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Perkinsiella amoebae-like endosymbionts of *Neoparamoeba* spp., relatives of the kinetoplastid *Ichthyobodo*

Iva Dyková[1,2,*], Ivan Fiala[1,2], Jiří Lom[1] and Julius Lukeš[1,2]

[1]Institute of Parasitology, Academy of Sciences of the Czech Republic, Branišovská 31, 370 05 České Budějovice, Czech Republic, Fax: 0042-385 310 388; E-mail iva@paru.cas.cz
[2]Faculty of Biological Sciences, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic

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Eukaryotic endosymbionts ("parasomes") were studied in *Neoparamoeba* strains of different origin including two strains of *N. pemaquidensis* (Page, 1970), the agent of amoebic gill disease (AGD). Comparative study of the endosymbionts revealed their mutual ultrastructural similarity and also similarity with the endosymbiont *Perkinsiella amoebae* Hollande, 1980 described from *Janickina chaetognathi* and *J. pigmentifera*. Morphological features supported Hollande's hypothesis on the kinetoplastid origin of *P. amoebae* but the first conclusive results were obtained in this study using marker genes. The recognition of euglenozoan spliced leader RNA (SL RNA) gene sequences in the genomic DNA of endosymbionts from five *Neoparamoeba* strains together with the acquisition of one SSU RNA gene sequence allowed us to specify the relationship of the endosymbionts under study with kinetoplastids. Phylogenetic analyses of SSU rRNA gene sequence data currently available revealed close relationship of the first sequenced *Perkinsiella amoebae*-like organism with *Ichthyobodo necator*. Sequence comparisons disclosed that *P. amoebae*-like organisms possess SL RNA genes with a significant level of divergence from other kinetoplastids. However, the sequences are more closely related to kinetoplastids and *Diplonema* spp. than to euglenids. Three types of the SL RNA gene sequences obtained from *P. amoebae*-like organisms were congruent with phylogeny of their *Neoparamoeba* host strains.

Key words: *Neoparamoeba* spp.; parasome; *Perkinsiella amoebae*-like organisms; spliced leader RNA genes; SSU rRNA gene; Kinetoplastida, kinetoplast; *Ichthyobodo*.

Introduction

Since the description of *Paramoeba eilhardi* Schaudinn, 1896, the so-called parasome (i.e., "Nebenkörper" or "Amphosome"), a conspicuous formation localised in the proximity of the amoeba nucleus, has been taken as the pivotal diagnostic feature of the genus *Paramoeba* Schaudinn, 1896. Although the parasome has been known for a long time in *Paramoeba* species, understanding of its nature has progressed very slowly. The parasome was taken first for a cell organelle, but later considered to be a "secondary" or "parasitic" nucleus (Schaudinn 1896; Janicki 1912; Chatton 1953; Grell 1961). Only by means of electron microscopy was it possible to obtain more detailed descriptions. The study of *P. eilhardi* trophozoites resulted in a cautious interpretation of the para-

*corresponding author

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some as a cell, no longer as a mere cell organelle, by Grell and Benwitz (1970). Similarly in an ultrastructural study of *P. perniciosa* Sprague, Beckett and Sawyer, 1969, Perkins and Castagna (1971) characterised the parasome as a "discrete organism", "not an organelle of amoeba". Based on ultrastructural data, the hypothesis that the parasome was a symbiotic organism of unknown origin was cautiously formulated for the first time by Grell and Benwitz (1970) and then by Page (1973).

The first paper that interpreted the "parasome" using ultrastructural findings was that of Hollande (1980). He described the parasome in parasitic amoebae of chaetognaths [*Janickina pigmentifera* (Grassi, 1881) and *J. chaetognathi* (Grassi, 1881)] and defined it as a symbiotic organism with a eukaryotic nucleus but lacking kinetosomes, mitochondria, Golgi apparatus and endoplasmic reticulum. He interpreted the DNA-containing structure in the symbiont's centre as a kinetoplast and proposed new generic and specific names (*Perkinsiella amoebae*) for this organism. Analysing individual characters of its cell organisation and using an elimination method of comparison, Hollande (1980) concluded that the closest relatives of *Perkinsiella amoebae* were kinetoplastid flagellates. As evidence he quoted *Cryptobia vaginalis*, which according to him had a kinetoplast of a type similar to the DNA structure in *P. amoebae*.

Organisms morphologically similar to *P. amoebae* Hollande, 1980 (PLOs) have been described in the cytoplasm of seven named species of amoebae that, according to the recent concept of the family Paramoebidae Poche, 1913 (Page 1987), belong to the genera *Paramoeba* Schaudinn, 1896, *Neoparamoeba* Page, 1987 and *Janickina* Chatton, 1953 (Grell and Benwitz 1970; Perkins and Castagna 1971; Page 1973; Hollande 1980; Jones 1985). Amoebae containing PLOs were isolated in far distant localities, from water and sand as well as from vertebrate and invertebrate hosts. These observations, plus the pathogenicity clearly proven for three species containig PLOs [*Paramoeba perniciosa*, *P. invadens* Jones, 1985 and *Neoparamoeba pemaquidensis* (Page, 1970)] provided strong motivation for detailed study of PLOs. In addition, the understanding of the "parasome" (*Perkinsiella amoebae* and PLOs) represents an exceptional challenge from the phylogenetic point of view. In symbiotic associations in which symbionts are modified due to morphological and functional integration with the host organism, one of the most interesting topics is the evolutionary origin of the symbiont, i.e., its relationship to "free-living" organisms.

The purpose of this investigation was to characterise PLOs that we have found in trophozoites of *Neoparamoeba* strains of different origin, compare them with data published on PLOs from other species of the family Paramoebidae and use molecular approaches to verify the hypothesis on the kinetoplastid origin of such endobionts proposed by Hollande (1980) for *Perkinsiella amoebae*. The morphology of PLOs living together with *Neoparamoeba* trophozoites was briefly presented and its close resemblance to that of *P. amoebae* was briefly mentioned in Dyková et al. (2000).

Since *Janickina* spp., hosts of *Perkinsiella amoebae*, are not available in culture collections of protists, we could not compare the type species of the endosymbiont with our symbionts. This, together with genetic differences disclosed among PLOs from different *Neoparamoeba* strains (see below), explains the rigorous usage of *Perkinsiella amoebae*-like organism/s (PLO/PLOs) in the current text.

Materials and methods

Nucleotide sequence data reported in this paper have been submitted to the GenBank™ and received the accession numbers AY163355 (SSU rRNA gene), AY163350–AY163354 (SL RNA genes).

Abbreviations

ISH – *in situ* hybridisation
FISH – fluorescent *in situ* hybridisation
PLO/PLOs – *Perkinsiella amoebae*-like organism/organisms
SL-RNA gene – spliced leader RNA or mini-exon gene
PLO/denomination of *Neoparamoeba* strain: PLO/AFSM2V, PLO/AFSM3, PLO/AFSM11, PLO/SM53, PLO/SM68.

Trophozoites of ten *Neoparamoeba* strains were tested for the presence of *Perkinsiella amoebae*-like organisms (PLOs) and used for morphological studies. Five strains of this set were used also for molecular studies. *Neoparamoeba* strains characterised in previous papers (Dyková et al. 2000; Fiala and Dyková 2003) were isolated from gills of farmed fishes *Scophthalmus maximus* (turbot, 6 strains) and *Dicentrarchus labrax* (sea bass, 3 strains). One seawater strain (CCAP 1560/7) was obtained from Culture Collection of Algae and Protozoa (Ambleside, UK). Trophozoites harvested from agar plate cultures (maintained as described in Dyková et al.

2000) represented the essential source of material for light microscopical, ultrastructural and molecular studies. These samples were supplemented with trophozoites cultured for a short period of time in 75% seawater (Sea salts, Sigma 9883) added to FHM (Fat Head minnow) cell culture (ECACC 88102401) strained off culture medium.

Nucleic acid staining

Four representatives of three distinct subclasses of DNA stains were used for detection of PLOs: the cationic dye acridine orange (Lachema C.I. 46005), Hoechst 33258 (Sigma B 2883) and DAPI (Sigma D 9542) of the group of indoles and imidazoles, and dimeric cyanine dye YOYO®-1 iodide (Molecular Probes Y-3601). The cells washed from agar plate cultures with 75% seawater (Sea salts Sigma 9883) were left to attach to slides coated either with Biobond (Electron Microscopy Sciences 71304) or with 0.1% Poly-*L*-lysine hydrobromide (Polysciences 9730). After 2 to 3 h in a dark wet chamber the cells were fixed according to the requirements of staining protocols (Vickerman 1977; Gicquaud and Tremblay 1991). Various fixatives (four mixtures of paraformaldehyde and glutaraldehyde with different concentrations of both components) and permeabilizing pretreatments that differed in exposure time were applied for YOYO-1 staining. Slides were mounted using 50% glycerol or antifade mixture (Traut et al. 1999). Fluorescence images were obtained in and photomicrographs were taken with an Olympus BX51 microscope and a Zeiss Axioplan 2.

Transmission electron microscopy

Over a long period of culturing (all strains under study were subcultured continuously for more than 1 year), 46 samples of *Neoparamoeba* trophozoites (20 samples from sea bass and 26 from turbot strains in 135 blocks) taken consecutively were used for ultrastructural studies. Trophozoites of *Neoparamoeba* strains were fixed with 3% glutaraldehyde in 0.1 M cacodylate or phosphate buffer and postfixed in 1% osmium tetroxide solution. Mollenhauer's Epon-Araldite resin, Poly/Bed 812-Araldite mixture or Spurr's resin were used for embedding. Ultrathin sections (as a minimum, 4 grids prepared of each block) were stained with 2% uranyl acetate in 50% methanol, post-stained with Reynold's lead citrate and examined with a JEOL JEM 1010 electron microscope operating at 80 kV.

Molecular characterisation of PLO

SL RNA gene

Since several attempts which aimed to isolate PLO from *Neoparamoeba* trophozoites failed, total DNA extracted for molecular characterisation of *Neoparamoeba* strains (Fiala and Dyková 2003) was subjected to a PCR amplification of potential spliced leader RNA (SL RNA or mini-exon) gene repeat known to be characteristic for Euglenozoa (euglenids, kinetoplastids and *Diplonema* spp.). PCR was performed with oligonucleotides ME-L (5′-cccgaattCTGTACTATATTGGT-3′) and ME-R (5′-catagctgtttcctcAATAAAGTACA-GAAACTG-3′) (Grisard et al. 1999) using conditions described by Fernandes et al. (1997). Gel-purified amplification products were cloned into the TOPO-TA vector (Invitrogen) and sequenced on an automatic sequencer CEQ™ 2000 (Beckman Coulter). The CEQ DTCS Dye Kit (Beckman Coulter) was used according to the manufacturer's protoc≠ol. Mini-exon repeat sequences of 11 organisms compared with sequences of PLOs from five *Neoparamoeba* strains were obtained from the GenBank database. Secondary structure of SL RNA was predicted using program Mfold v. 3.0 available on http://mfold.burnet.edu.au.

In situ hybridisation

In situ hybridisation was applied to visualise specific nucleic acid sequences at cellular and subcellular levels. The oligonucleotide IF1(5′AATTTCTGCTATAATAG TTCAG-3′) prepared by Generi Biotech was designed as complementary to the first 22 sites of SL RNA exon of PLO from *Neoparamoeba* strains AFSM2V and AFSM11. About 100 pmol of the IF1 primer was labelled with digoxigenin according to the manufacturer's instructions (DIG oligonucleotide Tailing Kit) (Roche). After being treated as described above, the cells on poly-*L*-lysine coated slides were fixed at room temperature (RT) for 30 min in a solution of 4% (w/v) formaldehyde, 5% (v/v) acetic acid, and 0.9% (w/v) NaCl, fixation was stopped by washing the slides with phosphate buffered saline (PBS), and the slides were stored in 70% ethanol at 4 °C. Next, the cells were dehydrated in a graded series of ethanol, washed in 100% xylene, rehydrated, and transferred into PBS. After a brief treatment with 0.1% (w/v) pepsin in 0.1 M HCl, the slides were washed in PBS and submerged for 10 min into 1% formaldehyde for post-fixation. In a humidity chamber, hybridisation was performed in the hybridisation mix (60% formamide; 300 mM NaCl; 30 mM sodium citrate; 10 mM EDTA; 25 mM NaH_2PO_4, pH 7.4; dextran sulphate; 250 ng/µl salmon sperm DNA; and 5 ng of probe per slide) for 20 h at 37 °C. Afterwards, the slides were washed in a solution of 60% formamide; 300 mM NaCl and 30 mM sodium citrate for 5 min at RT (three times) and at 37 °C (once), for 5 min in PBS, and for 30 min in a blocking solution (100 mM Tris, pH 7.5; 150 mM NaCl; 0.5% [w/v] blocking reagent). Detection was as follows: incubation in the anti-DIG-fluorescein antibody (1:5000) in blocking solution for 45 min at RT; wash in 100 mM Tris, pH 7.5; 150 mM NaCl; 0.05% Tween-20 three times for 10 min; dehydrated in a graded series of ethanol; air dried;

stained with DAPI (0.1 µg/ml) for 3 min at RT, rinsed with distilled water and mounted in the antifadant Dabco (Fluka). The slides were examined with a Zeiss Axioplan 100 microscope and images were recorded with a cooled charge-coupled device camera Meg F-View II (Soft Imaging Systems, Munster).

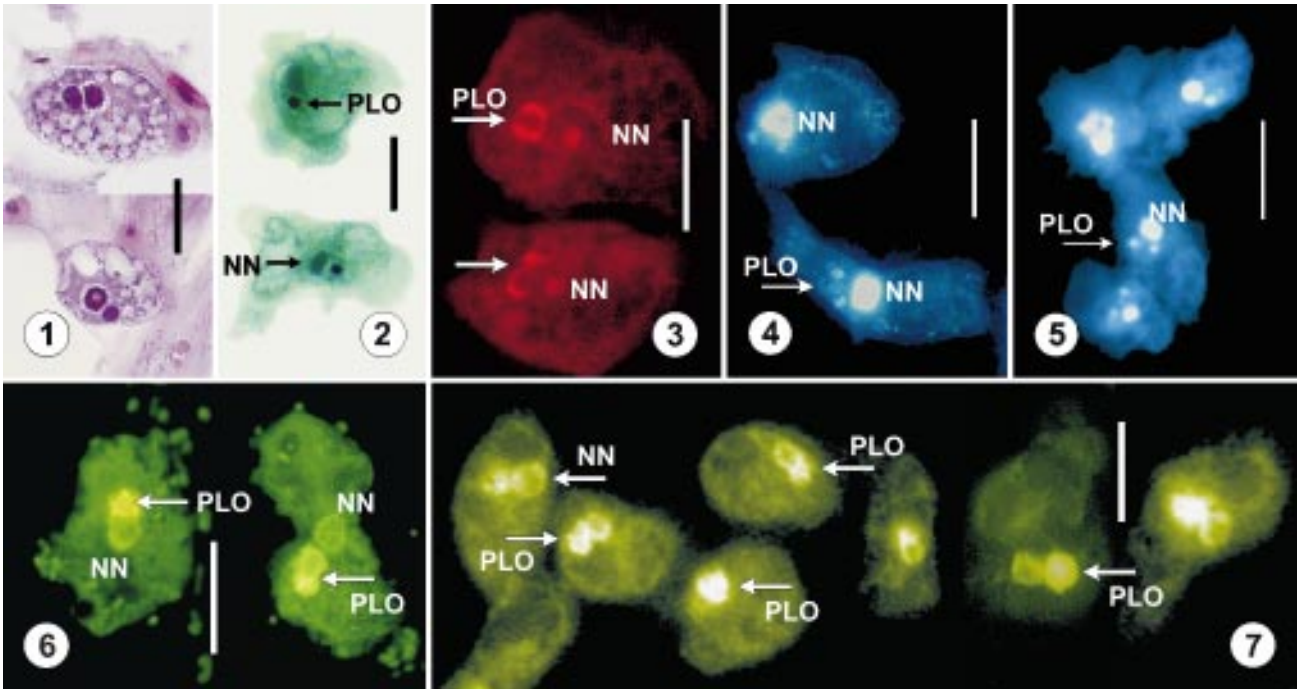
Electron microscope *in situ* hybridisation

The oligonucleotide probe prepared for the whole cell *in situ* hybridisation was used also for *in situ* hybridisation on ultrathin sections of pelletted cells embedded in hydrophilic Lowicryl K4M and on cryosections. Electron microscope techniques followed the methods of Morel et al. (2001) and Chevalier et al. (1997) and used EM sheep anti-digoxigenin: 10 nm Gold (BBINTERNATIONAL).

SSU rRNA gene

Universal eukaryotic SSU rRNA oligonucleotides (5′-ACCTGGTTGATCCTGCCAG-3′ and 5′-CTTCCGCTGGTTCACCTACGG-3′) (Medlin et al. 1988) were used to amplify the target genes in total DNA extracted from the *Neoparamoeba* cells. PCR amplification, cloning and sequencing were performed as described elsewhere (Fiala and Dyková 2003).

Phylogenetic analyses were based on alignment that contained sequences of 16 bodonids, 18 trypanosomatids, 4 diplonemids and 4 euglenids. The following taxa were considered as outgroups: i) euglenids only, ii) both euglenids and diplonemids, and iii) diplonemids only. Sequences were aligned in the Clustal X program (Thompson et al. 1997). Alignment consisted of 2420 nucleotides, from which 562 ambiguously aligned sites were removed. Supplementary analyses with a dataset of 905 highly conserved positions (1515 excluded) were also performed. Phylogenetic analyses were performed using maximum parsimony (MP) and maximum likelihood (ML). Both methods were carried out with the program package PAUP*, Version 4.0b10a (Swofford 2001). The MP analysis was done using heuristic search with random addition of taxa (10 replications) and the ACCTRAN-option. Gaps were treated as missing data. Transversion/transition (Tv/Ts) ratios were 1:1, 1:2 and 1:3. For the ML analysis, the likelihood ratio test (LRT) implemented in the Modeltest v. 3.06 (Posada and Crandall 1998) was used to determine the best model of evolution. Based on the LRT, the ML was performed with the GTR+G+I model of evolution. The estimated α-parameter was 0.428, the number of substitution types was 6 and the proportion of invariable sites was 0.029. The best tree was searched with Tree Bisection-Reconnection (TBR) rearrangements. Genetic distances were calculated with the K2P algorithm. Clade support was assessed with bootstrapping (500 replicates for MP, 100 replicates for ML).



Figs 1–7. PLOs as observed in the light microscope when stained with haematoxylin and eosin in histological sections of gills of *Scophthalmus maximus* (Fig. 1), Feulgen nuclear reaction (Fig. 2), acridine orange (Fig. 3), Hoechst 33258 (Fig. 4), DAPI (Fig. 5), YOYO-1 (Fig. 6) and when visualised by *in situ* hybridisation with digoxigenin-labelled oligonucleotide IF1 (Fig. 7). **NN** – nucleus of *Neoparamoeba* trophozoite; **PLO** – *Perkinsiella amoebae*-like organism. Scale bars = 10 µm.

Results

In all samples examined consecutively during the long-term culturing of ten *Neoparamoeba* strains, trophozoites contained organisms closely resembling the endosymbiont *Perkinsiella amoebae* Hollande, 1980. In the course of culturing of amoebae the number of these organisms (PLOs) per amoeba trophozoite gradually diminished, but as long as the cultures grew well each trophozoite contained one PLO as a minimum.

Detection of PLOs with nucleic acid stains

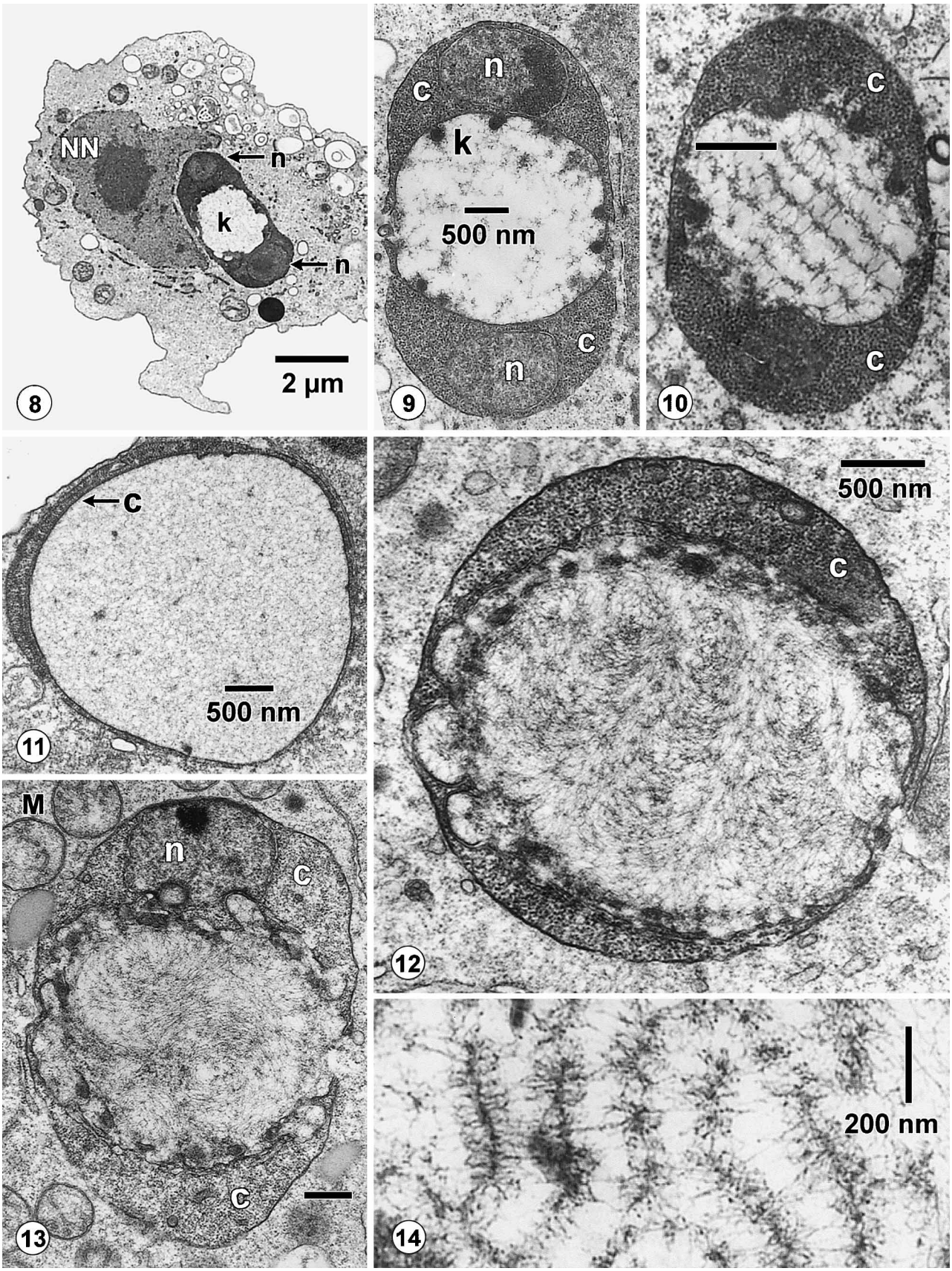
In order to visualise PLOs, various nucleic acid stains were employed for the first time, thus extending the diagnostic tools available for members of the family Paramoebidae and their endosymbionts (Figs 1, 2). They confirmed the light microscopical observations of PLOs in trophozoites of all samples of *Neoparamoeba* strains under study. However, images achieved with individual fluorochromes gave variable results (Figs 1–6). In preparations stained with Hoechst 33258 or DAPI, bipolarly symmetrical PLOs were recognised by a bright signal of two nuclei localised on the poles (Figs 4, 5) with only a weak signal being associated with putative kinetoplast. The nucleus of the *Neoparamoeba* host cell was stained evenly by the other dyes, with the exception of acridine orange which stained only the *Neoparamoeba* nucleolus and polar parts of the PLOs cytoplasm (Fig. 3).

Ultrastructure of PLOs

Comparative ultrastructural study based on a series of sections through amoeba trophozoites cut in different incident planes allowed us to characterise the morphology of PLOs and their relation to the host cell. Since no strain-related differences were detected in the fine structure, the features common for PLOs living together with trophozoites of the *Neoparamoeba* strains under study were summarised and documented. Best images were obtained with the double-fixation method followed by dehydration through an acetone series and embedding in Spurr's resin.

PLOs were localised in the cytoplasm of the trophozoites with no vacuole membrane around them. Most frequently, they were observed as bipolarly symmetrical oval structures with dense poles and conspicuous electron lucent middle part (Figs 8, 9). The dense poles were constituted by cytoplasm, rich in ribosomes, and membrane-bound eukaryotic nuclei (Fig. 9). The middle part surrounded by a thin layer of cytoplasm corresponded to the structure previously described as similar to a nucleoid (Perkins and Castagna 1971) and later, as a kinetoplast (Hollande 1980) (Figs 10–13). The observed morphology of the loose and fragile structure, the kinetoplast, in the middle part of these PLOs was obviously dependent on the plane of sectioning and on the condition of the cell.

Using the same method of fixation, the fine structure was not preserved well in many sections (Figs 8, 9) or the inner organisation was tufted and hard to discern (Fig. 11). Nevertheless, an arc-shaped arrangement of fibrils (Figs 12, 13) and nodes of electron-dense material with a repetitive motif of spring-like coiled fibrils were recognised in the kinetoplast located in the lumen of a single giant mitochondrion (Figs 10, 14). In the thinnest layer of cytoplasm surrounding the kinetoplast, as its equatorial part (Fig. 15), an array of transversely sectioned microtubules (a maximum of 14) was detected (Figs 16, 17). In this zone of the PLO, a loss of integrity or slight constriction of the mitochondrion was observed (Figs 15–18). Towards the poles, the mitochondrion-cytoplasm interface was characterised by interlocked protrusions of both components (Figs 19, 20). A three-dimensional model of bipolarly symmetrical PLO helped us to interpret remaining types of images lacking the prominent middle part: densely stained rounded forms of small diameter (1.5–2.0 µm) (Fig. 22) represented early stages of PLO development. Large rounded images containing nuclei surrounded by cytoplasm corresponded to the polar part of ovoid PLO, sectioned transversely to the plane of its long axis (Fig. 23). Semiserial sectioning revealed in early stages of the development of a PLO a dense zone corresponding to mitochondrion-cytoplasm interface with numerous interlocked protrusions (Figs 24, 25) and multilocular appearance of the kinetoplast (Fig. 26). The most difficult to interpret were double membrane-bounded structures resembling cytoplasmic protrusions into the mitochondrion-kinetoplast organelle but detached from cytoplasm (Fig. 21). In the cytoplasm of PLOs from three *Neoparamoeba* strains a membrane-bounded cytoplasmic organelle (Figs 27–29) with a highly ordered pattern of closely spaced, parallel, dense lines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(Fig. 28) was found. Once, the position of this organelle evoked potential exocytosis of material it contained (Fig. 29).

The localisation of PLOs within *Neoparamoeba* trophozoites was highly restricted to the close vicinity of the amoeba nucleus. Due to the intimacy of morphological association, amoeba nuclei revealed impressions, from shallow, enclosing part of a PLO, to very deep, encircling a PLO with lobular extensions (see also in Dyková et al. 2000). The significant predominance of the bipolarly symmetrical form among all others observed in thin sections suggested that this form of PLO develops rapidly and, most probably, is long lasting. The number of PLO division stages was extremely low compared to other stages observed and the number of sections examined. The presence of two nuclei on each of the two poles was observed in the PLOs from *Neoparamoeba* strains from turbot quite frequently (Figs 30, 31) but the whole process of division could not be traced. In the initial phase of culturing of a *Neoparamoeba* strain from *Dicentrarchus labrax*, predivision stages of PLO (Fig. 32) contained up to eight nuclei in one section. In addition, bipartition of the kinetoplast was observed in the same material (Fig. 33).

Spliced leader RNA (mini-exon) gene of PLOs

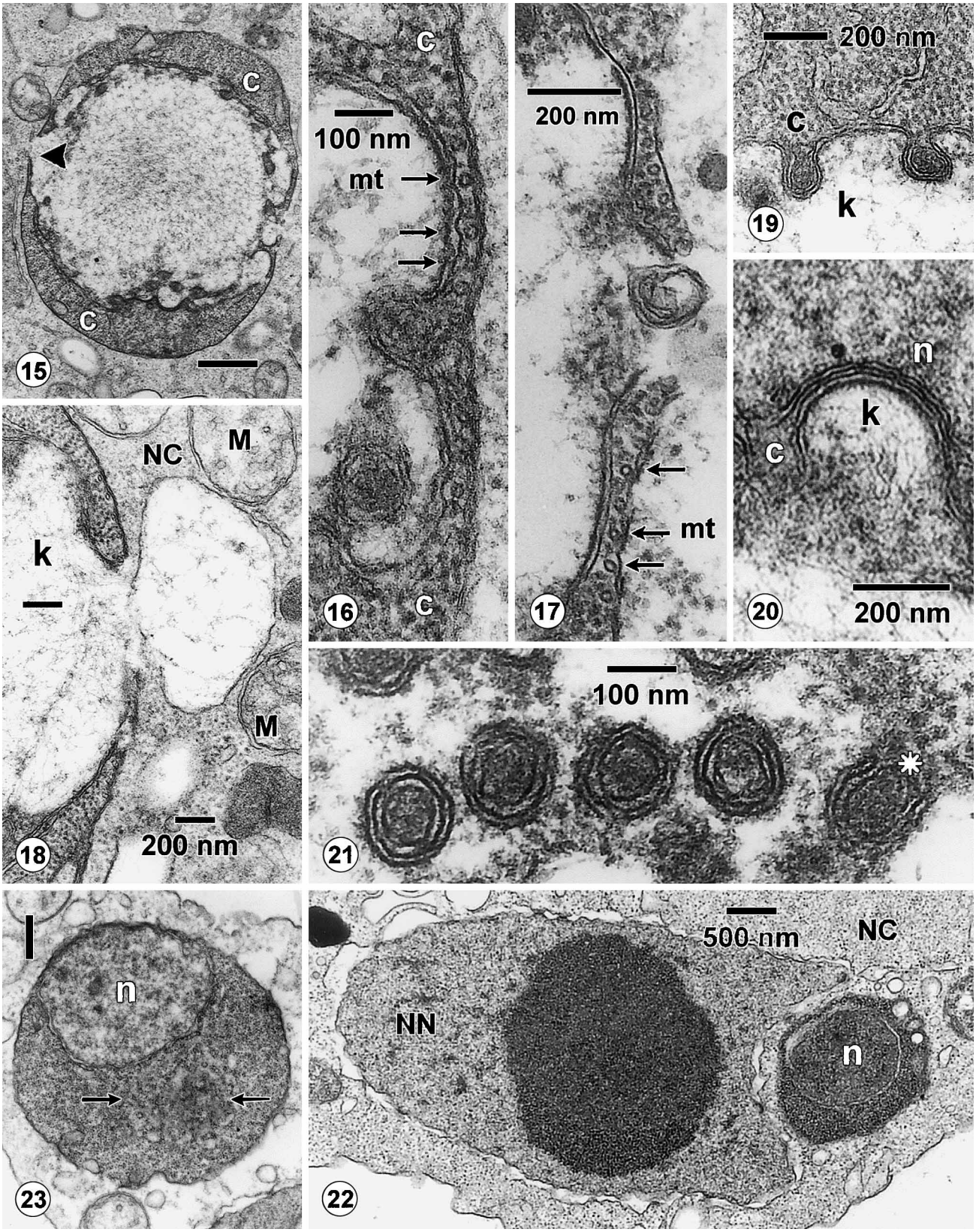
The DNA of five *Neoparamoeba* strains was subjected to a PCR assay for amplification of the SL RNA gene. The sequence of amplicons, that ranged in size from 450 to 700 bp, revealed the presence of SL RNA gene repeats that consisted of a 38 bp-long exon, intron and non-transcribed spacer. In the studied PLOs, the size of the SL RNA gene ranged between 111 and 165 nucleotides. The 5S rRNA gene was not associated with the sequenced SL RNA tandem repeats.

Two sequence patterns were found within the exon, that differed in two positions only (12 and 14) (Fig. 34), thus subdividing our SL RNA dataset into two subsets with identical exons. The first subset included SL RNA gene sequences from PLO inhabiting two strains (AFSM2V and AFSM11) identified previously as *N. pemaquidensis* (Fiala and Dyková 2003). Both sequences differed only in two positions in the intergenic region (Fig. 35). The second subset included SL RNA sequences of PLOs retrieved from strains AFSM3, SM53 and SM68. According to their SSU rRNA gene sequences their *Neoparamoeba* hosts are different from *N. pemaquidensis* and *N. aestuarina* (Fiala and Dyková 2003). The identity of these sequences within the exon region was 100%. In the intron region four changes were found between PLO/SM53 and PLO/AFSM3. The SL RNA intron of PLO/SM68 differed significantly in length as well as in the sequence from the other PLOs.

Comparison of the SL RNA exon sequences of our PLOs set with representatives of kinetoplastids, euglenids and *Diplonema papillatum* revealed the following unexpected divergences from the consensus sequence: i) all common leaders are 38 bp-long in contrast to the 39–41 bp and 26 bp-long homologues in kinetoplastids and euglenids, respectively; ii) in position 3 of the most conserved region of the exon, where all kinetoplastids described so far contain a C residue, the PLOs sequences possess a T; iii) positions 6 and 7 are also specific for PLOs, since they contain CT instead of AC, present in all other kinetoplastids; iv) all PLO exons shared a T in position 5 with *Trypanoplasma borreli* and *Bodo caudatus*. The first 13 nucleotides at the 5' end of the intron are highly conserved in kinetoplastids while the same region is unique in diplonemids and *Euglena gracilis* (Fig. 34). Based on this region, PLOs are clearly affiliated with Kinetoplastida rather than with other flagellates.

The SL RNA of PLO/AFSM3 was folded by the program Mfold into a secondary structure reminiscent of that described for kinetoplastids (Fig. 36). It is composed of three stem loops and a single-stranded region containing a putative "Sm"-binding site in contrast to the four stem loops structure of euglenids.

Figs 8–14. Transmission electron micrographs of *Perkinsiella amoebae*-like organisms (PLOs). **8–9.** The most frequently observed developmental stage. Overview of a bipolarly symmetrical PLO stage localized in the vicinity of a *Neoparamoeba* nucleus (Fig. 8) and higher magnification of the similar stage (Fig. 9). **10–13.** Four images of PLO sectioned longitudinally (Figs 10, 13) and transversely (Figs 11, 12) showing different arrangement of the kDNA network. **14.** The spring-like coiled fibrils of kDNA.
Scale bars: for Figs 10 and 13 = 500 nm. **n** – nucleus of PLO, **NN** – nucleus of *Neoparamoeba* trophozoite, **k** – kinetoplast, **c** – cytoplasm of PLO, **M** – mitochondrion of *Neoparamoeba* trophozoite.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Using *in situ* hybridisation we have explored a reasonable assumption that PLOs would contain a SL gene, the transcript of which is added post-transcriptionally to all mRNAs. A single oligonucleotide complementary to the SL RNA exon of PLO/AFSM2V and PLO/AFSM11 was labelled with digoxigenin, and visualised with corresponding antibody. Although non-specific staining of low intensity was seen in host cells, we were able to detect the highest concentration of FITC in PLOs, where it was bound to digoxigenin-labelled IF1 (Fig. 7).

While the digoxigenin-labelled kDNA-targeted synthetic oligonucleotide probe gave good and consistent images of PLOs in whole cells, the labelling density achieved in ultrathin sections did not satisfy us although conditions for *in situ* hybridisation were modified several times.

The PLOs SL RNA gene sequences were registered in GenBank database under the accession numbers AY163350–AY163354.

SSU rRNA gene

It proved rather difficult to obtain the PLO SSU rRNA gene. Out of about 50 clones containing SSU rRNA genes obtained with the degenerate oligonucleotides from the *Neoparamoeba* strains (about 10 per strain), only one was of clearly non-amoebic origin. Along with the SSU rRNA sequence of the *Neoparamoeba* host cell (see Fiala and Dyková 2003), it was obtained from PLO/AFSM3. The BLAST search revealed closest homology of this sequence with the kinetoplastid *Ichthyobodo necator*. The length of the entire sequence was 1982 bp including the regions corresponding to the forward and reverse primers. The G+C content was 52.5%.

The PLO sequence was added to the alignment of SSU rRNA sequences used for analysis of kinetoplastid phylogeny. It consisted of 39 taxa and contained 1858 characters. The number of parsimony informative sites was 1052. The similarity between PLO/AFSM3 and *I. necator* was 85.5%, while its similarity with other kinetoplastids dropped to about 65%. In both MP [Tv/Ts 1:1 (4803 steps), 1:2 (6977 steps), and 1:3 (9142 steps)] and ML analyses [–ln = 23816.915] PLO/AFSM3 and *I. necator* formed a monophyletic branch supported by 100% bootstrap value (Fig. 37). Moreover, early branching of this clade from the kinetoplastid lineage gathered maximum bootstrap support. While *Procryptobia sorokini* constituted the next basal branch, the branching order of the bodonid clades 1 to 3 (sensu Simpson et al. 2002) was not resolved. At the same time, the monophyly of trypanosomatids remained well supported. No major changes in the tree topology were observed when different outgroup taxa were used (data not shown).

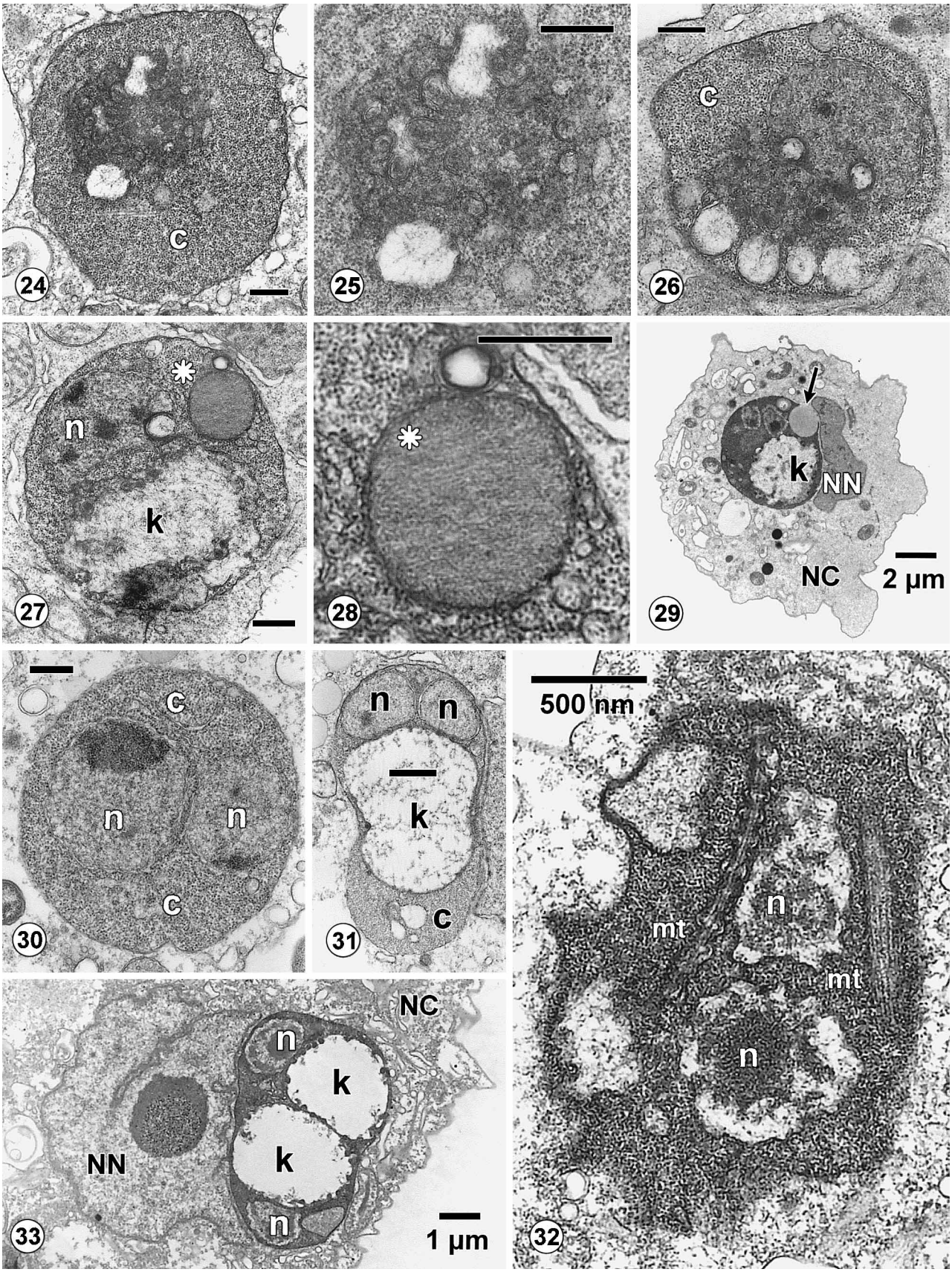
The SSU rRNA gene sequence of PLO/AFSM3 was deposited in GenBank under accession number AY163355

Discussion

Morphology of PLOs

The subjects of this study, the endosymbionts present in a perinuclear position in *Neoparamoeba* species, seem to be highly unusual members of the order Kinetoplastida.

Figs 15–21. Details of ultrastructure of *Perkinsiella amoebae*-like organisms (PLOs) on the periphery of the kinetoplast. **15.** PLO sectioned in the middle part, between the levels of nuclei, overview. Arrowhead indicates the thinnest layer of cytoplasm. **16–18.** The longitudinal sections through the equatorial part of bipolarly symmetrical PLOs. **16.** The thinnest layer of cytoplasm reinforced with microtubules (arrows). **17, 18.** The loss of PLO integrity observed frequently in the equatorial level, accompanied sometimes by the "leakage" of PLO material into the cytoplasm of *Neoparamoeba* (Fig. 18). **19, 20.** The kinetoplast-cytoplasm interface with interlocked protrusions. **21.** The structures resembling cytoplasmic protrusions into the kinetoplast network that are detached from cytoplasm (compare with the structure marked with asterisk). **22.** Small, early stage of PLO in the vicinity of *Neoparamoeba* nucleus. **23.** Developmental stage of PLO with the nucleus and the structure resembling endoplasmic reticulum (arrows).
Scale bars: for Fig. 15 = 1 μm, for Fig. 23 = 500 nm. **c** – cytoplasm, **k** – kinetoplast, **M** – mitochondria of *Neoparamoeba*, **mt** – microtubules, **n** – nucleus of PLO, **NC** – cytoplasm of *Neoparamoeba*, **NN** – nucleus of *Neoparamoeba*.


c
24
25
c
26
n
k
27
28
k
NN
NC
2 µm
29
c
n
n
c
30
n
n
k
c
31
500 nm
n
mt
mt
n
32
NC
n
k
NN
k
n
1 µm
33

With a certain level of experience, PLOs are detectable in living amoeba trophozoites and in routinely stained histological sections (Fig. 1). In trophozoites attached to slides, PLOs can be distinctly visualised also with various fluorochromes.

The fact that even a broad comparison of ultrastructure did not help in discriminating among PLOs from ten *Neoparamoeba* strains (of different origin) warrants caution when comparing with very scant data on PLOs from *Paramoeba* and *Janickina* spp. Since we found that even in regularly passaged cultures of *Neoparamoeba* trophozoites, PLOs do not divide synchronously, the statistical evaluation of their size is extremely difficult and the data by Hollande (1980) on the size differences of *P. amoebae* related to the host cell species are not convincing.

Concerning the relation of ultrastructural features of PLO to features characterisic of Kinetoplastida, several points have to be stressed. PLOs are aflagellate, usually binuclear cells with a single giant kinetoplast-mitochondrion. Another unusual feature is the extremely reduced number of subpellicular microtubules in an incomplete microtubular corset. Although the kinetoplast of PLOs resembles to a certain extent the poly-kDNA nucleoids with the DNA fibrils radiating from a dense core (Lukeš et al. 2002), one can easily propose that it represents a novel type of kDNA organisation, and thus extends the known diversity of kDNA structures in bodonids. The kDNA

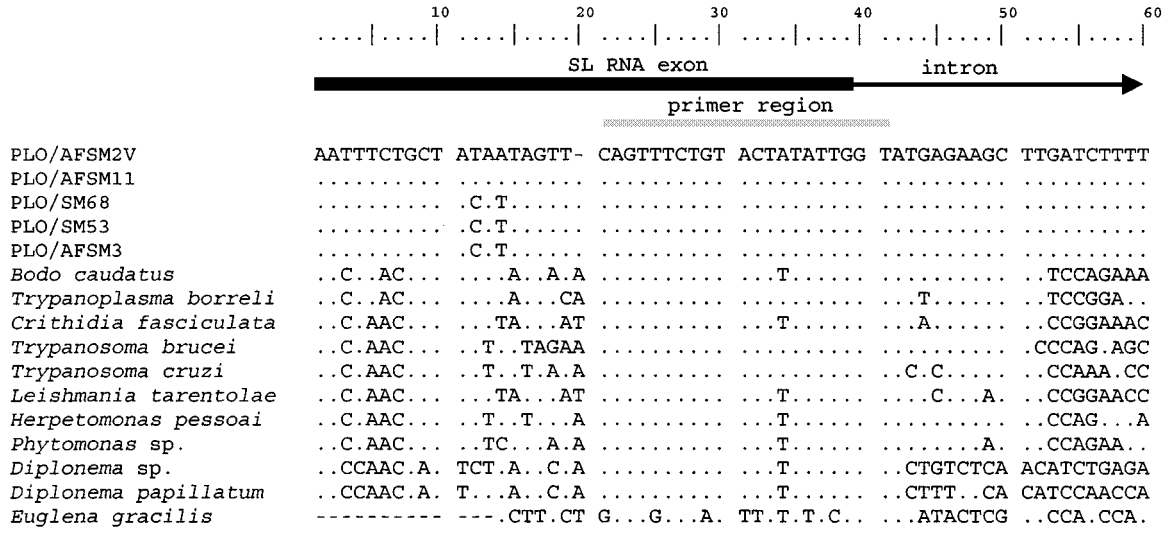


Fig. 34. Alignment of SL RNA exon and part of intron sequence from PLOs and representatives of kinetoplastids, *Diplonema* spp. and *Euglena gracilis*. Nucleotides identical to that of PLO/AFSM2V are represented by dots. Gaps introduced into sequences are denoted by dashes. GenBank accession numbers are as follows: *Bodo caudatus* X63467, *Trypanoplasma borreli* L08172, *Crithidia fasciculata* U96170, *Trypanosoma brucei* X00935, *T. cruzi* X62674, *Leishmania tarentolae* X73121, *Herpetomonas pessoai* X623319, *Phytomonas* sp. X87136, *Diplonema* sp. AY007787, *D. papillatum* AY007785, *Euglena gracilis* X63153.

Figs 24, 25. The polar part of a PLO with an electron dense labyrinth-like structure beneath the nucleus. **26.** The semiserial section of the same PLO as in Fig. 24, showing multilocular appearance of the kinetoplast. **27–29.** The cytoplasmic organelles with a highly organised content (in Figs 27 and 28 marked with asterisks) that in Fig. 29 seems to be exocytosed (arrow). **30, 31.** Two stages of PLO after the division of nuclei at the poles. **32, 33.** Dividing stages of PLO. Bipartition of the kinetoplast is illustrated in Fig. 33.
Scale bars: for Figs 24–28 and 30 = 500 nm, for Fig. 31 = 1 µm. **c** – cytoplasm of PLO, **k** – kinetoplast, **mt** – microtubules, **n** – nucleus of PLO, **NC** – cytoplasm of *Neoparamoeba* trophozoite, **NN** – nucleus of *Neoparamoeba* trophozoite.

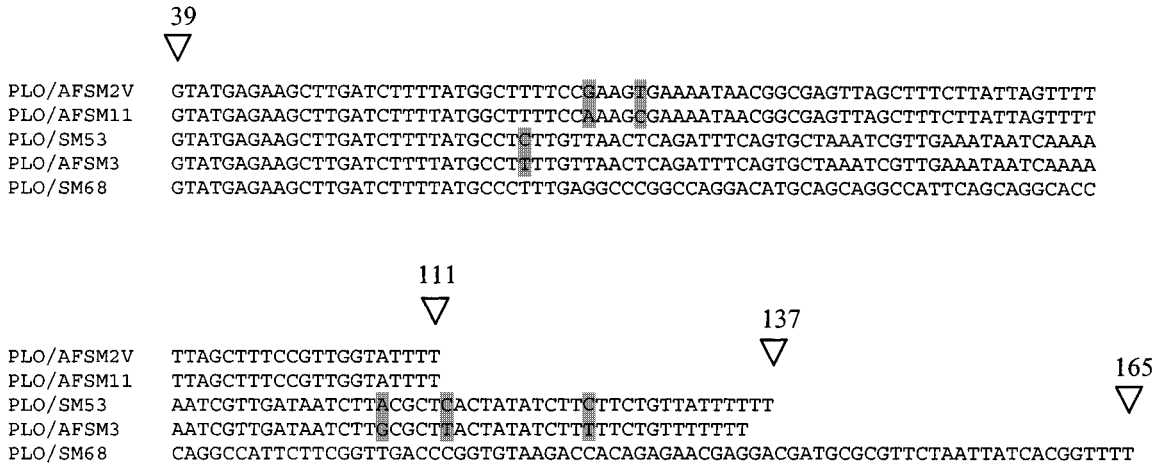


Fig. 35. DNA sequence comparison of the introns of SL RNA genes from PLOs. Numerals with arrowheads indicate position in SL RNA gene sequence. Shaded nucleotides show the differences between two related subsets of PLOs.

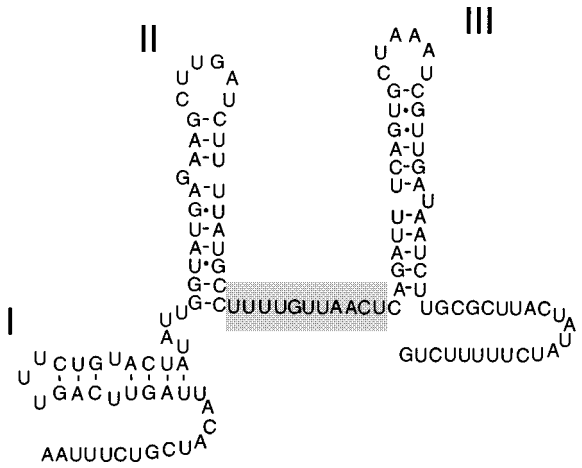


Fig. 36. Possible secondary structure of SL RNA of PLO/AFSM3. Shaded box indicates putative Sm-binding site. The stem loops are numbered I, II, and III.

formed a fibrous mass in the mitochondrion of bipolarly symmetrical forms of PLOs, with a labyrinth near the poles of the organelle, reminiscent of that described by Brooker (1971) in *Crithidia fasciculata* and by Vickerman and Preston (1976) and Vickerman et al. (1991) in *Cryptobia vaginalis*, *C. keysselitzi* and *Trypanosoma raiae*. It was interpreted by the authors mentioned above as a mitochondrial labyrinth formed by intrusions of the endoplasmic reticulum into a localised region of the mitochondrion near the kinetoplast. Variability of coiling of DNA fibres has also a parallel among kinetoplastid flagellates, where it is associated with developmental stages (Brugerolle et al. 1979).

The only other example we have found for the bipolar symmetry of PLOs with nuclei on poles is the parallel with diplozoic forms described as a result of incomplete separation of the karyomastigonts of diplomonads. Perkins and Castagna (1971) originally classified the bipolar form of PLO as a predivision form. Interestingly, predivision individuals with already duplicated flagellar apparatus are known to occur frequently in *I. necator* (Joyon and Lom 1969), an organism closely related to PLO.

The spherical cytoplasmic inclusion with a remarkably regular pattern of parallel dense lines resembled crystalline or paracrystalline inclusions that were presumed by Fawcett (1966) to be the storage form of a protein synthesized by the cell. So far, in kinetoplastids, paracrystalline inclusions have been described in the glycosomes, (Vickerman et al. 1991), another unique organelle of this group of flagellates. Unfortunately, we do not have enough data to further explore certain resemblances between the cytoplasmic inclusions in PLOs and glycosomes. Similarly, when trying to study the process of PLO division, we have faced the same problem as Hollande (1980). Not even our material was ample enough to clarify the whole process.

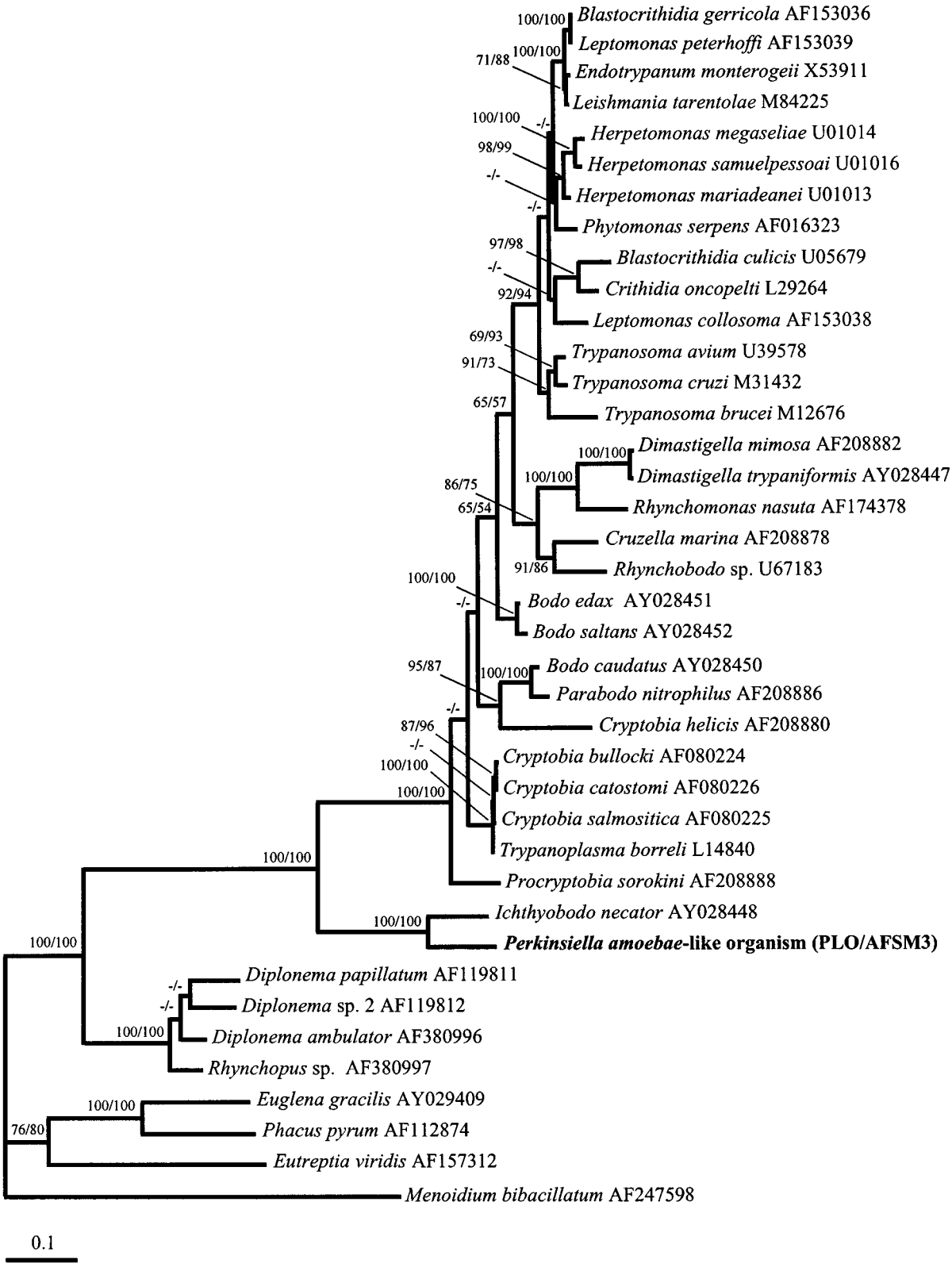


Fig. 37. Maximum likelihood tree (GTR+G+I; –ln = 23816.915) based on 18S RNA sequences. The tree is rooted on four euglenid sequences. Bootstrap values (ML and MP Tv/Ts = 1:2) are indicated for nodes gaining more than 50% support. The distance scale is given under the tree.

Molecular analyses

Amplification of the SL RNA gene in DNA extracted from *Neoparamoeba* strains and its localisation by *in situ* hybridisation in cultured cells confirmed the hypothesis on the kinetoplastid origin of PLOs. A closer relationship of PLOs to kinetoplastids and *Diplonema* spp. rather than to euglenids was supported by predicted kinetoplastid-type secondary structure of the SL RNA transcript as well as by the sequence of the intron.

When compared with kinetoplastids, PLOs have one unique substitution in the first four nucleotides of the SL exon sequence. The first four nucleotides are modified by methylation and form the characteristic cap structure (Perry et al. 1987), which is highly conserved among all kinetoplastids but not in *Diplonema* spp. (Sturm et al. 2001). PLOs have substitutions in positions 6 and 7, while sequences of *Diplonema* spp. possess the same nucleotides as all kinetoplastids. In addition, an association of the SL RNA and 5S rRNA genes that is frequently encountered in early-branching kinetoplastids (Santana et al. 2001) is absent from the obtained sequences. In total, these data suggest a rather distinctive position of PLOs within the Euglenozoa.

Since the SL RNA gene is not suitable for robust phylogenetic analyses, the more appropriate SSU rRNA gene was used for this purpose. Due to preferential amplification of the SSU rRNA gene of the host amoeba, only one SSU rRNA gene sequence was obtained for a PLO. Its close relationship with *I. necator*, an early-branching kinetoplastid (Callahan et al. 2002), is of particular interest. *I. necator* and PLO/AFSM3 constitute a well supported clade that represents a sister group to all other kinetoplastid taxa. We assume that the addition of the PLO SSU rRNA sequence, which subdivides the long branch of *I. necator*, confirms the ancestral character of both flagellates within the Kinetoplastida. Based on these results we may speculate that the endosymbiotic association of PLO and *Neoparamoeba* might have been established in an early phase of the kinetoplastid evolution.

Symbiotic system *Neoparamoeba*-PLO

In amoebae, especially in freshwater species, the best-studied endosymbionts are bacteria. In addition to the frequent occurrence of facultative endosymbionts, stable symbiotic relationships have also been described between amoebae and bacteria (Daniels et al. 1966; Lee at al. 1985; Bradley and Marciano-Cabral 1996). Only a few studies have been devoted to symbiotic associations of amoebae with algae (Lee et al. 1985; Karpov et al. 1991). The other protistan endobionts of amoebae have been neglected as is apparent from the lack of data in a review on amoebae, flagellates and ciliates as host organisms of various symbionts (Ossipov et al. 1997). Since free-living amoebae are constantly exposed to other microorganisms and are able to ingest most of them, phagocytosis is considered the most common way of establishing an endosymbiotic association. It might be taken into consideration also for the ancestors of PLO and *Neoparamoeba* spp. In accordance with definitions by Reisser et al. (1985), the symbiotic association of PLOs and *Neoparamoeba* trophozoites ("eukaryote within eukaryote") was found to belong to a stable and hereditary endosymbiotic system: neither can trophozoites be set free from PLOs nor the PLOs from trophozoites. In addition, during host cell division the PLOs become distributed into daughter cells. This, together with site selection of PLO within the host cell and lack of morphologically detectable regulations of growth rate of PLO by the host cell indicate an advanced coevolutionary process, which is underlined by a certain degree of specificity found in PLO-*Neoparamoeba* systems in the present work.

The nature of association of PLOs and amoebae of the genera *Neoparamoeba*, *Paramoeba* and *Janickina* is far from being understood. Some hundred years after its discovery we are only starting to achieve basic knowledge of the eukaryotic PLOs that form mutualistic associations with amoebae of the family Paramoebidae. The question worded already by Page (1970) – whether or not the genera containing enigmatic "parasomes" are monophyletic or whether similar symbionts became established in more than one group of amoebae – has been partly answered also in a previous paper (Fiala and Dyková 2003). The congruence between phylogeny of the *Neoparamoeba* strains (Fiala and Dyková 2003) and homology of the PLO SL RNA genes allows anticipation that not only *Neoparamoeba* spp. but also amoebae belonging to other genera of the family Paramoebidae can host specific PLOs. The influence of endosymbionts on the host cell physiology remains unknown, and many other interesting questions should be addressed. Are PLOs derived from an amastigote stage, or from organisms that lost the flagellum as a result of adaptation to endobiosis? Did PLOs retain some

components of the paraflagellar rod or structures related to it? Do PLOs contain true glycosomes; what is the structure of the kDNA network; what is the role of PLO in pathogenicity of *Neoparamoeba* strains? The assumed genetic relationship of PLO with the host nuclear genome as well as the role of PLOs in the evolutionary history of kinetoplastids opens a wide field for future research. However, the above mentioned topics will most likely require modifications of methodology employed in research on kinetoplastids that are culturable apart from their host organisms.

I. necator, a cosmopolitan dangerous ectoparasite of most species of freshwater fishes, has been recorded several times also from marine fishes and from salmonids that transfer from freshwater to seawater (Lom and Dyková 1992). The early position of both PLOs and *I. necator* in the SSU rRNA-based phylogenetic tree of kinetoplastids may bring about speculation on their mutual relationship. It is difficult to assume that it was *I. necator* that became an endosymbiont/endoparasite (?) in amoebae, although one can assume that both flagellates (PLO and *I. necator*) had a common ancestor.

The establishment of a symbiotic relationship, which has been considered an important motor of evolution in general (Bardele 1997), resulted in the case of PLOs in the origin of unique endosymbiotic organisms in which of course it is difficult to presume further evolution.

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