Real-time observations on the development of intranuclear 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 plasmidium. 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

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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 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. terricola* (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
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

| Host organism | Parasite | Reference |
|---------------|----------|-----------|
| *Thecamoeba verrucosa* (Ehrenberg, 1838) Schaeffer, 1926 | Nucleophaga amoebae Dangeard, 1895 | Dangeard, 1895 |
| *Thecamoeba quadrilineata* (Carter, 1856) Leppi, 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) Leppi, 1960 | Nucleophaga terricolae Corsaro et al., 2016 | Michel et al., 2012; Corsaro et al., 2016; MycoBank & Index Fungorum 816522; GenBank KX017226 |
| *Thecamoeba sphaeronnucleolus* (Greeff, 1891) Schaeffer, 1926 | Nucleophaga cf. amoebae | Penard, 1902, 1905 |
| *Thecamoeba sphaeronnucleolus* (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 butschilii* (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 gregariformis" 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 hypotrophica, 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.
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 nucleus 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 maturated 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 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.
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

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**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.
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 plasmodium 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 filled 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 karyoplasma 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 gregariformis”. These observations indicate that the pattern of parasite development may be species-specific and could be utilised for species distinction.

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