovis*. *Tricholimax hylae* shares morphological features both with Pelomyxidae and Mastigamoebidae (see Brugerolle 1982, 1991; Walker *et al.*, 2001) and was recently assigned as a sole genus and species in the family Tricholimacidae Cavalier-Smith, 2013. *Mastigella bovis* was described from the rumen of cattle as a member of the genus *Mastigamoeba* (Liebentanz, 1910) and listed as a probable member of the genus *Mastigella* by Ptáčková *et al.* (2013); we transferred it to the genus *Mastigella*, see above. All these three lineages could possibly represent other independent transitions between free-living and endobiotic lifestyle. However, their presumed phylogenetic positions have to be clarified by analyses of molecular data.

3.5.**Flagellar apparatus of Conosa and Archamoebae**

The last common ancestor of Conosa (= Variosea, Macromycetozoa, and Archamoebae) was very probably an aerobic, biflagellated protist equipped with both anterior and recurrent flagellum (Cavalier-Smith, 1998, 2013; Cavalier-Smith *et al.*, 2015). Assuming that what is now known to be the most phylogenetically-widespread flagellar morphology is ancestral, the basal bodies of its flagella may have been associated with five different microtubular elements defined as MTA1–MTA5 (Wright *et al.*, 1979); the basal body of the anterior flagellum would have been associated with MTA1–3, while the posterior basal body would have been associated with MTA4 and 5. Yubuki and Leander (2013) synonymized MTA3 with the eukaryotic root R3 and MTA2 with superficial microtubules that originate on it. Further, they hypothesized that MTA4 is homologous to the eukaryotic root R2, and MTA5 corresponds to the root R1**.** These four cytoskeletal elements must have arisen very early in the evolution of eukaryotes (Yubuki and Leander, 2013). Some flagellated members of the Conosa also possess MTA1, which arises from a microtubule organizing center (MTOC) located at the proximal part of the basal body of the anterior flagellum. In those conoseans that have the flagellar apparatus associated with the nucleus, MTA1 microtubules (if present) extend from the MTOC towards the apical part of the nucleus and follow its surface (*e.g.* Wright *et al.*, 1979; Spiegel, 1981).

The clade Conosa was morphologically defined by a monolayer of microtubules partially or completely surrounding the anterior basal body and diverging towards the nucleus and cell posterior as a half or three-quarters open cone (Variosea and Mycetozoa) or a complete cone (Archamoebae) (Cavalier-Smith, 1998, 2013, 2015). The cone as defined by Cavalier-Smith is the same structure as MTA2 in Wright's terminology, and the superficial microtubules of Yubuki and Leander's terminology (Yubuki and Leander, 2013). However, conoseans equipped with both posterior and anterior flagella possess a more complex cone, formed not only by microtubules of MTA2, but also MTA3–MTA5 (Wright *et al.* 1979). In addition, the MTA1 of Macromycetozoa *sensu* Berney *et al.* (2015) and protosteloid Variosea usually form an inner cone associated with the apical part of the nucleus (e.g. Wright *et al.*, 1979; Spiegel, 1981; Walker *et al.*, 2001, 2003). During the evolution of Conosa, the ancestral flagellar apparatus has been transformed into many different variants. In some lineages, the posterior flagellum and associated cytoskeleton (Pelobiontida, *Planoprotostelium*, *Cavostelium*, *Phalansterium*), or even the whole flagellar apparatus (Entamoebidae, '*Endolimax* and *Iodamoeba*' clade in the Pelobiontida, *Flamella*, *Acramoeba*, *Grellamoeba*, *Filamoeba*) have been lost (see Spiegel, 1981; Appendix 3 in Walker *et al.*, 2001; Cavalier-Smith *et al*., 2015, Ptáčková *et al*., 2013).

Walker et al. (2001), Cavalier-Smith (2013) and Yubuki and Leander (2013) have all suggested that the microtubular cone is homologous to the MTA2. This interpretation is, however, problematic in two main aspects: (1) the superficial microtubules of other Conosa and eukaryotes form a sheet anchoring close to the dorsal side of the anterior basal body, so MTA-2 would have had to undergo intricate rearrangements during the evolution of Archamoebae. (2) Radiating microtubules arise in multiple layers from the basal body of several Archamoebae (see Brugerolle 1982, 1991; Simpson et al 1997; Walker *et al.*, 2001; Frolov *et al.* 2011). Such architecture is highly unusual for superficial microtubules since they form a monolayer in other eukaryotes. The other possible homology would be with MTA1 as defined by Wright et al. (1979). However, if this were correct, then the cone of Archamoebae would not be homologous to the cone of other Conosa as defined by Cavalier-Smith (2013). In our opinion, it is currently impossible to decide between these two interpretations of homology of the archamoebean cone because the flagellar apparatus of Archamoebae is too simplified and derived. Individual microtubular elements cannot be unequivocally homologized with microtubular ribbons of other Conosa and Eukaryota.

Regardless of which scenario is correct, it is clear that flagella of most *Pelomyxa* spp. and *Tricholimax hylae* have lost motility secondarily, and their non-'9+2' pattern of axoneme microtubules (see Walker *et al.*, 2001) is aberrant. Besides, members of the genus *Pelomyxa* have multiplied the flagellar apparatus and nucleus (*e.g.* Chistyakova *et al.*, 2014). Ancestors of Entamoebida and '*Endolimax* + *Iodamoeba*' clade have completely lost the flagellar apparatus.

We conclude that the last common ancestor of Pelobiontida, or possibly of all of the Archamoebae, resembled members of the genera *Mastigamoeba* and *Mastigella*: possessing a single motile, anterior flagellum with the classical '9+2' pattern of axoneme microtubules, lacking a posterior flagellum, and outer dynein arms in the anterior flagellar axoneme; and with the anterior basal body having a cone of radiating microtubules and lateral microtubular ribbon.

4. **CONCLUSIONS**

Our work provides the first robust evidence for the primary divergence at the base of Archamoebae between Entamoebida and a major clade containing all flagellate Archamoebae (Pelobiontida). Based on these results, we revised the higher classification of Archamoebae and concluded that the bacterial nitrogen fixation system was present in the last common ancestor of Archamoebae (LCAA), mitochondrial derivatives of the LCAA contained a sulfate activation pathway, and that the endobiotic life-style has arisen at least three times during the evolution of the group. Our comparative ultrastructural analysis of Mastigamoebidae „A" and „B" showed synapomorphies of these two clades and indicates that *Mastigamoeba aspera*, the type species of the genus, belongs to the Mastigamoebidae „A". Future studies on individual lineages included in the present study may help us to elucidate the evolution of anaerobic metabolism, via lateral gene transfer; as well as to understand the transition from free-living to endobiotic and parasitic lifestyles.

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Legends to tables and figures

Figure 1: Bootstrap support of Archamoebae and its internal nodes as seen in single-gene and seven-gene phylogenetic analyses. In the lower side of the table, all alternative groups that were recovered with bootstrap support >50 in at least one single gene tree are also presented.

Figure 2: Phylogenetic tree of eukaryotes based on concatenation of seven protein-coding genes: actin, α-tubulin, β-tubulin, EF1α, EF2, HSP70, HSP90. The tree is based on alignment of 3585 positions and 78 taxa. The topology was constructed in PhyloBayes under CAT Poisson model. The values at nodes represent PhyloBayes posterior probabilities, RAxML non-parametic bootstraps, and IQ-tree bootstrap support. The values lower than 50% or 0.5 are marked by „*“; branches that were missing in the best ML tree topology are marked by „-“. Clades supported by statistical support higher than 0.98/90/90 are marked by thick branches. Taxa whose ESTs were newly sequenced are in bold. Photos: (A) – *Rhizomastix libera* strain IND8, (B) – *Mastigella eilhardi* strain ATCC 50342. Scale Bar = 10 µm. With respect to results published by Derelle et al. (2015), we did not mark *Malawimonas* as a member of Excavata.

Figure 3: Mastigamoebidae A taxa show more morphological variation and are generally larger than Mastigamoebidae B taxa. Microtubules of the cone in Mastigamoebidae A arise laterally, from along the sides of the basal body; whereas those in Mastigamoebidae B arise longitudinally, close to the base of the basal body. (A–J) show a representative of Mastigamoebidae A, *Mastigamoeba balamuthi*. (A–F) Light microscopy, DIC optics (A, B) Small amoebae with 2–4 nuclei, the dominant life cycle stage of *M. balamuthi*; (C) "Giant" amoeba form with ca. 50 nuclei; (D) Binucleate gliding flagellate; (E) Uninucleate flagellate; (F) Swimming form with posterior pseudopodia. (G–J) Transmission electron-microscopy of the flagellar apparatus, serial sections, 90 nm apart: Flagellar apparatus, showing lateral emergence of cone microtubules from the basal body (Bb), transitional cylinder at the base of the transition zone (TC), and electron-dense column (DC) at the top of the transition zone, from which the central pair of axonemal microtubules emerges. A microtubular root (MR) emerges laterally from the basal body, with a bilaminar root sheet (RS) on its distal edge. **(K–P) show a representative of Mastigamoebidae B, *Mastigamoeba guttula*.** (K, L) Gliding cells, strains

LUH2NS4 and HRADANAN respectively; (M) Aflagellate cell, LUH2NS4; (N–P) Strain HRADANAN, transmission electron-microscopy of the flagellar apparatus; (N) Transverse section through the basal body, close to the base, showing longitudinal alignment of cone microtubules (MC) and the lateral emergence of the microtubular root (MR); (O–P) Longitudinal sections through the flagellar apparatus, showing the laterally-emerging microtubular root (MR), the transitional cylinder (TC), and the longitudinally-emerging microtubular cone. The transition zone is ca. 200 nm long, which is short, similar to that seen in *Mastigamoeba simplex* (Walker et al. 2001). Scale bar in K = 20 µm for a, b; 25 µm for c; 15 µm for d; 20 µm for e; 750 nm for f–j; 10 µm for k, l, m; 500 nm for n; 750 nm for o, p. Micrographs K, L, M are reproduced from Ptáčková *et al*. 2013 with permission from Elsevier.

Figure 4: Representative flagellar apparatuses from Mastigamoebidae A and B. (A) Schematic diagram of the microtubular flagellar apparatus of *Mastigamoeba punctachora*, a representative of Mastigamoebidae A. Note that the cone of microtubules (MC) arises laterally from both the sides and the base of the basal body. The flagellar transition zone (TZ) is long and contains a dense column (DC). An MTOC below the basal body has not been confirmed in *M. punctachora* so this characteristic of some members of Mastigamoebidae A is not shown here. Fl, flagellar axoneme; TC, transition zone cylinder; Bb, basal body; RS, bi-laminar root sheet; MR, microtubular root; SMt, side microtubules (part of the microtubular cone). **(B) Schematic diagram of the microtubular flagellar apparatus of Mastigamoeba simplex, a representative of Mastigamoebidae B.** Note that the cone of microtubules (MC) arises longitudinally from near the base of the basal body. The flagellar transition zone (TZ) is short and contains no extra elements. Both figures are reproduced from Walker *et al*. 2001, with permission from Elsevier.

Table 1: Flagellar apparatus characteristics of representatives of Mastigamoebidae A and B clades.

Figure 1 (RGB)
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CLADE	Bootstrap support in phylogenetic trees (RAxML)							
	ACT	ATUB*	BTUB	EF1A	EF2	HSP70	HSP90*	ALL
Archamoebae			57			83	66	100
Entamoebida	99	100	100	100	100	100	100	100
Pelobiontida					91		85	100
Pelomyxina	76			96	50	99		100
Mastigamoebina				53		97		96
Mastigamoebidae			85		93			93
Rhizomastixidae	100	100	100	100	100	100	100	100
Pelomyxina + Mastigamoebinae	52							
Pelomyxina + Rhizomastixidae					51			
Pelomyxina + Entamoebida						69		
Mastigella + Mastigamoebidae							52	
Mastigamoeb. B + Rhizomastixidae						59		

* Sequences of these genes are missing in *Pelomyxa* data

0 50 70 80 90 100%

Figure 2 (RGB)
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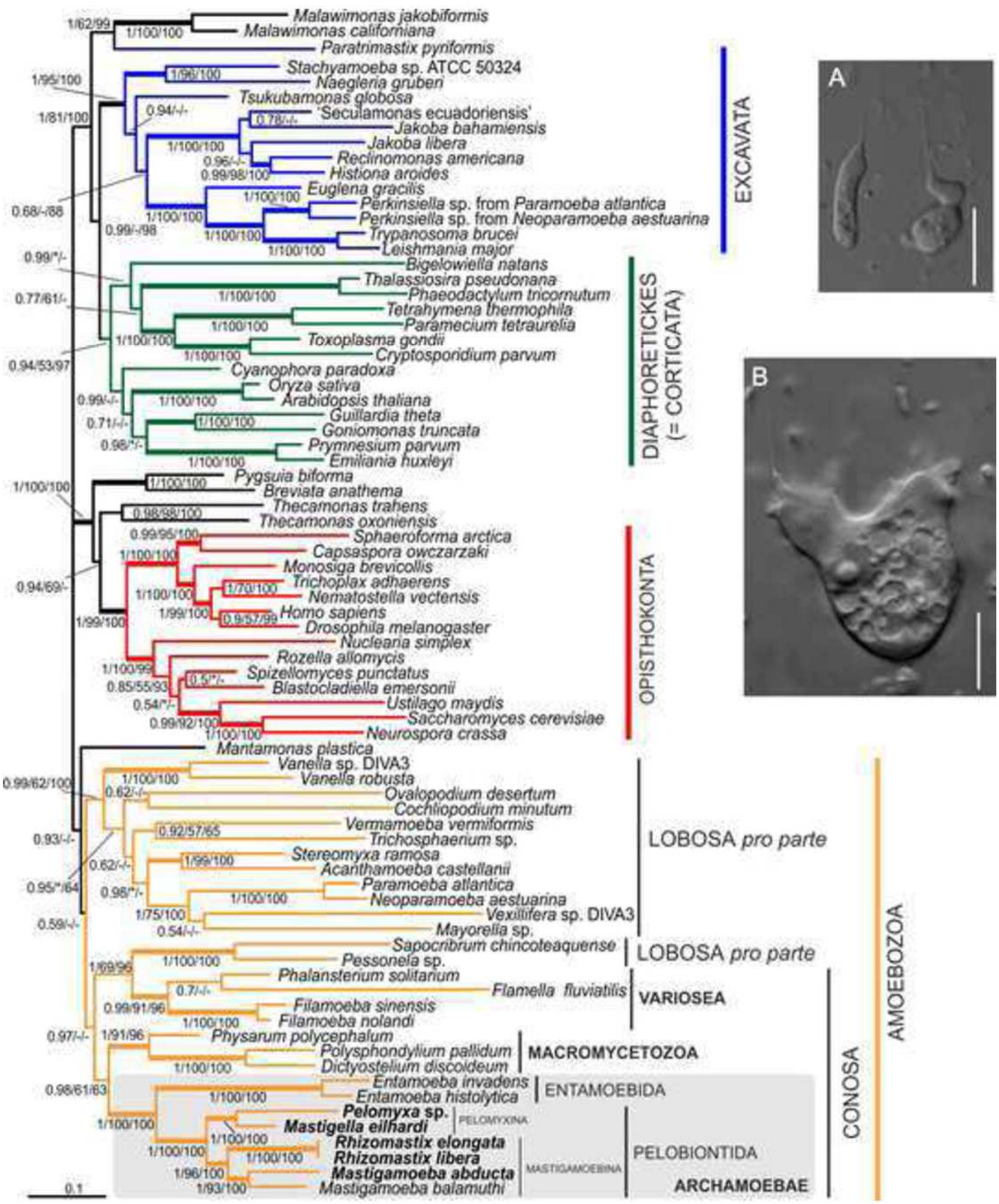

Figure 3
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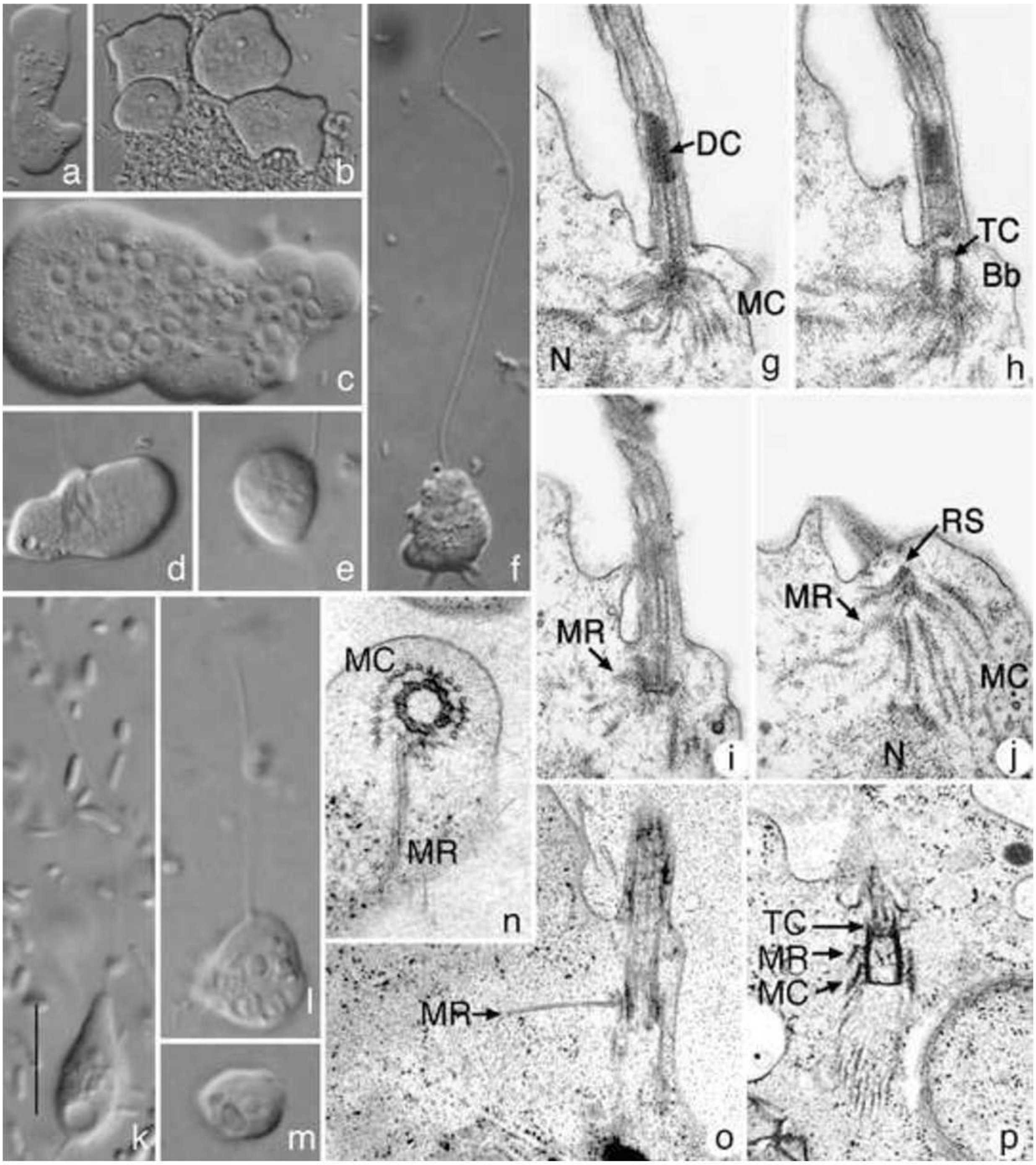

Figure 4
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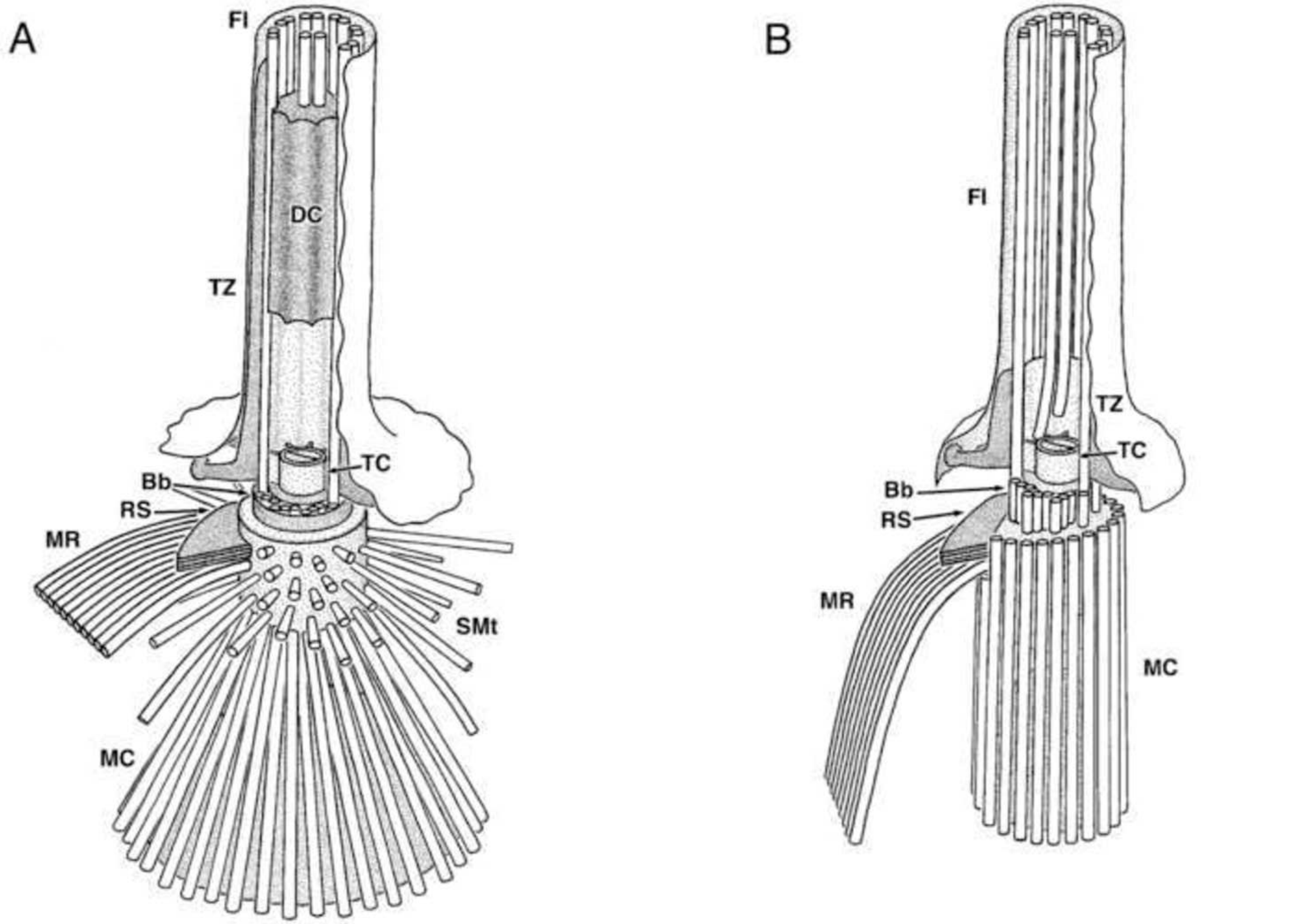

Table 1

Larger group/ Species	Flagellum	9+2 axoneme	Dynein arms missing	Transition Zone length	Transitional Dense Column or Spiral	Transition Zone cylinder	Cartwheel in base of Bb	Bb length	Lateral root of MT	Bi-laminar root sheet	MT of cone emerging from sides of Bb	MT of cone emerging from Bb base	Electron-dense material around Bb	Electron-dense MTOC below Bb	References
Mastigamoebidae "A"															
Mastigamoeba aspera	Long (>2× cell)	9+2	?	"Short"	?	?	?	250nm	Yes	?	Many	Most of cone	No	Triangle	Chystyakova *et al.* 2012
Mastigamoeba balamuthi	Long (>2× cell)	9+2	Yes, outer	700nm	DC	Yes	?	250nm	Yes	Yes	Most of cone	Few	No	No	Chavez *et al.* 1986, Brugerolle 1991, **this paper**
Mastigamoeba punctachora	Long (>2× cell)	9+2	Yes, outer	1000nm	DC	Yes	No	250nm	Yes	Yes	Most of cone	Few	No	Possibly?	Bernard *et al.* 2000, Walker *et al.* 2001
Mastigamoeba schizophrenia	Long (>2× cell)	9+2	Yes, outer	700nm	DC	Yes	Yes	250nm	Yes	Yes	Single layer at base, lateral	No	No	No	Simpson *et al.* 1997
Mastigamoeba sp.	Long (>2× cell)	9+2	?	700nm	Spiral + central filament	Yes	?	250nm	Yes	Yes	Single layer at base, lateral	No	No	Ring	Brugerolle 1991
Summary	Long	9+2	Yes	Long	Transit-ional element present	Yes	?	250nm	Yes	Yes	MT arise laterally from sides of BB	Some taxa	No	Some taxa	
Mastigamoebidae "B"															
Mastigamoeba simplex	Long (>2× cell)	9+2	Yes, outer	200nm	No	Yes	Yes	250nm	Yes	Yes	Single layer at base, in longitudinal axis	No	No	No	Walker *et al.* 2001
Mastigamoeba guttula	Long (>2× cell)	9+2	?	200nm	No	Yes	?	250nm	Yes	Yes	Single layer at base, in longitudinal axis	No	No	No	**This paper**
Summary	Long	9+2	?	Short	No	Yes	?	250nm	Yes	Yes	MT arise longitudinally	No	No	No	

8. Závěrečné shrnutí

Během několika posledních let se nám podařilo nashromáždit unikátní sbírku kultur, která čítá stovky izolátů volně žijících a endobiotických protist. Nedílnou součást předstvaují izoláty archaméb, které jako jedni z mála dokážeme dlouhodobě kultivovat. Za velký úspěch mimo jiné považujeme, že některé izoláty druhu *Pelomyxa schiedti* v našich podmínkách přežily až dva roky a konkrétně izolát SKADARSKE dokonce přežívá doteď. Připomeňme, že rod *Pelomyxa* je všobecně velmi těžko kultivovatelný. Díky stabilním kulturám bylo možné získat velké množství nových sekvenčních dat z převážně volně žijících archaméb, které přispěly k detalnější představě o vzájemných příbuzenských vztazích ve skupině. Právě kvůli chybějícím datům zůstávala doposud převážná část fylogenetického stromu archaméb nerozřešena. Pomocí molekulární fylogeneze a za současné podpory morfologických znaků se nám podařilo prokázat, že málo probádaný rod *Rhizomastix* patří mezi archaméby. Stejně tak jsme odhalili, že SSU rDNA sekvence původně přiřazovaná druhu *Mastigella commutans* pravděpodobně patří druhu *Mastigamoeba punctachora* a sekvence SSU rDNA prezentovaná jako *Pelomyxa palustris* patří ve skutečnosti *P. stagnalis*. Celkem jsme popsali 13 nových druhů archaméb a získali jsme 31 dosud nepublikovaných sekvencí genu pro SSU rRNA a 22 nových sekvencí genu pro aktin.

Z výsledků fylogenetických analýz vyplývá, že se archaméby dělí na čtyři hlavní čeledí: Rhizomastixidae, Entamoebidae, Pelomyxidae a Mastigamoebidae. Analýzy založené pouze na genu pro SSU rRNA a genu pro aktin ale spolehlivě nevyřešily vzájemné vztahy mezi těmito hlavními liniemi. Pro získání přesnějších výsledků bylo potřeba provést muligenovou analýzu. Aby byly všechny linie dostatečně zastoupeny, bylo nutné rozšířit dostupná data pro tvorbu datasetu, a proto byly analyzovány transkriptomy z nových druhů archaméb. Jednalo se o druh izolovaný ze septiku *Rhizomastix elongata* a volně žijící *R. libera* (oba čeleď Rhizomastixidae) a volně žijící druhy *Mastigella eilhardi* a *Pelomyxa* sp. (oba čeleď Pelomyxidae) a *Mastigamoeba abducta* (Mastigamoebidae B). Jako první jsme provedli multigenovou analýzu archaméb, ve které byly zastoupeny všechny hlavní linie. Kromě vyřešení vzájemných vztahů mezi jednotlivými rody jsme ze získaných dat dále zjistili, že poslední společný předek archaméb již měl jak ε-proteobakteriální NIF systém, tak dráhu aktivace sulfátu lokalizovanou v mitochondrii.

Z našich dat vyplývá, že rod *Rhizomastix*, který tvoří čeleď Rhizomastixidae, je monofyletický a rozpadá se na volně žijící a endobiotickou linii. Původně jsme se na základě našich výsledků domnívali, že je tato čeleď blízce příbuzná rodu *Entamoeba*, ale multigenová analýza založená na sedmi genech (aktin, α-tubulin, β-tubulin, EF1α, EF2, HSP70, HSP90) tuto hypotézu vyvrátila. Ukázalo se, že rod *Rhizomastix* je ve skutečnosti sesterský čeledi Mastigamoebidae, která zahrnuje především volně žijící druhy. Jeho buňky mají navíc unikátní ultrastrukturu, která představuje nový typ cytoskeletární organizace u archaméb. Jedná se především o přítomnost tzv. rhizostylu, který je pravděpodobně modifikací mikrotubulárního koše ostatních bičíkatých archaméb. Ultrastruktura *R. elongata* izolovaného ze septiku je dokonce ještě komplexnější než u volně žijícího *R. libera*.

Skupina Mastigamoebidae se na fylogenetických stromech člení na dvě linie Mastigamoebidae A a Mastigamoebidae B, což je podpořeno také odlišnými znaky, které jsou pro jednolivé skupiny charaktristické. Mastigamoebidae A zahrnují morfologicky více variabilní a obecně větší druhy a mikrotubuly, které vytváří konus, vystupují po stranách celé délky bazálního tělíska. Na druhou stranu zástupci skupiny Mastigamoebidae B mají menší a uniformnější buňky a mikrotubuly koše vychází podélně z báze bazálního tělíska. Z multigenové analýzy vyplývá, že až na výjimky parazitická skupina Entamoebidae představuje hlubohou linii archaméb, která je sesterská skupině Pelobiontida (původní pelobionti), nyní tvořené čeleděmi Pelomyxidae, Rhizomastixidae a Mastigamoebidae.

Čeleď Pelomyxidae sestává z rodů *Mastigella* a *Pelomyxa*, přičemž první jmenovaný je parafyletický. Na společnou evoluční historii ukazují také některé morfologické znaky, které oba dva výše zmíněné rody sdílí. Jedná se např. o tvar buňky, pomalý pohyb bičíku, počet jader, uspořádání heterochromatinu v jádře nebo přítomnost endosymbiotických prokaryot v buňce.

Na základě našich dat lze říci, že parazitismus se u archaméb objevil v evoluci nejméně třikrát nezávisle na sobě, a to u posledního společného předka čeledi Entamoebidae, v čeledi Mastigamoebidae B u posledního společného předka rodů *Iodamoeba* a *Endolimax* a v rámci rodu *Rhizomastix*. Tato hypotéza je ale podmíněna předpokladem, že volně žijící druhy archaméb sekundárně neopustily endobiotický způsob života. Navíc stále neznáme fylogenetickou pozici parazitického rodu *Endamoeba* a druhů *Tricholimax hylae* a *Mastigamoea bovis*, které můžou spadat mezi výše jmenované parazitické linie nebo přestavovat další nezávislé parazitické taxony. Doposud se nepodařilo získat žádná molekulární data ani z rodu *Mastigina*.