Real-time observations on the development of intra-nuclear parasite *Nucleophaga amoebae* (Rozellomycota) in the culture of *Thecamoeba quadrilineata*

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Summary

Nucleophaga amoebae belongs to the phylum Rozellomycota (Opisthokonta), a widespread clade of parasites, considered as intermediate link between fungi and microsporidia. This organism is an obligate intranuclear parasite of the free-living amoeba *Thecamoeba quadrilineata*. The life cycle of this organism is difficult to study, many details require further clarification, and available light-microscopic images are limited in number and quality. We performed real-time observations on the process of parasite propagation in amoeba culture using Eppendorf Cell Imaging Plates and Differential Interference Contrast (DIC) microscopy. Development of the parasite was traced from the engulfment of spores by the amoeba cell to the production of a new generation of spores. *Nucleophaga* cells proliferate inside the host nucleus. The earliest intranuclear developmental stages that we observed were rounded uninucleate cells located at the margin of the host nucleolus. Growth resulted in formation of a large multinucleate plasmodium, which further became segregated into numerous individual uninucleate sporoblasts. After a period of maturation, sporoblasts transformed into the rounded spores enclosed in the sporophorous vesicle, probably formed by the remnants of the membrane of the plasmodium. At the final stage of the developmental cycle the amoeba cell died, its envelope, as well as the nuclear membrane broke, and the spores were released into the environment. The developmental cycle took approximately 5 days. Infected amoebae never divided, so we can suggest that the infection suppressed mitosis in the host cell.

Key words: amoeba, life cycle, microsporidia, *Nucleophaga*, nucleus, parasite, Rozellomycota

Introduction

The Rozellida (Cryptomycota Jones and Richards, 2011, Rozellomycota Corsaro et al., 2014, Rozellosporidia Karpov et al., 2017) are a widespread environmental clade of parasites, an intermediate link between fungi and microsporidia (Lara et al., 2010; Jones et al., 2011; Karpov et al., 2014, 2017; Bass et al., 2018). Most of the diversity of rozellids remains cryptic. Around 300 environmental sequences are available in GenBank, but only a few species belonging to this group have been isolated and studied at the organismal level. Among the described species of rozellids there are remarkable intranuclear parasites of amoebae – the species of the genera *Nucleophaga* and *Paramicrosporidium*. They possess certain microsporidia-like traits in their morphology. Some authors considered them as microsporidia-like rozellids (Corsaro et al., 2014a, 2014b, 2016), while others suggested an extension of the microsporidia, with the inclusion of these groups as 'short-branched' ones (Bass et al., 2018). Studies on these organisms are of key importance for understanding the early evolution of the Holomycota and the origin of the microsporidia – a unique group of highly reduced intracellular parasites.

The genus *Nucleophaga* was established by Dangeard (1895). The type species, *N. amoebae* was detected in the karyoplasm of *Thecamoeba verrucosa* (Ehrenberg, 1838, as *Amoeba*) Schaeffer, 1926. Similar parasites were reported from a number of amoeba species as well as from other protists (Table 1). More recently, these intranuclear parasites of amoebae were found in the environment by Rolf Michel with colleagues and became an object of active studies (Michel, 1997, 2006; Hoffmann et al., 1998; Michel et al., 2000, 2009a, 2009b, 2012). A strain of *T. quadrilineata* infected with a parasite resembling *N. amoebae* was isolated from the mesh-work of the roots from moss collected near the entrance of the grotto at Tannheim in Austria (Michel, 2006; Michel et al., 2009a). Another parasite, found in a culture of *T. terricola* established from a sample of the bark from a Sycamore tree in Andernach (Germany), was assigned to a new species of *Nucleophaga* – *N. terricolae* (Michel et al., 2012; Corsaro et al., 2014b). Phylogenetic studies demonstrated rather close relationships of *Nucleophaga* spp. with another group of intranuclear parasites of amoebae, belonging to the genus *Paramicrosporidium*. Both these groups emerged as separate lineages within the highly supported rozellids (Corsaro et al., 2014a, 2014b, 2016).

Most records of *Nucleophaga*-like organisms belong to the late 19th – early 20th centuries. They were mainly based on incidental observations made on fixed and stained materials. In contrast, the strain isolated by Michel (2006) is still maintained in a culture of *T. quadrilineata*. The original host strain CCAP 1583/10 was studied by LM and EM and confirmed to belong to this species (Kamyshatskaya et al., 2018). In the present study, we performed real-time continuous observations on the culture of *T. quadrilineata* CCAP 1583/10 infected with *N. amoebae* strain KTq2, cultured in Eppendorf Cell Imaging Plates with fine (0.17 mm) glass bottom. This allowed us to apply Differential Interference Contrast (DIC) optics to get higher resolution images of the developmental stages of this parasite inside the host nucleus. We traced the parasite's life cycle from the engulfment of the spores by an amoeba cell to the production of a new generation of spores and their release into the environment. The results provide better knowledge on the development of this organism. Our approach may be used to study the development of similar parasites in a variety of unicellular hosts.

Material and methods

The culture of *Thecamoeba quadrilineata* CCAP 1583/10, a host species of *Nucleophaga amoebae* strain KTq2, was maintained in 60 mm Petri dishes, half-filled with wMY agar (Spiegel et al., 1995) at +18 °C. Amoebae in cultures fed on accompanying bacteria. To maintain *Nucleophaga* infection during subculturing, a small piece of agar with free spores was added to the Petri dish together with the agar piece containing the amoebae. Within 10–14 days a dense culture containing both, healthy and infected amoeba cells, was observed. Mature spores, released from the destroyed cells in culture were used as a spore source for further experiments. Both, infected and non-infected amoeba cultures, were maintained in parallel.

Real-time observations on the development of *N. amoebae* were performed using 24-well Eppendorf Cell Imaging Plates (#0030741021, Eppendorf, Germany) with glass bottom made of 0.17 mm coverslips. A single healthy amoeba cell from a non-infected culture was washed three times with sterile PJ medium (Prescott and James 1955) to eliminate the bacteria and then transferred into

Table 1. Host range and occurrence of *Nucleophaga* species in amoebozoans and some other groups of protists.

Host organism	Parasite	Reference
Thecamoeba verrucosa (Ehrenberg, 1838) Schaeffer, 1926	*Nucleophaga amoebae* Dangeard, 1895	Dangeard, 1895
Thecamoeba quadrilineata (Carter, 1856) Lepşi, 1960	*Nucleophaga amoebae* Dangeard, 1895	Michel, 2006; Michel et al., 2009a; Corsaro et al., 2014b; MycoBank & Index Fungorum 172878; GenBank JQ288099
Thecamoeba terricola (Greeff, 1866) Lepşi, 1960	*Nucleophaga terricolae* Corsaro et al., 2016	Michel et al., 2012; Corsaro et al., 2016; MycoBank & Index Fungorum 816522; GenBank KX017226
Thecamoeba terricola (Greeff, 1866) Lepşi, 1960	*Nucleophaga* sp. as "*Sphaerita nucleophaga*"	Mattes, 1924
Thecamoeba sphaeronucleolus (Greeff, 1891) Schaeffer, 1926	*Nucleophaga* cf. *amoebae*	Penard, 1902, 1905
Thecamoeba sphaeronucleolus (Greeff, 1891) Schaeffer, 1926	*Nucleophaga* sp. as "*Sphaerita nucleophaga*"	Mattes, 1924
Mayorella viridis (Leidy, 1874) Harnisch, 1968	*Nucleophaga* sp.	Gruber, 1904
"*Amoeba vespertilio*"* Penard, 1902	*Nucleophaga* sp. as "*Sphaerita nucleophaga*"	Mattes, 1924
Endolimax nana Wenyon et O'Connor, 1917	*Nucleophaga* sp.	Nöller, 1921, 1922
Endolimax nana Wenyon et O'Connor, 1917	*Nucleophaga nana* Brumpt et Lavier, 1935	Brumpt and Lavier, 1935
Iodamoeba butschlii (Prowazek, 1912) Dobell, 1919	*Nucleophaga* sp.	Nöller, 1921, 1922
"*Endolimax williamsi*"* Prowazek, 1911	*Nucleophaga intestinalis* Brug, 1926	Brug, 1926; Index Fungorum 628247
Entamoeba ranarum Grassi, 1879	*Nucleophaga ranarum* Lavier, 1935	Lavier, 1935; MycoBank & Index Fungorum 276112
Endamoeba blattae Bütschli, 1878	*Nucleophaga* sp.	Mercier, 1907, 1910; Janicki, 1909
Arcella vulgaris Ehrenberg, 1830	*Nucleophaga* sp.	Elpatiewsky, 1907
Endamoeba disparata Kirby, 1927	*Nucleophaga* sp.	Kirby, 1927
Endamoeba majestas Kirby, 1927	*Nucleophaga* sp.	Kirby, 1927
Endamoeba simulans Kirby, 1927	*Nucleophaga* sp.	Kirby, 1927
Endolimax termitis Kirby, 1927	*Nucleophaga* sp.	Kirby, 1927
Endamoeba beamonti Kirby, 1932	*Nucleophaga* sp.	Kirby, 1932
Entamoeba citelli Becker, 1926	*Nucleophaga* sp.	Sassuchin, 1931
"*Pygolimax gregariniformis*"* Tyzzer, 1920	*Nucleophaga* sp.	Tyzzer, 1920
"*Amoeba vespertilio*"* Penard, 1902	"*Nucleophaga*-like intranuclear parasite"	Doflein, 1907
"*Naegleria* sp." (probably *Endolimax nana*)	"*Nucleophaga*-like intranuclear parasite" (*Nucleophaga hypertrophica*, Epstein 1922)	Epstein, 1922
Peranema trichophorum Ehrenberg, 1838 (Euglenida)	"*Nucleophaga*-like intranuclear parasite" (as *Nucleophaga peranema* Hollande et Balsac, 1942)	Hollande and Balsac, 1942; MycoBank & Index Fungorum 628243
various parabasalids	"*Nucleophaga*-like intranuclear parasite"	Kirby, 1941
Pseudospora volvocis Cienkowski, 1865 (Cercozoa)	"*Nucleophaga*-like intranuclear parasite"	Robertson, 1905; Kirby, 1941

Notes: * – the validity of species name remains doubtful.

a well of the Cell Imaging Plate filled with sterile PJ medium. Mature spores of *Nucleophaga* were collected from the agar of an infected culture, washed three times as described above and added to the well of the plate. The density of experimental infection depended on the number of spores added to the healthy amoeba cells. To receive low-infected cells with prevalence of individual infections, approximately 10 spores per cell were added. In experiments on "hyperinvasion" approximately 100 spores per cell were transferred. After inoculation, plates were maintained at +18 °C. In every series of experiments, two plates were inoculated with 12 hrs shift. The development of infection was monitored every 3 hrs during the day (10 am − 10 pm). This scheme ensured the continuous monitoring of parasite development with 3 hrs steps. Plates were observed and photographed using an inverted Leica DMI3000 microscope equipped with DIC optics and Leica DFC 295 photo camera. To get higher quality images, a number of infected cells at different time points of development were transferred to

the object slides and photographed using upright microscope Leica DM2500 equipped with DIC optics and a DS-Fi-3 camera (Nikon, USA).

Results

Non-infected amoebae were oblong or nearly rounded, with characteristic dorsal folds and wrinkles. They had spherical nuclei with a single central nucleolus. The latter often possessed several lacunas (Fig. 1, A−C). Amoeba cells engulfed *Nucleophaga* spores by phagocytosis. During the first 24 hrs, food vacuoles containing spores could be observed in the host cytoplasm (Fig. 1, D). In most cases, the parasites infected the amoeba nucleus during the following 12 hrs. The moment of transfer of the parasite from the phagosome to the nucleus was never observed. The earliest intranuclear stages that we could detect were small, rounded cells of the parasite localized at the margin of the nucleolus of the amoeba nucleus (Fig. 1, D−F). During their growth, cells of the parasite, which we recognised as 'sporonts' started to show irregularities on their surface, visible at the optical level as an uneven enlightenment along the surface of the parasite cell (Fig. 1, F). The size of the uninucleate sporont varied from 2.7 to 6.7 μm, depending on the age. During the following 2 days we observed a rapid enlargement of the host nucleus and the formation of the multinucleate plasmodium of the parasite inside the nucleolus (Fig. 1, G). Within 36 hrs after infection the multinucleate plasmodium of *N. amoebae* occupied almost the entire internal space of the host nucleus (Fig. 1, H). Uneven fringe enlightenments as observed in early sporonts were seen on the surface of the plasmodium as well (Fig. 1, G, H). The maximal observed size of the mature plasmodium was approximately 20 μm. Within 48 hrs after infection, the plasmodium started to fragment into sporoblasts; this was the onset of the sporogenesis (Fig. 1, I). At the final stage of development, the sporogonial plasmodium transformed into a sporophorous vesicle with numerous rounded sporoblasts, which further matured into the spores. Their number was variable, and due to tight packing of the spores in the vesicle could not be calculated precisely. However, we estimated between ~50 to ~350 spores in one sporophorous vesicle in the case of individual infection. Throughout the development of the parasite, the host cell maintained integrity and mobility, yet it never divided, but died soon after the formation of spores (Fig. 1, J). Mature spores had a regular spherical shape, their size varied from 2.7 to 3.0 μm (average 2.8 μm). The development of the parasite from the moment of invasion until the maturation of the next generation of spores took around 5 days under the conditions tested. After the death of the amoeba, the spores remained inside the cell remnants, being enclosed in the 'sporophorous vesicles', supposedly formed by the membrane of the plasmodium and further surrounded with the derivatives of the host nuclear envelope and its cytoplasmic membrane. However, all these envelopes were rather fragile and the spores were soon found to occur freely in the environment (Fig. 1, J).

In our experiments, cases of infection of an amoeba by a single *Nucleophaga* cell were rare. Usually, we observed co-infections of an amoeba with several cells of the parasite. When the number of added spores exceeded 100 per host cell, the infection was always multiple ('hyperinvasion'). In this case, 2 to 9 parasites infected one amoeba nucleus (Fig. 2, A−E). In such cases the development of the individual parasites occurred asynchronously (Fig. 2, D−E). These multiple infections resulted in the formation of several sporophorous vesicles within one host nucleus at the end of sporogenesis. The size of the vesicles and the number of spores produced in each of them accordingly was smaller (Fig. 2, F). The final size of the infected nucleus in case of 'hyperinvasion' was slightly larger than in case of individual infection, but it was occupied with several smaller plasmodia instead of a single large one. In the smallest vesicle observed we counted only 12 spores; the largest ones contained more than a hundred spores.

Discussion

In previous studies, the development of *Nucleophaga amoebae* in living amoeba cells was observed using phase-contrast light microscopy in cultures and on microscope slides (Michel et al., 2009a). We applied DIC microscopy, which allows better resolution of the inner structure of the cell. Observations were performed in living cultures, using Cell Imaging Plates with a thin glass bottom. This allowed us to apply high aperture lenses to trace the entire cycle of the development of parasites in an individual amoeba cell and to get DIC images of the parasite at different stages of the life cycle.

Generally, our study confirmed the observations by Michel et al. (2009a), Corsaro et al. (2014b)

Fig. 1. Light microscopic observation of the development of *Nucleophaga amoebae* KTq2 in the culture of *Thecamoeba quadrilineata* CCAP 1583/10 in case of individual infection, DIC. A – Non-infected amoeba; B, C – non-infected nucleus of amoeba under higher magnification; D – spores of the parasite in vacuoles; E – young sporont inside amoeba nucleus; F – uninucleate sporont with irregular surface; G, H – subsequent stages of growth of parasite plasmodium; I – sporogenesis; J – mature spores in the dead amoeba cell. *Abbreviations*: n – amoeba nucleus, nu – amoeba nucleolus, p – parasite, *black arrowheads* – parasite nucleus. Scale bars: A, D – 10 μm, B, C, E–J – 5 μm.
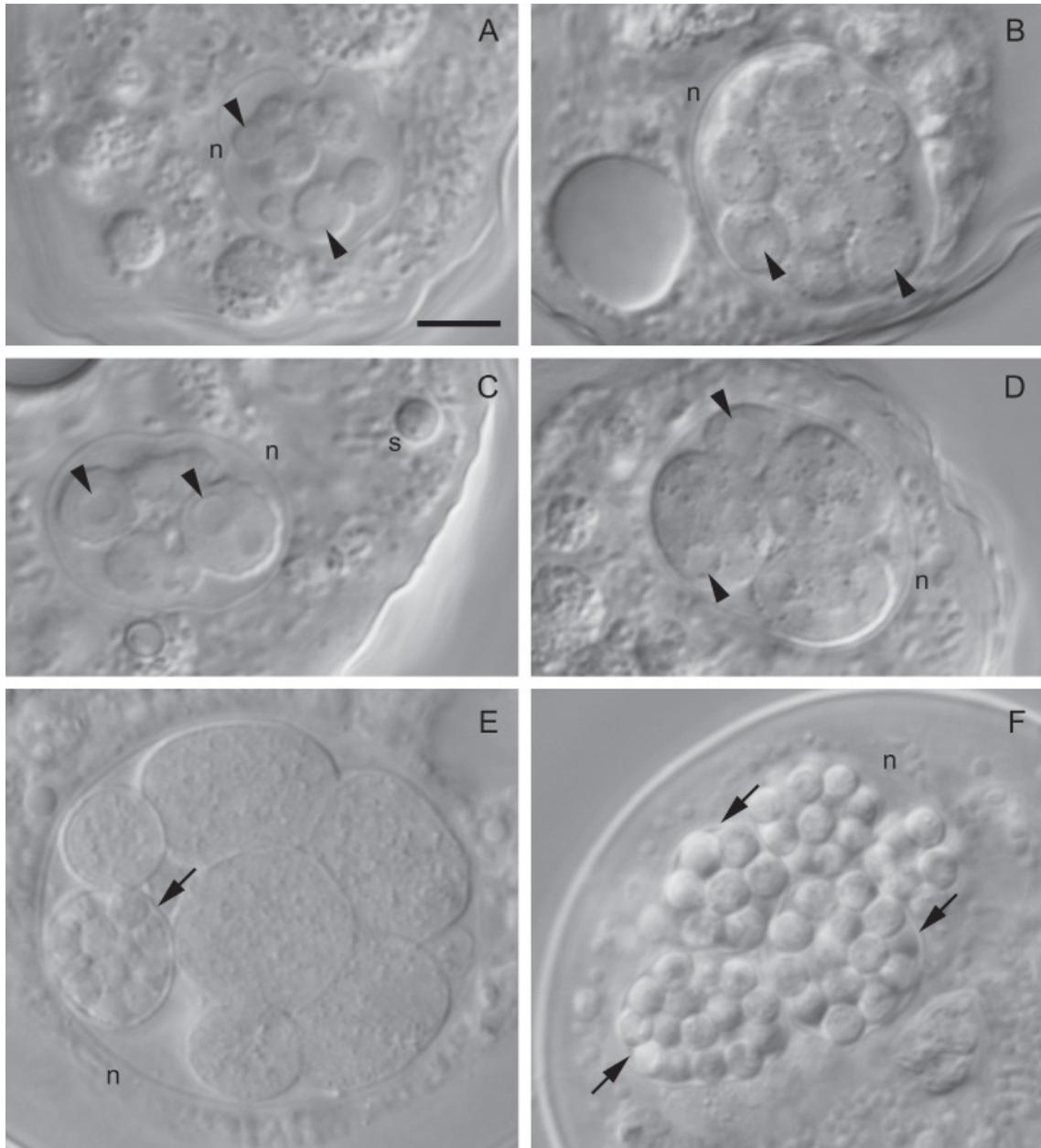
Fig. 2. Light microscopic observation of the development of *Nucleophaga amoebae* KTq2 in the culture of *Thecamoeba quadrilineata* CCAP 1583/10 in case of multiple infection ('hyperinvasion'), DIC. A–C – Young sporonts; D – plasmodia of the parasite; E – several plasmodia of different size and a sporophorous vesicle; F – several sporophorous vesicles. *Abbreviations*: n – amoeba nucleus, s – engulfed spore in the phagosomes, *black arrowheads* – parasite nucleus, *black arrow* – sporophorous vesicle. Scale bar: A–F – 5 µm.

and by the earlier authors (Dangeard,1895) on the development of this parasite, however, some new details were recovered. The general outline of the *Nucleophaga* life cycle (Fig. 3) resembles the development of many other spore-forming parasites and, in particular – microsporidia (Cali and Takvorian, 2014). The division of early uninucleate stages (the process, which can be interpreted as merogony) was never observed, so we conclude that it does not appear in the life cycle of this organism. This conclusion is in a good agreement with the observations of other authors suggesting the absence of merogony in the life cycles of phylogenetically close groups, e.g. metchnikovellids
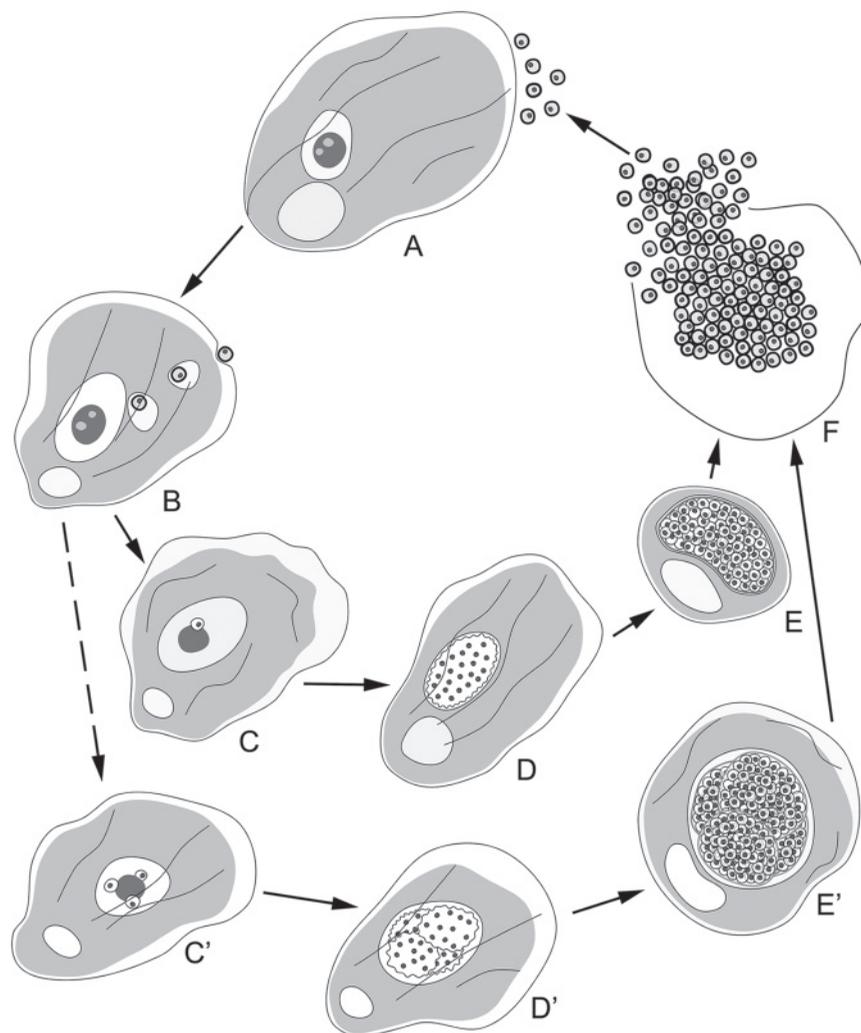
Fig. 3. Life cycle of *Nucleophaga amoebae* in the culture of *Thecamoeba quadrilineata* in case of single (C−E) and multiple infections (C'−E'); schematic drawing. A − Non-infected amoeba; B − phagocytosis of the spores of parasite; C, C' − early stage of parasite development: young uninucleate sporont(s) localized at the margin of the nucleolus of host nucleus; D, D' − proliferation of the parasite: multinucleate plasmodium(-ia); E, E' − sporogenesis: formation of the sporophorous vesicle(s) with numerous spores in the hypertrophied nucleus; F − mature spores within the degenerated amoeba nucleus.

and chytridiopsids (Larsson, 2014; Sokolova et al., 2014). Hence, uninucleate developmental stages of *Nucleophaga* may be interpreted as sporonts. Thus, the multinucleate plasmodium formed at a later stage of the life cycle may be interpreted as a sporogonial plasmodium.

The irregular surface of the sporonts, well visible under the DIC optics, attracts special attention. We suppose that this feature correlates with the formation of tubular structures and finger-like outgrowths of the envelopes of late sporonts and plasmodia observed in TEM micrographs (Figs 6−7

in Michel et al., 2009a; Fig. 1h in Corsaro et al., 2014b; Kamyshatskaya et al., 2019). These structures are probably participating in the intensification of the host-parasite exchange.

In earlier studies, a rapid and significant enlargement of the host nucleus was considered to be the first sign of infection with *Nucleophaga* spp. (Dangeard, 1895, Brumpt and Lavier, 1935, Michel et al., 2009a). In our experiments, in cases of individual infection, the hypertrophy of the host nucleus at the initial stages of parasite development was not so obvious. The plasmotomy begun when

the plasmodium occupied the entire volume of the nucleus, probably at this moment the resources of the host cell nucleus were entirely depleted. It is interesting that the invasion of the nucleus with multiple parasites did not result in a higher efficiency of spore formation; the total number of spores after hyperinvasion was generally lower than after the single invasion. Apparently, the parasites start to compete for the resources and this results in lower growth efficiency. Another point may be mainly a physical one — the need to place several rounded plasmodia inside the nucleus results in a less efficient use of its volume, thus the total volume of the plasmodia is lower.

In our cultures used to maintain *Nucleophaga*, about 80% on average of the infected amoeba cells were invaded by two or more parasites. During the experiments in cell plates it was difficult to achieve the invasion of an amoeba cell by a single parasite, and this required decreasing the number of spores used to the lowest limit when a cell has the chance to meet and engulf a spore. Dangeard (1895) also reported cases of multiple (up to 4) and sometimes asynchronous infections. This is probably related to the fact that in culture amoebae engulfed many spores simultaneously as the spores did not immediately disperse in the environment but remained in groups within the remnants of the dead amoeba cells. It is unlikely that this is the same in nature. The density of amoeba populations is generally much lower in natural habitats than in cultures as could be observed when isolating infected amoebae from the environment for the first time. The envelopes surrounding the spores are rather fragile, and most probably the spores disperse in the environment over long distances rather quickly after the death of amoeba cell.

The pattern of parasite development may differ between *Nucleophaga* species (Blackwell et al., 2019). In our observations on *N. amoebae*, after the infection of the nucleus, young parasites were always localized at the margin of the nucleolus. Further developmental stages of the parasite were located inside the nucleolus, and finally the plasmodium fil-led almost the entire nucleus. Kirby (1927) described that *Nucleophaga* sp. surrounded the nucleolus inside the nucleus, 'centralizing' it. In contrast, in the case of a *Nucleophaga*-like intranuclear parasites found in *Trichonympha* sp., the parasite develops in the centre of the nucleolus and displaces the nucleolar material to the periphery of the nucleus (Kirby, 1941). Epstein (1922) mentioned a similar process for a *Nucleophaga*-like intranuclear parasite

infecting the amoeba "*Naegleria* sp." Mattes (1924) described that the parasite in *Thecamoeba* (*Amoeba*) *sphaeronucleolus* pushed the remains of the nucleolus to the periphery of the nucleus. In contrast, the species *N. intestinalis* developed in the karyoplasm outside of the nucleolus, subsequently flattening it (or its remnants) against the nuclear envelope (Brug, 1926). The same pattern was observed by Tyzzer (1920) for *Nucleophaga* sp. developing in "Pygolimax gregariniformis". These observations indicate that the pattern of parasite development may be species-specific and could be utilised for species distinction.

Acknowledgements

References

Bass D., Czech L., Williams B.A.P., Berney C., Dunthorn M., Mahé F., Torruella G., Stentiford G.D. and Williams T.A. 2018. Clarifying the relationships between Microsporidia and Cryptomycota. J. Eukaryot. Microbiol. 65, 773−782. doi:10.1111/jeu.12519.

Blackwell W.H., Letcher P.M. and Powell M.J. 2019. Review of *Nucleophaga* (a primitive, 'cryptomycotan' genus): Summary of named and unnamed species, with discussion of contemporary and historical observations. Phytologia. 101, 1−18.

Brug S.L. 1926. *Nucleophage intestinalis* n. sp., parasiet der Kern van *Endolimax williamsi* (Prow.) = *Endolimax bütschlii* (Prow.). Mededelingen van den Dienst der Volksgezondheit in Nederlandisch-Indië. 4, 466−468.

Brumpt E. and Lavier G. 1935. Sur une *Nucleophaga* parasite d'*Endolimax nana*. Ann. Parasitol. Hum. Comp. 13, 439−444.

Cali A. and Takvorian P.M. 2014. Developmental morphology and life cycles of the microsporidia. In: Microsporidia - Pathogens of Opportunity, Vol. 2 (Eds: Weiss L.M. and Becnel J.J.). Wiley Blackwell Press, Hoboken, New Jersey, pp. 71−133.

Corsaro D., Walochnik J., Venditti D., Steinmann J., Müller K.D. and Michel R. 2014a. Microsporidia-like parasites of amoebae belong to the

early fungal lineage Rozellomycota. Parasitol Res. 113, 1909−1918. doi:10.1007/s00436-014-3838-4.

Corsaro D., Walochnik J., Venditti D., Müller K.D., Hauröder B. and Michel R. 2014b. Rediscovery of *Nucleophaga amoebae*, a novel member of the Rozellomycota. Parasitol Res. 113, 4491−4498. doi:10.1007/s00436-014-4138-8.

Corsaro D., Michel R., Walochnik J., Venditti D., Müller K.D., Hauröder B. and Wylezich C. 2016. Molecular identification of *Nucleophaga terricolae* sp. nov. (Rozellomycota), and new insights on the origin of the Microsporidia. Parasitol Res. 115, 3003−3011. doi:10.1007/s00436-016-5055-9.

Dangeard P.A. 1895. Memoire sur les parasites du noyau et du protoplasma. Le Botaniste. 4, 199−248.

Doflein F. 1907. Studien zur Naturgeschichte der Protozoen. V. Amöbenstudien. Arch. Protistenk. 1, Suppl. 1.

Elpatiewsky W. 1907. Zur Fortpflanzung von *Arcella vulgaris* Ehrbg. Arch. Protistenk. 10, 441−466.

Epstein H. 1922. Über parasitische Infektion bei Darmamöben. Arch. Rus. Soc. Protistol. 1, 46−78 (in Russian with German summary).

Grüber. 1904. Über *Amoeba viridis* Leidy. Zool. Jahrb., Festschr. Weissmann, Suppl. 7. p. 67.

Hoffmann R., Michel R., Schmid E.N. and Müller K.D. 1998. Natural infection with microsporidian organisms (KW19) in *Vannella* spp. (Gymnamoebia) isolated from a domestic tap-water supply. Parasitol Res. 84, 164−166. doi:10.1007/s004360050377

Hollande A. and de Balsac H.H. 1942. Parasitisme du *Peranema trichophorum* par une Chytridinée du genre *Nucleophaga*. Arch. Zool. Exp. Gén. 82, 37−46.

Janicki C. 1909. Über Kern und Kernteilung bei *Entamoeba blattae* Bütschli. Biol. Zbl. 29, 381−393.

Jones M.D., Forn I., Gadelha C., Egan M.J., Bass D., Massana R. and Richards T.A. 2011. Discovery of novel intermediate forms redefines the fungal tree of life. Nature. 474 (7350), 200−203. doi:10.1038/nature09984.

Kamyshatskaya O., Mesentsev Y., Smirnov A., Michel R., Walochnik J. and Nassonova E. 2018. Fine structure of *Thecamoeba quadrilineata* strain CCAP 1583/10 (Amoebozoa, Discosea, Thecamoebida), the host of *Nucleophaga amoebae* (Opisthosporidia). Protistology. 12, 191−201. doi:/10.21685/1680-0826-2018-12-4-4.

Kamyshatskaya O., Gordetskaya O., Mezentsev Y., Walochnik J., Corsaro D., Michel R., Smirnov

A. and Nassonova E. 2019. Ultrastructural affinities of *Nucleophaga amoebae* (Opisthokonta: Rozellomycota): from Rozella to Microsporidia. Abstr. VIII Europ. Congr. of Protistol. − ISOP joint meeting. Rome, Italy. P. 57. http://www.ecop2019.org/abstract-book/.

Karpov S.A., Mamkaeva M.A., Aleoshin V.V., Nassonova E., Lilje O. and Gleason F.H. 2014. Morphology, phylogeny, and ecology of the aphelids (Aphelidea, Opisthokonta) and proposal for the new superphylum Opisthosporidia. Front Microbiol. 5, 112. doi:10.3389/fmicb.2014.00112

Karpov S.A., Torruella G., Moreira D., Mamkaeva M.A. and López-García P. 2017. Molecular phylogeny of *Paraphelidium letcheri* sp. nov. (Aphelida, Opisthosporidia). J. Eukaryot. Microbiol. 64, 573−578. doi:10.1111/jeu.12389.

Kirby H.Jr. 1927. Studies on some amoebae from the termite Mirotermes, with notes on some other Protozoa from the Termitidae. Quart. J. Micro. Sci. 71, 189−222.

Kirby H.Jr. 1932. Protozoa in termites of genus *Amitermes*. Parasitology. 24, 289−304.

Kirby H.Jr. 1941. Organisms living on and in Protozoa. In: Protozoa in Biological Research (Eds: Calkins G. N. and Summers F. S.). Colombia University Press, New York, pp. 1009−1113.

Lara E., Moreira D. and López-García P. 2010. The environmental clade LKM11 and *Rozella* form the deepest branching clade of fungi. Protist. 161, 116−121. doi:10.1016/j.protis.2009.06.005.

Larsson J.I.R. 2014. The Primitive Microsporidia. In: Microsporidia: Pathogens of Opportunity. 1st ed. (Eds: Weiss L.M. and Becnel J.J.). John Wiley and Sons, Inc., Ames, Iowa, pp. 605−634.

Lavier G. 1935. Sur une *Nucleophaga* parasite du noyau d'*Entamœba ranarum*. Ann. Parasitol. Hum. Comp. 13, 351−361.

Mattes, O. 1924. Über Chytridineen im Plasma und Kern von *Amoeba sphaeronucleolus* und *Amoeba terricola*. Arch. Protistenk. 47, 413−430.

Mercier L. 1907. Un parasite du noyau d'*Amoeba blattae* Bütschli. C.R. Soc. Biol. Paris. 62, 1132−1134.

Mercier L. 1910. Contribution a l'etude de l'amibe de la Blatte (*Entamoeba blattae* Bütschli). Arch. Protistenk. 20, 143−175.

Michel R. 1997. Freilebende Amöben als Wirte und Vehikel von Mikroorganismen. Mitt. Österr. Ges. Tropenmed. Parasitol. 19, 11−20.

Michel R., Schmid E.N., Böker T., Hager D.G., Müller K.D., Hoffmann R. and Seitz H.M. 2000. *Vannella* sp. harboring Microsporidia-like

organisms isolated from the contact lens and inflamed eye of a female keratitis patient. Parasitol. Res. 86, 514−520. doi:10.1007/s004360050704.

Michel R. 2006. Isolierung und Darstellung von intranukleären Parasiten aus *Thecamoeba quadrilineata* und *Saccamoeba limax*. Mikrokosmos. 97, 101−107.

Michel R., Hauröder B. and Zöller L. 2009a. Isolation of the amoeba *Thecamoeba quadrilineata* harbouring intranuclear spore forming endoparasites considered as fungus-like organisms. Acta Protozool. 48, 41−49.

Michel R., Müller K.D. and Hauröder B. 2009b. A novel microsporidian endoparasite replicating within the nucleus of *Saccamoeba limax* isolated from a pond. Endocytob. Cell Res. 19, 120−126.

Michel R., Müller K.D., Schmid E.N., Theegarten D., Hauröder B. and Corsaro D. 2012. Isolation of *Thecamoeba terricola* from bark of *Platanus occidentalis* harbouring spore-forming eukaryotic endoparasites with intranuclear development. Endocytob. Cell Res. 22, 37−42.

Nöller W. 1921. Über einige wenig bekannte Darmprotozoen des Menschen und ihre nächsten Verwandten. Arch. Schiffs- u. Tropenhyg. 25, 35−46.

Nöller W. 1922. Die wichtigsten parasitischen Protozoen des Menschen und der Tiere. I Teil. Einführung in die allgemaine Kenntnis und die Abschnitt I: Die parasitischen Rhizopoden. Berlin.

Penard E. 1902. Faune rhizopodique du bassin du Léman. Henry Kündig, Genève.

Penard E. 1905. Observations sur les Amibes à pellicule. Arch. Protistenk. 6, 175-206.

Prescott D.M. and James T.W. 1955. Culturing of *Amoeba proteus* on *Tetrahymena*. Exp. Cell Res. 8, 256−258. doi:10.1016/0014-4827(55)90067-7.

Robertson M. 1905. *Pseudospora volvocis*, Cienkowski. Quart. J. Micro. Sci. 49, 213−230.

Sassuchin D. 1931. Zum Studium der Darmprotozoenfauna der Nager im Süd-Osten RSFSR. I. Darmprotozoen des *Citellus pygmæus* Pallas. Arch. Protistenk. 74, 417−428.

Sokolova Y.Y., Paskerova G.G., Rotari Y.M., Nassonova E.S. and Smirnov A.V. 2014. Description of *Metchnikovella spiralis* sp. n. (Microsporidia: Metchnikovellidae), with notes on the ultrastructure of metchnikovellids. Parasitology. 141, 1108−1122. doi:10.1017/S0031182014000420.

Spiegel F.W., Moore D.L. and Feldman J. 1995. *Tychosporium acutostipes*, a new protostelid, which modifies the concept of the Protosteliidae. Mycologia. 87, 265−270. doi:10.2307/3760912.

Tyzzer E.E. 1920. Amoebae of the caeca of the common fowl and of the turkey − *Entamoeba gallinarum*, sp. n., and *Pygolimax gregariniformis*, gen. et spec. nov. J. Med. Res. 41, 199−209.

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