

## Isolation of the Amoeba *Thecamoeba quadrilineata* Harbours Intranuclear Spore Forming Endoparasites Considered as Fungus-like Organisms

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**Summary.** Among a population of *Thecamoeba quadrilineata* (*Thecamoebidae*) isolated from moss samples some trophozoites harboured intracellular aggregates of round-oval parasites which turned out to be spores with a real nucleus. These organisms were supposed to be fungal endoparasites beginning their development within the nucleus of the host invaded by young parasitic stages after the host amoeba had engulfed free spores from the environment. The complete developmental cycle was studied by electron microscopy, showing the intranuclear growth of freshly invaded young stages into large spore forming parasites differentiating into a great number of spores – all within the border of the host's nuclear membrane. These spores were not released into the environment until the death and decay of the host amoeba, where they could be ingested as infective stages by hitherto not infected thecamoebae. Host range studies with various free-living amoebae (FLA) showed that *T. striata* and *T. terricola* were as permissive to infection as the original host *T. quadrilineata*. *Sappinia* was only susceptible to a certain extent and therefore not considered as possible natural host. Remarkably, this observation shows that both nuclei of the bi-nucleate amoeba became simultaneously infected. The present morphological description corresponds to early observations with fungal intranuclear parasites called *Nucleophaga* Dangeard, 1887. However, genetic and phylogenetic studies have to corroborate the supposed fungal nature.

**Key words:** Fungal endoparasite, *Nucleophaga*, *Thecamoeba*, *Sappinia*, ultrastructure, amoeba.

### INTRODUCTION

Since Rowbotham (1980) observed the intracellular replication of *Legionella pneumophila* within *Acanthamoeba polyphaga* many authors corroborated these observations (Harf and Monteil 1988; Rowbotham 1993; Adeleke *et al.* 1996, 2001; Fields *et al.* 1993; Newsome *et al.* 1998). Other investigators described

the intracellular growth of various pathogens within free-living amoebae (FLA) such as *Listeria* sp. (Ly and Müller 1990), *Pseudomonas aeruginosa* (Michel *et al.* 1995, Marciano-Cabral and Cabral 2003), *Burgholderia* sp. (Michel and Hauröder 1997, Marolda *et al.* 1999), “environmental Chlamydiae” (Amann *et al.* 1997; Birtles *et al.* 1997; Horn *et al.* 2000; Greub and Raoult 2002, 2004; Horn 2008). The first members of this novel group of microorganisms discovered within FLA, i.e., *Parachlamydia* and *Neochlamydia* were isolated in our laboratory and later named as *P. acanthamoebae* (Amann *et al.* 1997) and *N. hartmannellae* (Horn *et al.* 2000), respectively, by sequencing the 16S

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rRNA gene. In contrast to these meanwhile frequently observed prokaryotic endoparasites and endosymbionts of FLA, eukaryotic endocytobionts have been reported more rarely to occur within certain FLA, e.g., large fungal organisms like *Cochlonema* sp. (Drechsler 1942) developing within the cytoplasm of its host amoeba. Previously the interesting fungus was rediscovered in our laboratory (Michel 1999, Michel and Wylezich 2005) as well as yeast-like cells within thecamoebae (Michel 1997). Only very recently it was shown that *Cryptococcus neoformans*, a highly pathogenic fungus, is also capable of replicating within FLA (Steenbergen *et al.* 2001). In addition to intracytoplasmic growing endoparasites, in 1886 Dangeard observed endoparasites, which had infected the nuclei of certain amoebae, where they had started a sporogonic cycle. He classified those as “*Nucleophaga* sp.” They were affiliated to the fungal group Chytridiales, although it is still an unproven assumption since neither electron microscopy nor molecular biological methods were available at that time. Later, *Nucleophaga* was described within the nuclei of *Entamoeba histolytica* and *Jodamoeba bütschlii* (Doflein und Reichenow 1929).

*Nucleophaga*-like endoparasites replicating within vannellae, showed traits of microsporidiae such as polar filaments (Hoffmann *et al.* 1998, Michel *et al.* 2000). As a result of the sporogonic cycle, large spore-aggregates were formed. Recently, similar parasitic aggregates have been discovered within nuclei of *Thecamoeba quadrilineata*, a species of gymnamoebae, which is quite different to vannellae. This new *Nucleophaga*-like endoparasite was briefly described by the means of light microscopy (Michel 2007) and is now subject of the present article which focuses on characteristic traits using electron microscopy in order to decide to which group of eukaryotes it may belong.

## MATERIALS AND METHODS

*Thecamoeba quadrilineata* called strain “Tq-2”, harbouring the intranuclear parasite called “KTq-2” was isolated from the meshwork of the roots from moss derived from the entrance of the grotto at Tannheim/Austria. Samples of moss were transferred to non-nutrient agar plates (NNA) and seeded with *Enterobacter cloacae* as food bacteria, according to Page (1988). The plates were incubated at room temperature for 14 days and were inspected daily for the presence of free-living amoebae (FLA).

## Cocultivation assay

Mature spores of the parasites were collected by incubating agar plates with infected thecamoebae until they decayed. Small quadrangular pieces of agar containing free spores were cut out and streaked upside-down onto the surface of a fresh agar plate. In the same manner, blocks from the agar surface bearing numerous, as yet non-infected, amoebae were cut out and transferred onto the streak of spores where they could become infected by ingesting the spores applied previously. These cocultivation plates were inspected daily for a period of 14 days. A successful infection could be observed within three to five days of incubation at room temperature.

## Electron microscopy

For electron microscopic studies infected amoebae were harvested from ten plates after incubation for three to five days with amoeba saline (Page 1988) and pelleted by centrifugation [500 g (= 1800 rpm) for 10 min.]. The resulting pellets were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 hour, washed twice in the same buffer, postfixed for 1 hour in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2), and embedded in Spurr resin. Finally, the sections were stained with uranyl acetate and Reynolds’s lead citrate and examined by using a Leo EM 910 transmission electron microscope (Leo, Oberkochen). Semi-thin sections were stained with 0.1% Toluidine blue. This was necessary in order to localize areas with accumulation of numerous fixed amoebae within the embedded pellets more easily.

## RESULTS

Within a few days of incubation, a mixed population of various amoeba species appeared in the surrounding area of moss samples on NNA plates. Within very few larger trophozoites – later identified as *T. quadrilineata* – aggregates of coccoid parasites attracted our attention that were released into the environment as spores after death and decay of the host amoebae. In subcultures we succeeded in separating infected amoebae from other species of amoebae and eliminating saprophytic fungi from the culture plates with Nystatin discs. As a first sign of infection the nucleus appeared distinctly enlarged within the freshly infected trophozoites (Fig. 1b) compared to the normal size of the nucleus in non-infected cells (Fig. 1a). It became evident that the endoparasite invaded the nucleus *via* the cytoplasm. Within a period of two to three days, one (Fig. 1d) or even up to four parasites (Fig. 2a) which had invaded the nucleus increased considerably in size. They subsequently differentiated into numerous round spores, 1.5–2.0 in diameter (Fig. 2b) and were best visible when the cytoplasm of some host amoebae was slightly compressed under a coverslip (Fig. 3a). Each trophozoite prone to die if

free spores have been ingested and have invaded the nucleus. Spores previously ingested were observed within the cytoplasm of some amoebae (Fig. 3b) supposed to be transported to the nucleus. The rate of experimental infection depends on the number of spores added to the non-infected trophozoites and varies from 20–100%. As a result of experimental infection and daily inspection, the developmental period of time could be estimated. The first small changes of the nucleus could be distinguished within 24 hours. Within a period of two days, a significant enlargement of the nucleus was obvious (Fig. 1b). Larger parasites with increasing differentiation into spores appeared within three to five days (Figs 2b, 3a).

Since the number of non-infected trophozoites decreased continuously during serial transfers of the infected population, attempts were made to get rid of the aggressive endoparasites. This could be achieved by incubating the culture at 28°C for five days, resulting in stagnation and death of the endoparasites. It is true that the host amoebae died as well; however, since the formation of new spores was interrupted the few surviving hitherto non-infected amoebae could now propagate unaffected. This parasite-free population served in first instance as a reserve population when the infected one was in danger of extinction but also for all intended co-cultivation assays.

Among all other amoeba strains tested only the binucleate amoeba *Sappinia* sp. was susceptible to infection to a certain extent. It is true that it became infected the same way as *T. quadrilineata*, but in most cases, the development of the endoparasites could not be completed since the host cell was killed by their endoparasites before spores could be formed. Only within one strain, “Busnog,” few infected amoebae enabled the production of mature spores. Of special interest was the

question whether only one or even both nuclei became infected. Indeed, both nuclei acquired an infection simultaneously – leading to two discernible parasitic aggregates. Interestingly, infected nuclei were separated from each other compared to always closely attached nuclei in non-infected sappiniae. In an overview (Fig. 4) of an infected amoeba, four parasitic stages (P) filling the nucleus could be observed within the cytoplasm, containing many food vacuoles with remnants of ingested bacteria. At an early phase of the intranuclear development young parasitic stages can be observed starting growth on the expense of the endosome (nucleolus) (Fig. 5). As a result, the endosome will be exploited and disappears during further growth of the endoparasites (Figs 6, 7). They grow to intermediate sporogonic stages with the interior differentiated into numerous compartments with membranes and various vacuoles (Fig. 6). The surface forms many microvilli enabling an intimate contact with the karyoplasm. In all cases observed so far, the membrane stays intact. In the course of further development, few round or oval-shaped sporogonic areas appear (Fig. 7) with electron dense material forming a kalotte inside these structures. The microvilli are now replaced by a fuzzy coat that also mediates “close junctions” between the parasites as sites of close attachment to each other.

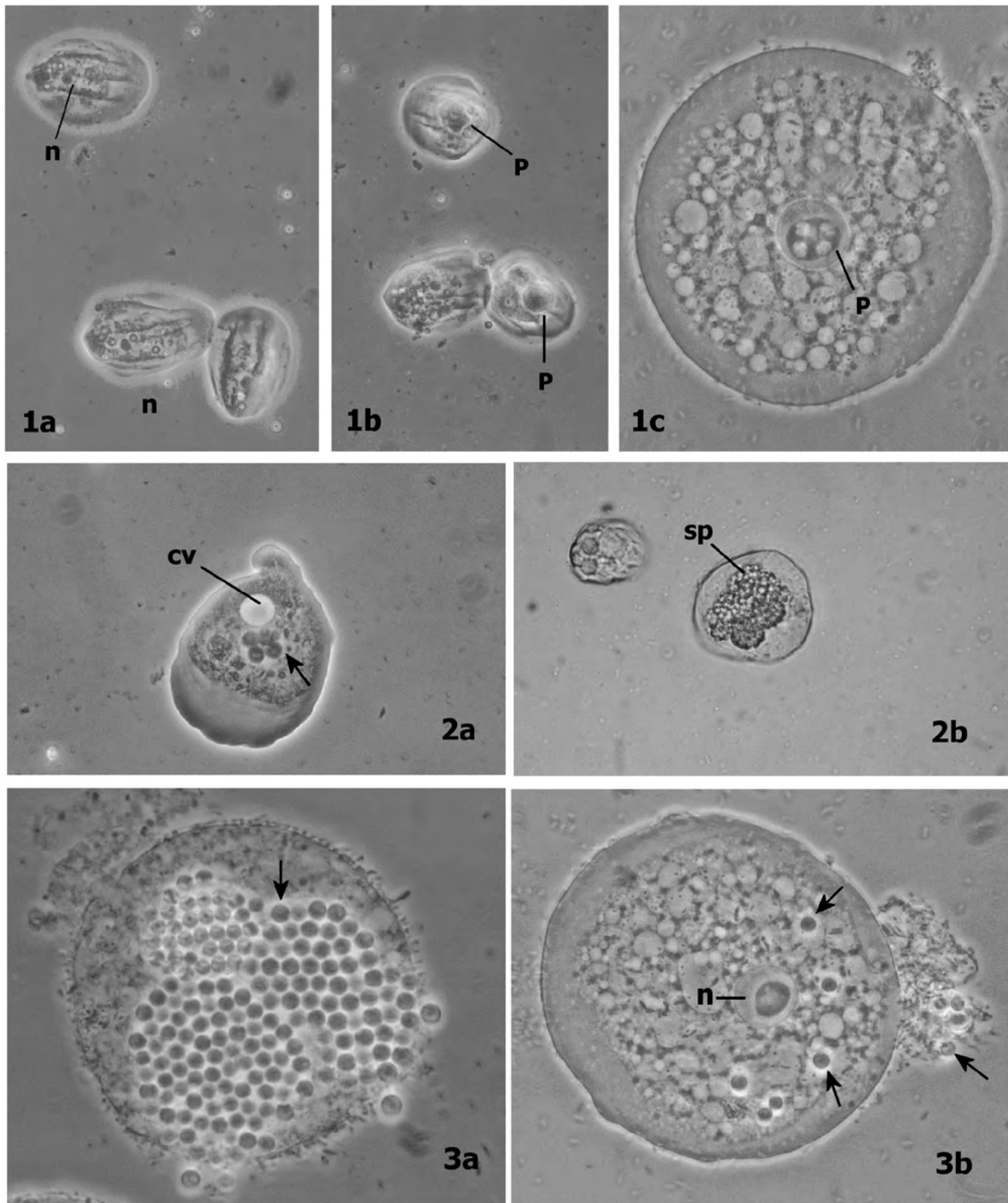
As a result of further differentiation processes, characteristic spores are formed (Fig. 8), which are still enclosed by the nuclear membrane (arrows) and by an inner membrane representing the former envelope of the single parasite. After death and rupture of the host amoebae, free spores (Fig. 9a) can be observed in the environment which will be ingested and enclosed within phagosomes of another *Thecamoeba* (Fig. 9b, c). At higher magnification, ultra-structural traits become visible more clearly comprising the nucleus, cytoplasmic

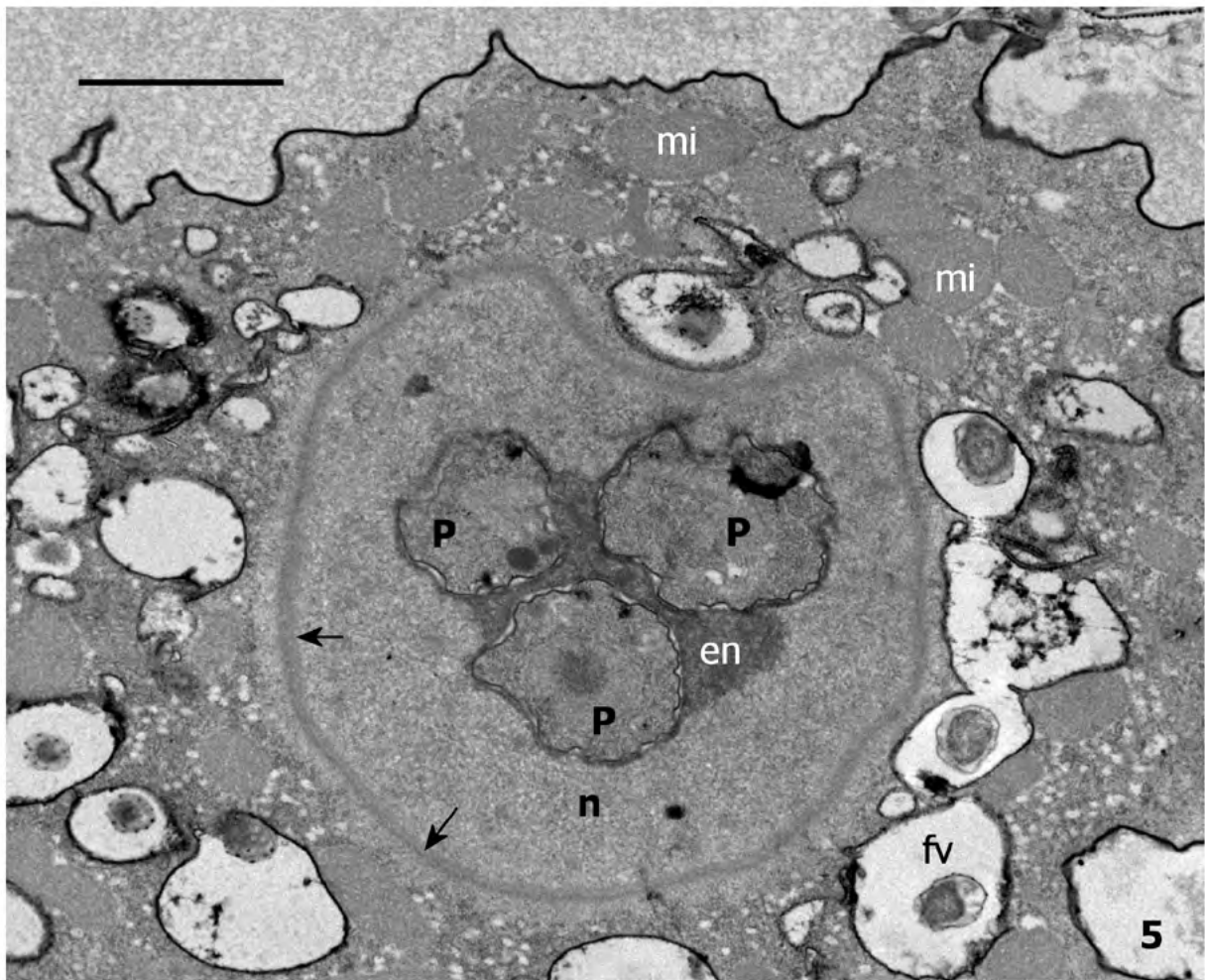
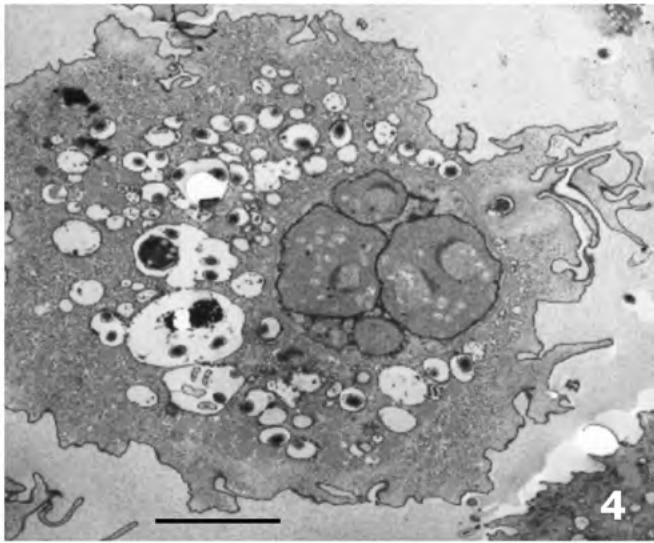


**Fig. 1. a** – *Thecamoeba quadrilineata*, strain Tq-2: three non-infected trophozoites with normal nucleus (n) forming characteristic dorsal folds (arrows) during unidirectional locomotion; **b** – three trophozoites – two of which showing enlarged nuclei caused by the developing intranuclear parasites (p), a, b – phase contrast, 450 ×; **c** – higher magnification of a trophozoite with one intranuclear parasite (p), nuclear membrane clearly visible, phase contrast, 1100 ×.

**Fig. 2. a** – *Thecamoeba quadrilineata*: one trophozoite with four intranuclear parasites (arrow), contractile vacuole (cv) in normal action. Phase contrast 450 ×; **b** – several intranuclear parasites differentiated into spores (sp), nuclear membrane has been disrupted by the growing parasite and the host amoeba has already died at this stage, bright field, 450 ×.

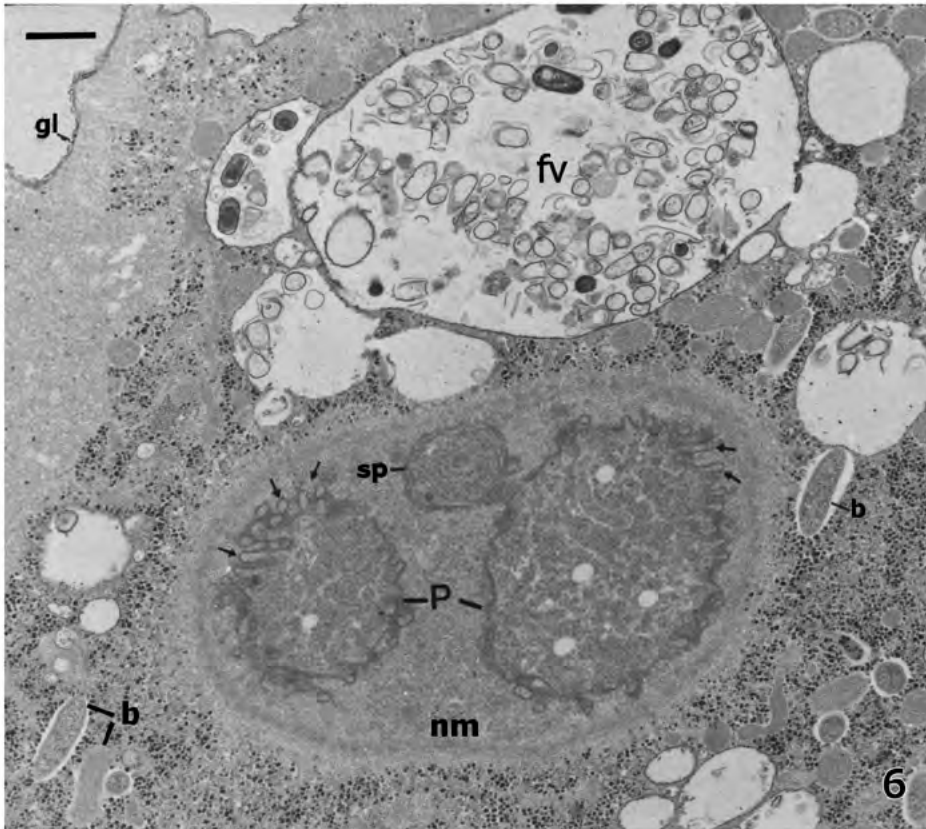
**Fig. 3. a** – Trophozoite of Tq-2: three mature intranuclear parasites after differentiation into numerous spores (arrows) partly liberated by rupture of the nuclear membrane of the host amoeba with disintegrated cytoplasm held together only by its pellicle. Phase contrast 1100 ×; **b** – trophozoite with a normal shaped nucleus showing intravacuolar spores (arrows) after ingestion from the environment – on the right hand few spores can be distinguished still outside the amoeba, phase contrast, 1100 ×.



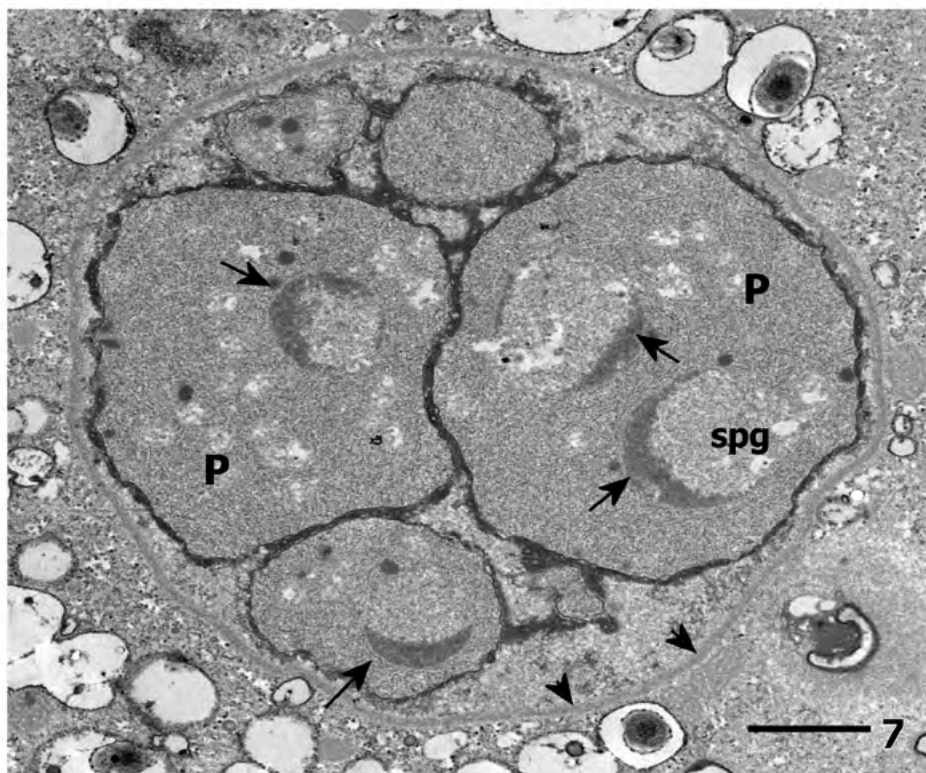


**Fig. 4.** Transmission electron micrograph (TEM) of an overview on a section of a *Thecamoeba quadrilineata* (T) parasitized by several endoparasites (p) occupying the enlarged nucleus. Fv-food vacuoles. Scale bar: 5.00  $\mu\text{m}$ .

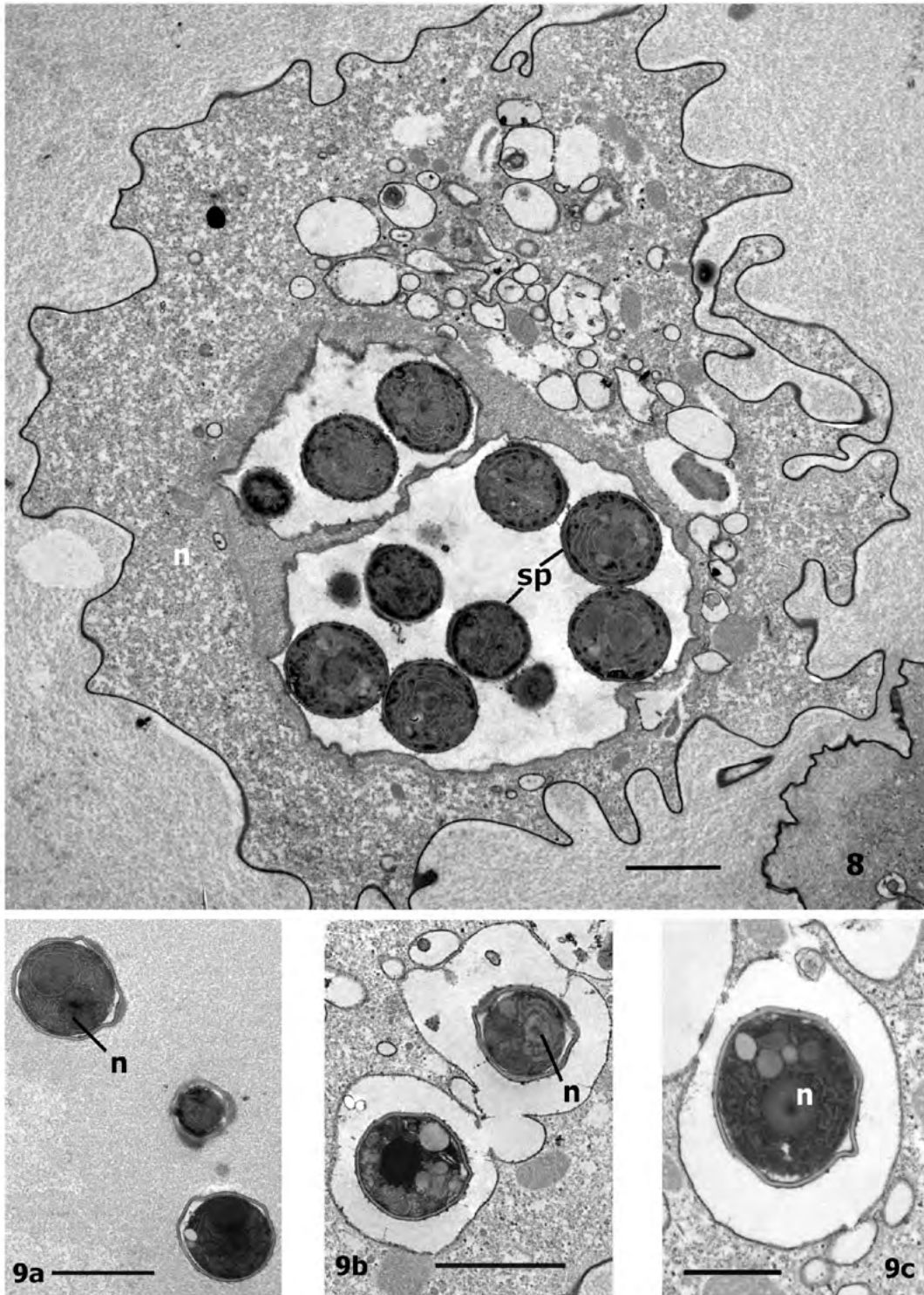
**Fig. 5.** TEM at higher magnification showing the nucleus (n) of an infected trophozoite of "Tq-2" harbouring three intranuclear parasites (p) obviously growing out from the margin of the significantly compressed endosome (en) of the nucleus. The nuclear membrane is clearly discernible (arrows), the nucleus is surrounded by various food vacuoles and also by a considerable number of mitochondria (mi). Scale bar: 1.00  $\mu\text{m}$ .



**Fig. 6.** TEM of a cytoplasmic area containing an infected nucleus with two more developed parasites (p) increased in size and a spore-like stage (sp) supposed to have intruded the nucleus. The older stages are bearing numerous microvilli at their surface allowing an intimate contact with the karyoplasm (arrows). The larger one has an extension of  $4.80 \times 3.80 \mu\text{m}$ . Together, they are enveloped by the nuclear membrane (nm). A large food vacuole contains some undigested bacteria and numerous remnants of them (fv). Few unidentified Gram-negative bacteria (b) within the cytoplasm represent endosymbionts. At this section especially a glycocalyx of the host amoeba is discernible (gl). Scale bar:  $1.00 \mu\text{m}$ .



**Fig. 7.** TEM of the hypertrophic nucleus at higher magnification showing further development of the spore-forming parasitic stages (up to  $5.50 \mu\text{m}$ ) within the nucleus surrounded by the nuclear membrane: distinct rounded areas with a diameter of  $1.40\text{--}2.00 \mu\text{m}$  in size lying in the range of mature spores (s. Fig. 8) are considered as spore generation centers (spg) with putative nuclear material arranged in form of a characteristic spherical calvarium (arrowheads). The parasites appear enveloped by a fuzzy coat of high electron density (arrows) enabling a close contact zone between the intranuclear parasites. Scale bar:  $1.00 \mu\text{m}$ .



**Fig. 8.** TEM of the parasitized nucleus (arrows) after differentiation of two mature parasites into spores (sp) of 1.50–2.00  $\mu\text{m}$  diameter. Cytoplasm appears still intact so far. Scale bar: 2.00  $\mu\text{m}$ .

**Fig. 9a** – Spores distributed in the environment, liberated by rupture of the host amoeba. Nucleus (n) can be easily distinguished, 10 500  $\times$ , scale bar: 2.00  $\mu\text{m}$ ; **b** – spores within food vacuoles (v) after ingestion by a hitherto not infected *Thecamoeba* 10 500  $\times$ , scale bar: 2.00  $\mu\text{m}$ ; **c** – spore at higher magnification (1.5  $\times$  1.9  $\mu\text{m}$ ) showing important details such as the nucleus and a vacuole with unknown function (star). It is enveloped by a distinct opaque wall of 42 nm limited by two elementary membranes. Scale bar: 1.0  $\mu\text{m}$ .

membranes, numerous ribosomes, and vacuoles of different electron density. They are encapsulated by a thick double-layered cell wall (Fig. 9c) with an inner space of 42 nm with low electron density.

## DISCUSSION

As a result of the detailed description of the various stages, the developmental cycle of the intranuclear parasite can be completely represented. By the possession of a distinct nucleus clearly discernible within the spores, it becomes evident that we discovered an eukaryotic endoparasite which we assume to belong to parasitic fungi. In order to corroborate this assumption, molecular biological methods are still necessary to identify the phylogenetic and taxonomic position of these intranuclear parasites as well. It can clearly be separated from previously described intranuclear parasites of vannellae affiliated morphologically to microsporidia-like organisms (Hoffmann *et al.* 1998, Michel *et al.* 2000).

Concerning fungi as endoparasites, we previously observed yeast-like fungi budding within cytoplasmic vacuoles of *T. similis* (Michel 1997). These yeasts served as intermediate between the parasitic and saprophytic way of life because on one side, they multiplied within vacuoles containing residues of digested bacteria, and on the other hand, they resisted lysosomal enzymes released into the phagosomes by the host amoeba (Michel 1997). Only recently it was proven that even the highly pathogenic *Cryptococcus neoformans* is able to grow within FLA such as acanthamoebae (Steenbergen *et al.* 2001). The observation that both the present and the yeast-like endoparasites were observed within thecamoebae as well as *Cochlonema euryblastum* suggests that the thecamoebae are preferred as host cells by various parasitic fungi. As was shown recently by cocultivation assays, “KTq-2” is not confined to its original amoebal host “Tq-2” alone but underwent a comparable development with production of infective spores within *Thecamoeba striata* and *T. terricola* as well (Michel 2007). Therefore, they are also considered as natural hosts of these intranuclear fungus-like parasites. This is not the case with *Sappinia* sp. because in most cases no mature spores were produced within the nucleus of this experimental host. However, it is a remarkable observation that both nuclei became infected at the same time leading to detachment of the otherwise closely attached nuclei.

As it is to a certain extent surprising that both, thecamoebae, *T. striata* and *T. terricola*, could be infected readily, despite the different structures of their nuclei, it is necessary to investigate the parasitic development within these experimental hosts by electron microscopy as well as the course of infection within *Sappinia* sp.

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