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Becnelia sigarae gen. n., sp. n. Isolated from Testes of the Water Boatmen, *Sigara lateralis* (Heteroptera: Corixidae) in the Czech Republic

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Summary. A microsporidian *Becnelia sigarae* gen. n., sp. n. (Microspora: Amblyosporidae) was isolated from testes of a water boatman *Sigara lateralis* (Heteroptera: Corixidae) near Bavorov, South Bohemia, Czech Republic. The life cycle of the pathogen includes a merogony with uninucleate stages, a meiotic sequence in part with binucleate stages (diplokarya) and a sporogony resulting in a persistent sporophorous vesicle containing eight spores. Spores are long oval, slightly curved, with broader basis and equally rounded ends, 5 ± 0.5 x 2.5 ± 0.5 µm in diameter. A series ending with early spores serving for autoinfection in the primary host and spread of the infection in other tissues differs in ultrastructure of spores. They are shorter and more constricted apically and measure 4 x 2.5 ± 3 µm. Both spore types have a polaroplast with a central part with multiple broad chambers enclosed anteriorly and posteriorly in circular layers of dense lamellae. The spore wall of both types is characterized by a thin exospore and an endospore of equal thickness all over the spore with exception of the attenuated apical pole. Mature spores have an anisofilar polar filament coiled in 9 - 11 turns with first 5 - 6 broader turns and 4 - 5 narrower turns. The early spores have the filament coiled in 7 turns, of which 4 are broader and 3 narrower. All characteristics of the new microsporidium reveal that it is similar to different *Amblyosporidae* and we therefore propose to include it into this family. The new genus *Becnelia* is proposed with *B. sigarae* as a type species. The taxonomic position as well as the relationships to other microsporidia described from Heteroptera are discussed.

Key words: *Becnelia sigarae* gen. n., sp. n., Heteroptera, life cycle, microsporidia, *Sigara*, taxonomy, ultrastructure.

Abbreviations: a - anchoring disc, D - disporous sporogony, e - exospore, E - endospore, er - endoplasmic reticulum, F - polar filament (F_1 - narrow part), g - gamet (planont), G - Golgi system, h - hinge, m - system of multiple membranes, M - meront, Me - meiont, n - nucleolus, N - nucleus, o - granular secretions, O - early spores, p - plasmalemma, P - lamellar polaroplast, P_1 - broad chamber polaroplast, Ps - polar sac, r - polysomes, R - rosette stage, S - mature spores, Sb - sporoblast, sc - synaptonematic complex, Sn - sporonts, Sp - sporogonial plasmodia, Sw - wrinkled sporoblast, t - tubular secretions, u - umbrella, v - sporophorous vesicle,V - system of vacuoles.

INTRODUCTION

From different Heteroptera at least 9 microsporidian species were described up-to-date, mainly from the aquatic bugs (Sprague 1977). None of them has been studied using electron microscopy, as far as we know and there are no data on their ultrastructures and a complete life cycle.

One infected adult water boatman, *Sigara lateralis* (Leach, 1817) (Heteroptera: Corixidae) was found in a large population collected in a temporary pool. The infected male had an orange coloured abdomen in the

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region of gonads and was dissected for identification of the parasite. A microsporidian with octosporous sporophorous vesicles was found in the testes of the male animal. *Toxoglugea gerridis* Poisson, 1941, *T. mercieri* (Poisson, 1924) Jírovec, 1936 both with horseshoe - like bent spores, *Thelohania veliae* Poisson 1928 with large oval spores, *Chapmanium nepae* (Lipa, 1966) Hazard and Oldacre, 1975 with navicular pansporoblasts and *Octosporea carlochagasi* Kramer, 1972 with tubular spores were other previously described octosporous species (Poisson, 1928; Jírovec, 1936; Hazard and Oldacre, 1975; Kramer, 1972). The first four were recorded from Heteroptera from Europe.

In this study we describe morphological features and the life cycle of a new microsporidian with octosporous sporophorous vesicle which differs from these mentioned above.

MATERIALS AND METHODS

The infected adult male water boatman *Sigara lateralis* was collected in a temporary pool near the village Bavorov, NW from České Budějovice, South Bohemia, Czech Republic, in September 1998. A large group of water boatmen was brought to the laboratory and checked for presence of microsporidia. One single male had an orange coloured abdomen due to a microsporidian infection. It was dissected and from infected tissues smears were prepared, fixed in methanol and stained with 10% (v/v) Giemsa solution for 20 min. for inspection under the light microscope.

For electron microscopy, pieces of infected tissue were fixed in Karnovsky (2.5% glutaraldehyde, 2% paraformaldehyde) overnight at 4°C. After several washes in cacodylate buffer (pH 7.4) they were postfixed with 2 % osmium tetroxide for 2 h. Pieces of tissue were dehydrated in a graded aceton series and embedded in Durcupan. Semithin sections were stained with toluidin blue while ultrathin sections were stained with uranyl acetate and lead citrate in routine process and examined with a JEOL 1010 TEM at 80kV.

RESULTS

Pathology in the host

In the inspected group of water boatmen one single adult male was infected, with the infection located in the testes. The abdomen of the infected animal was coloured orange due to staining of the peritoneal sheat contrasted by spore masses. The epithelial cover and the germarium were the seat of the infection in the testes and the developmental stages of the microsporidian were found in the mass of sperm cells. The infected gonad was hypertrophic, filled with developmental stages of the pathogen. In the sample of water boatmen there was no further infected adult, male or female.

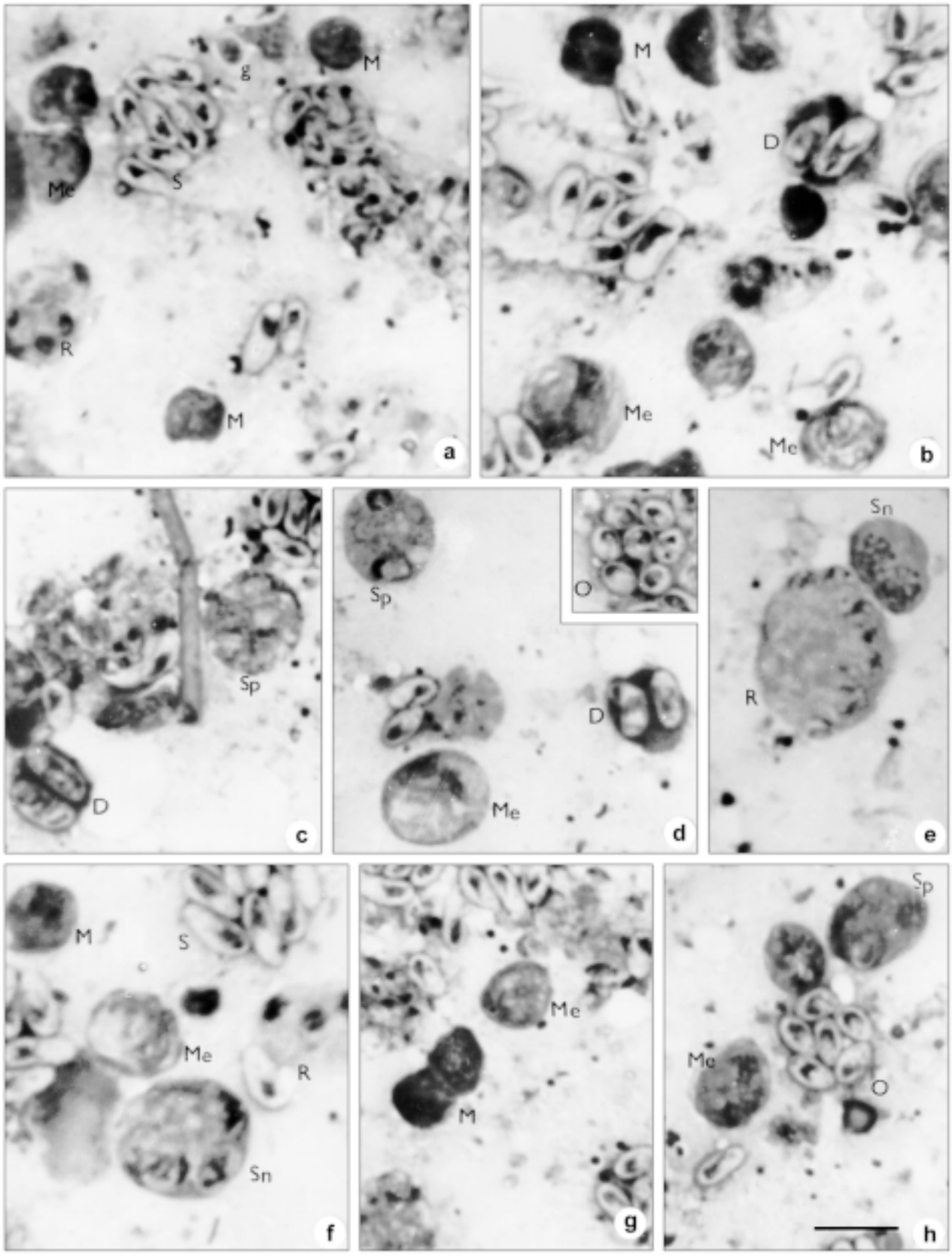
Light microscopy

On smears different vegetative stages were present together with mature spores in round sporophorous vesicles. The development was divided in four principal phases: the early planont - gamet stage, the merogonial cycle, the cycle of meiotic division, the sporogony and spore maturation (Figs. 1, 15). The distribution of individual phases was evaluated among 2000 counted stages in Giemsa stained smears. Early stages identified as gametes (Figs. 1a-g) were small round cells 2 - 3 µm in diameter with dense cytoplasm and minute single round nucleus l - 2 µm in diameter. These stages were rare or difficult to identify, in the analysed sample were just 0.4% of the group. Meronts as next step in development were usually presented as deeply stained oval or irregular bodies (M in Fig. l) with badly differentiated single nuclei and usually adhering in the smear to some sporophorous vesicles. They represented 2.8% of the evaluated group. Stages of meiotic division were the second series in the merogonial phase. They were represented by stages (Fig. l - Me) with a less stained cytoplasm and large nucleus. The size of these stages varied from 6 - l0 µm in diameter, usually the nucleus looked „empty“, with chromosomes in some phase of division. Nuclei were 3 - 6 µm in diameter and the series ends with binucleate stages where the two nuclei adhered to each other as diplokarya. This stage represented 3.4% of the total. It ended with binucleate stages representing the first step in sporogony.

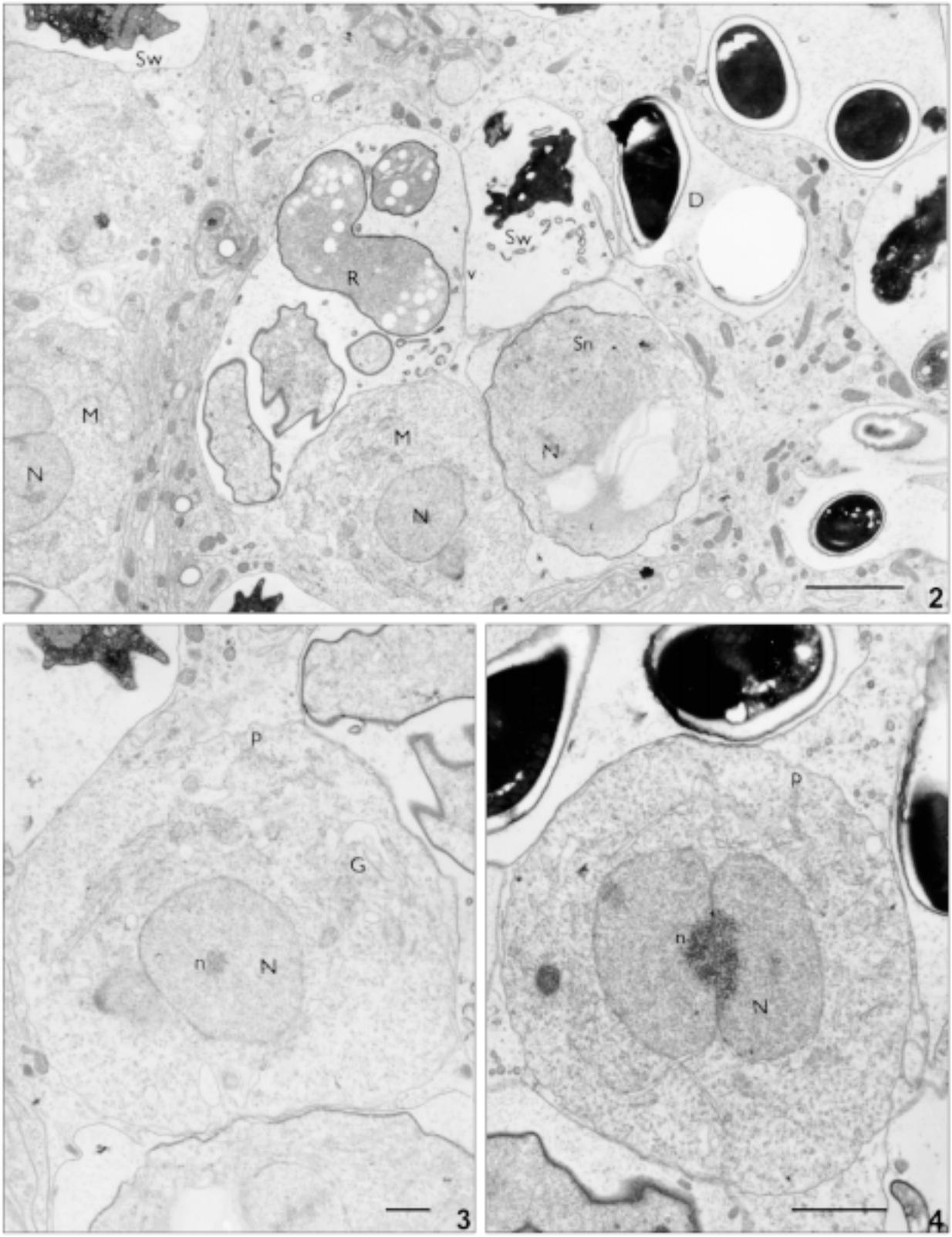
The next series, octonucleate plasmodia were produced in two subsequent nuclear divisions (l.5 %) (Fig. l - Sp). First the nuclei were distributed in the whole space of the stage, later (Fig. le - R) the nuclei were protruding on the border of the plasmodia and produced uninucleate budds which formed sporoblasts (rosettes, l.2%). This stage was usually oval, 10 x 15 µm in diameter.

The octospores were enclosed in a persistent thin sporophorous vesicle. The octospore stage with prominent metachromatic red granules in the posterior pole indicating the position of the posterosome was a specific stage in spore maturation (l5%). These young spores were oval, 5 x 2.5 µm, the granule was 0.5 - l µm in diameter.

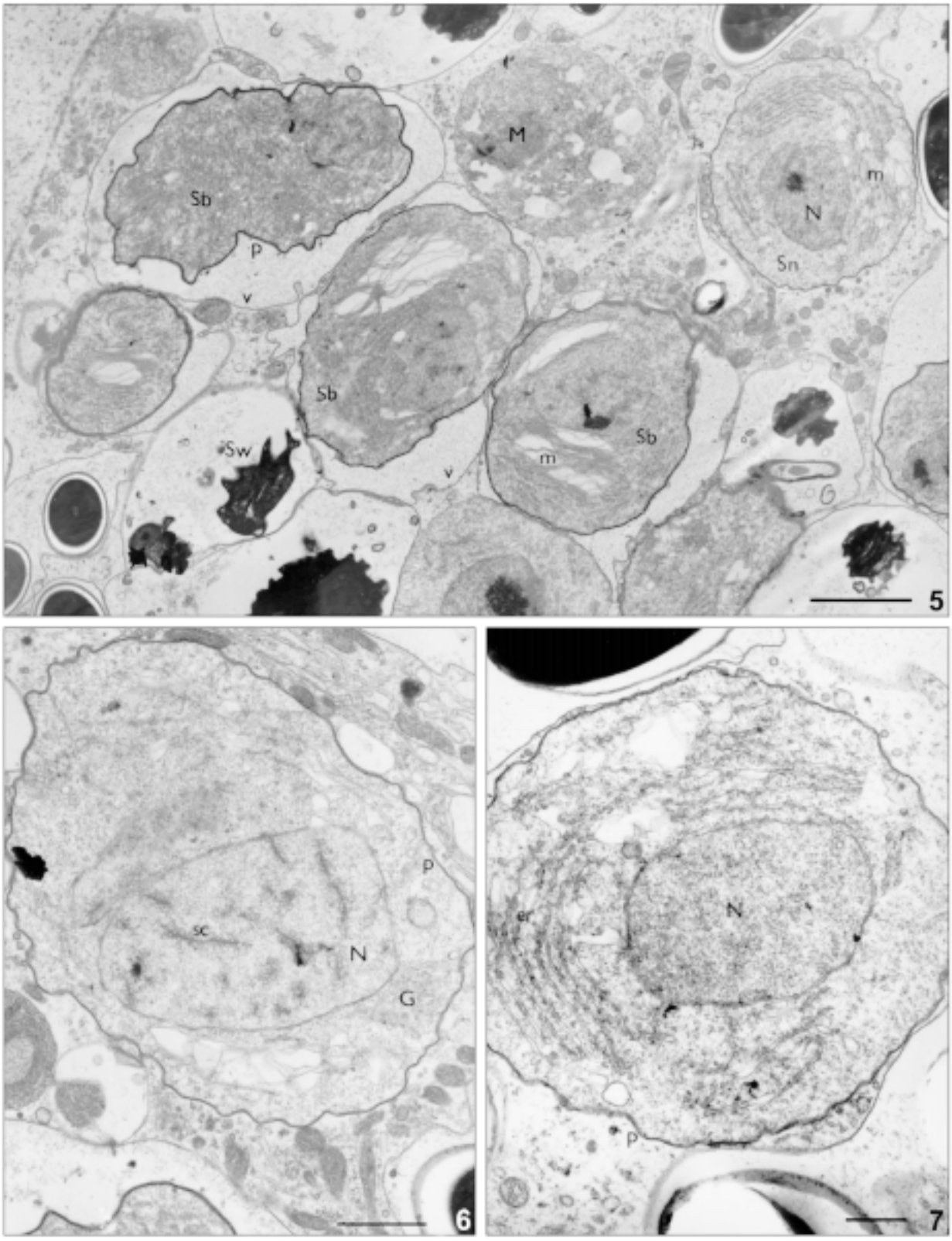
A sporophorous vesicle, 10-12 µm in diameter containing 8 slightly curved oval spores with broader basis and



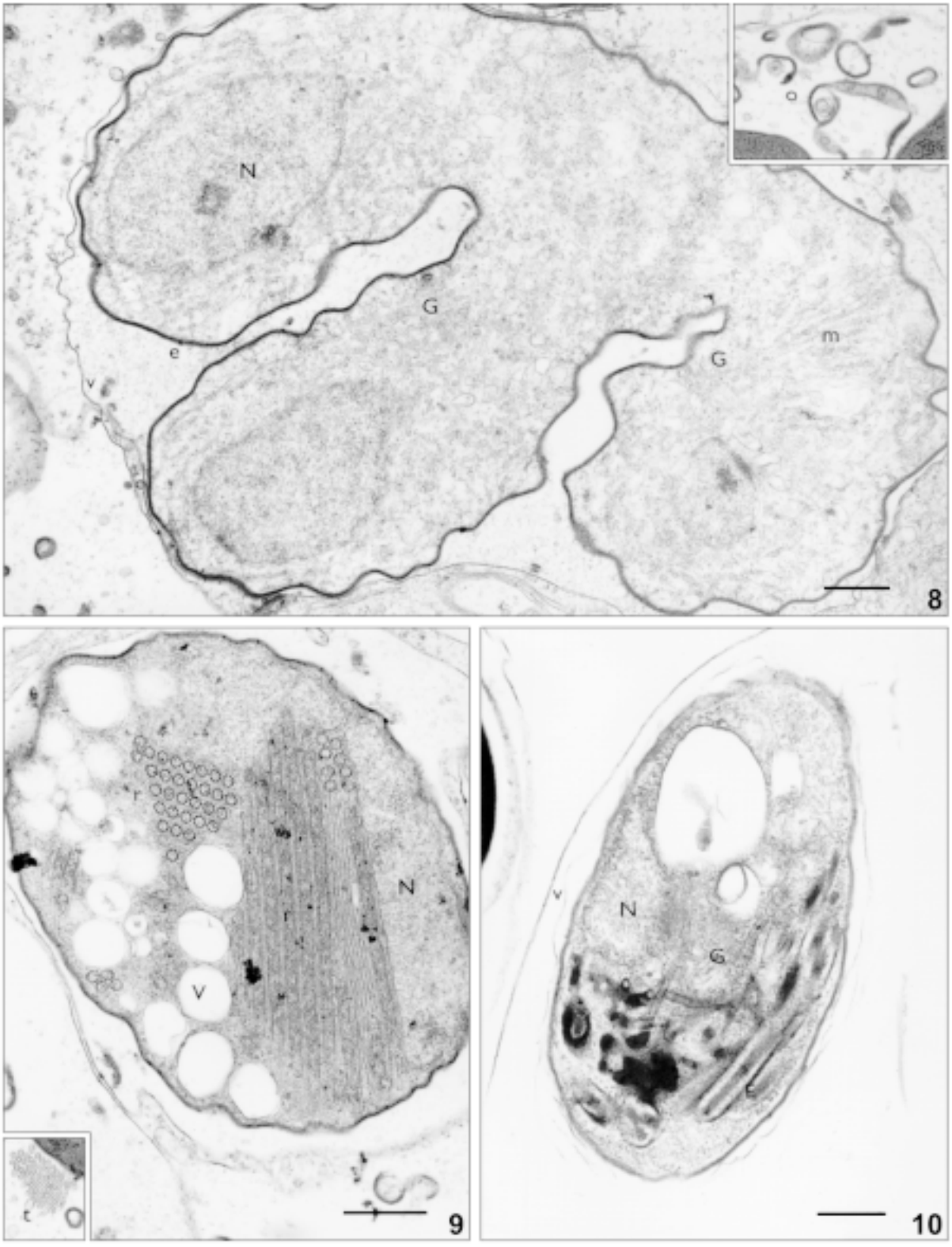
Figs. 1 a-h. Light micrographs of developmental stages of *Becnelia sigarae* in merogony, meiogony and sporogony. Symbols used see abbreviations. Scale bar - 5 µm



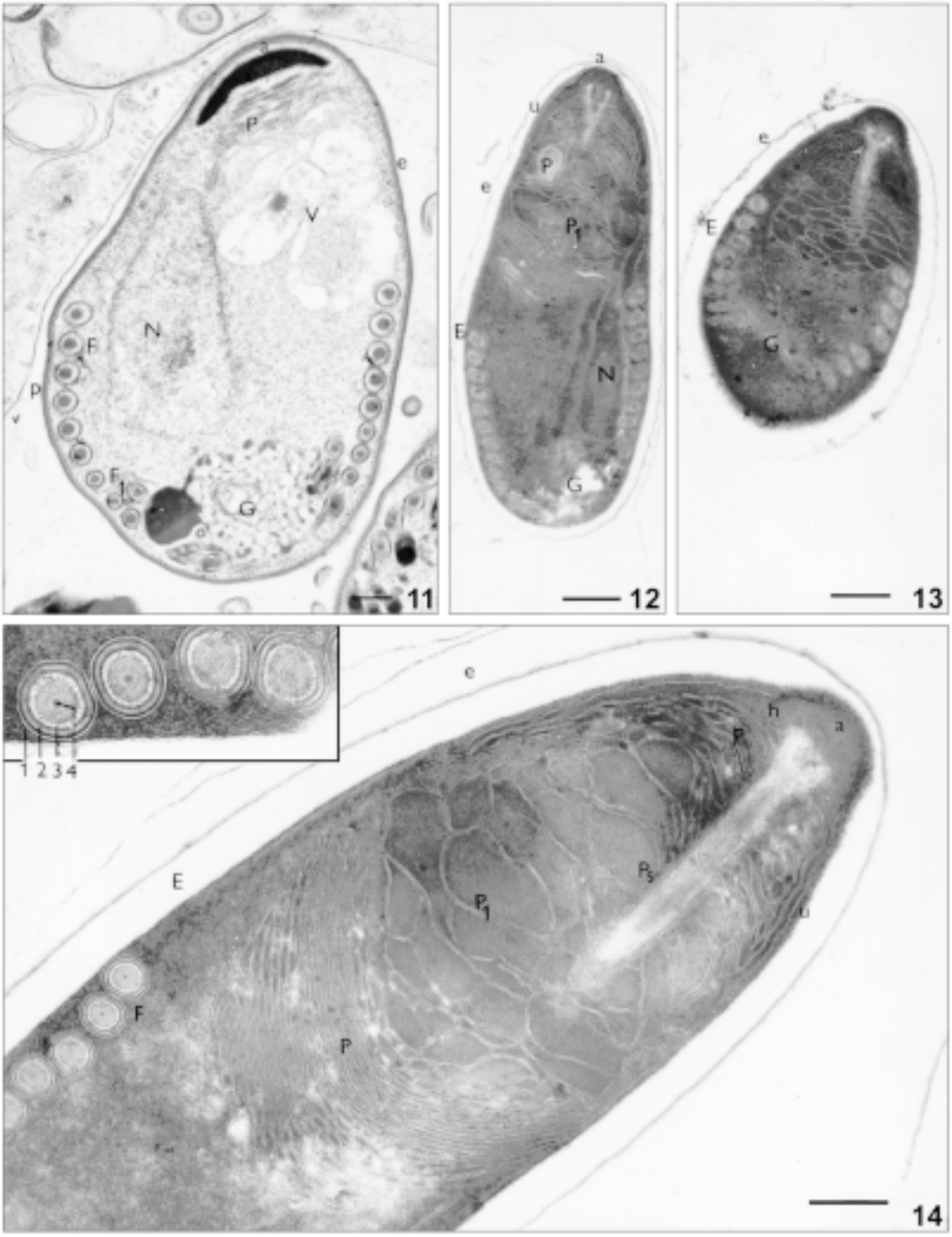
Figs. 2-4. Group of developmental stages of *B. sigarae*. **2** - disporoblastic sporophorous vesicle containing 2 spores (D). Close to the sporophorous vesicle (v) there are meronts (M), rosette stage (R), sporonts (Sn) and wrinkled sporoblasts (Sw). **3** - meront of *B. sigarae* with large vacuoles of Golgi (G) close to the nucleus (N) prepared for meiogonial division. **4** - meront with two adhering nuclei (N), exhibited a nucleolus (n). The cytoplasm is enclosed in the thin plasmalemma (p). Scale bars - **2** - 2 µm; **3** - 500 nm; **4** - 1 µm



Figs. 5-7. **5** - meronts (M) with thin plasmalemma, early sporont (Sn) without sporophorous vesicle and sporoblasts (Sb) within the sporophorous vesicle (v) with thickened plasmalemma (p) and system of multiple membranes (m). Wrinkled sporoblast with thickened plasma membrane and secretion tubules (Sw). **6** - sporont of the meiotic series. Its nucleus contains multiple synaptonematic complexes (sc). In the cytoplasm, enclosed in a thickened plasmalemma (p), there are several Golgi tubules (G). **7** - sporont with thickened plasmalemma (p), multiple lamellae of the endoplasmic reticulum (er) melting into a system of multiple smooth membranes. Scale bars - **5** - 2; **6** - 1 µm; **7** - 500 nm



Figs. 8-10. **8** - part of a sporogonial rosette of *B. sigarae* with nuclei (N) in finger - like buds. Close to the Golgi (G) there are system of multiple membranes (m). Inset: granular secretions (o) appearing in the episporontal space. **9** - sporont with multiple vacuoles (v) and strands of polysomes (r) adhering to the nucleus (N). Inset: tubular secretions (t). **10** - sporophorous vesicle (v) with immature spore. The Golgi (G) is connected with a immature polar filament (F). Scale bars - **8-10** - 500 nm



Figs. 11-14. **11** - young spore in the sporophorous vesicle (v) before formation of the endospore. The spore wall is formed from the plasmalemma (p) and an exospore (e) incrusted with electron - dense material. Flat anchoring disc (a) without lateral protrusions. Anterior polaroplast (P) is formed, the central and posterior part not yet constructed in a system of vacuoles (V). Polar filament in 6 broad (F) and 4 narow coils (F_1). On one side the Golgi system (G) is connected with the end of the filament, on the other side forming the metachromatic body of the posterosome. **12** - secondary (persistent) mature spore with internal structures. The polar filament (F) is fixed with anchoring disc (a) and prolonged with the umbrella (u) at the apical pole. Golgi system (G) is located near by the nucleus (N). Polaroplast bipartite, with lamellar parts (P) and with central located broad chambers (P_1). The spore wall is composed from the exospore (e) and a thick endospore (E). **13** - primary (early) spore with narrow apical end, thick endospore (E), thin exospore (e) and polar filament coiled in 4+3 turns close to the Golgi (G). **14** - anterior end of the secondary (persistent) spore with detailed view of the anchoring disc (a). The umbrella (u) connected with disc in a hinge (h) covers the lamellar polaroplast (P) which encloses the broad chambers of the polaroplast (P_1). The spore wall is composed from the outer exospore (e), middle endospore (E) and a inner plasmalemma. The mature polar filament is enclosed in a polar sac (Ps). Inset: cross section of the polar filament with four layers. Scale bars - **11** - 200; **12** - 500; **14** - 200 nm; **13** - 1 μm

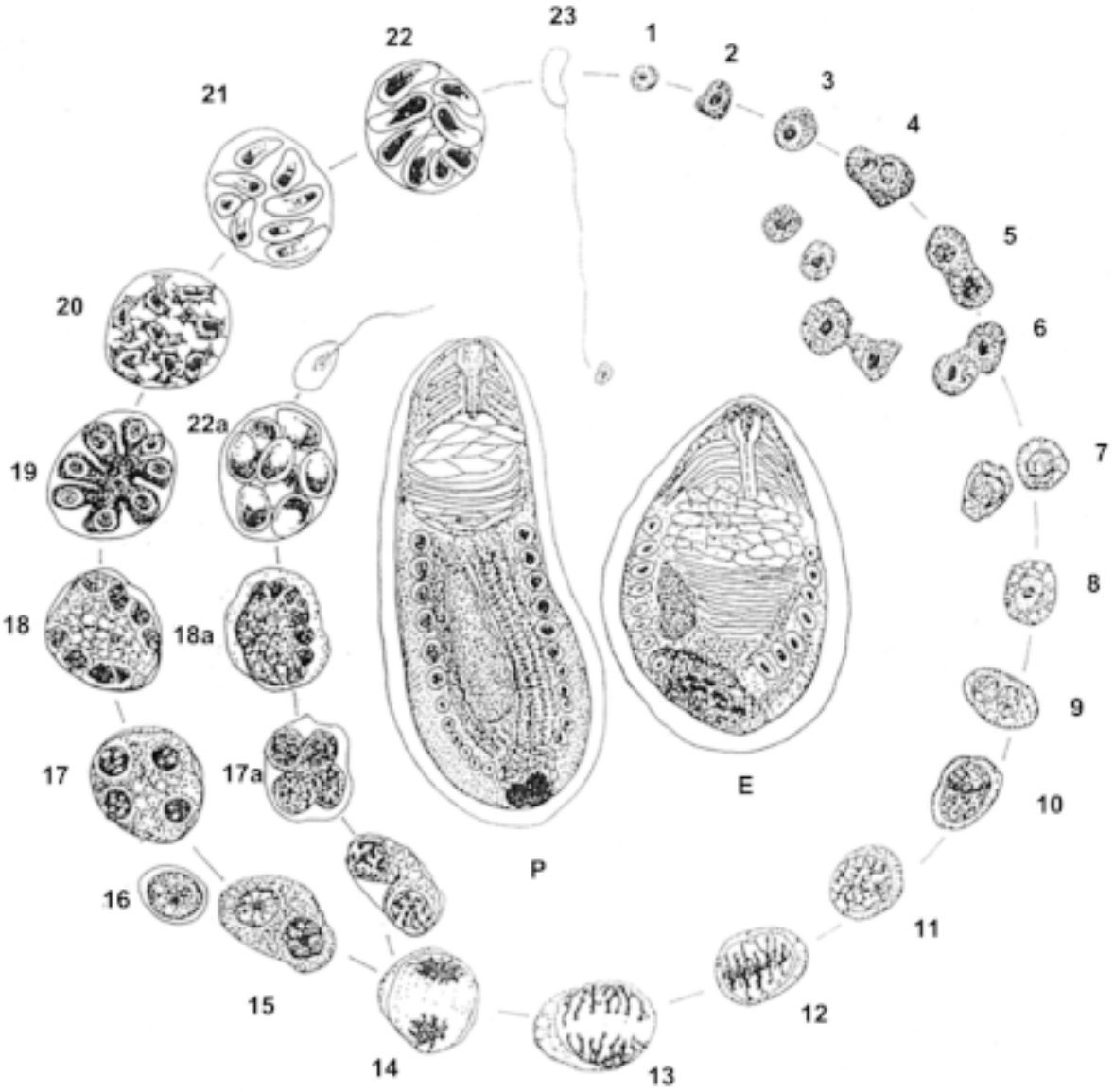


Fig. 15. The proposed life cycle of *Becnelia sigarae* (1) Gamet (planont). (2 - 6) Merogony. (7 - 14) Meiogony. (15 - 20) Sporogony, persistent series. (18) Plasmodium. (19) Rosette. (20) Crumpled sporoblasts. (21) Sporoblasts with metachromatic granule. (22) Mature sporophorous vesicle with octospores. (23) Mature spore. (16a - 22a) Sporogony of the early series. P - secondary (persistent) spore, E - primary (early) spore

equally rounded ends, 5 ± 0.5 x 2.5 ± 0.5 µm, was the final product of the sporogony. The spores represented in the evaluated group 71% of all stages. With the same morphology of sporogony we found octosporous pansporoblasts with shorter, broader spores measuring 4 x 2.5 - 3 µm (Fig. l - O) which probably represented the „early“ spores extruding their filaments in the host and injecting the germs into further tissues to spread the infection in the host. Rare were disporal sporophorous vesicles (Fig. l - D), 0.4% of all with spores of irregular size. They eventually represented teratospores varying in shape.

Electron microscopy

In infected host cells the vegetative stages were common in groups which were formed by dividing merogonial stages. Meronts (Figs. 2-4) were enclosed in a thin plasmalemma (p). In the cytoplasm containing many free ribosomes there were multiple vacuoles and circular lamellae of the rough endoplasmic reticulum. Several groups of vacuoles and tubular formations were part of the Golgi system (Fig. 3). The uninucleate meronts were undergoing a final nuclear division and their nuclei remained sticking together and formed diplokaryons (Fig. 4). These nuclei grow in size, and electron - dense nucleoli (n) appeared to indicate the initial phase of meiotic division. The nuclei of the meiotic series were large and synaptonematic complexes (Fig. 6) signalized the meiotic chromosomes. The plasmalemma of these stages was already thickened but the sporophorous vesicle was not yet formed. At that stage the system of parallel lamellae of the ER disappeared and was transformed in a vacuole with multiple smooth membranes (Fig. 7). With further division of nuclei in the sporonts, the sporophorous vesicle was extended and it enclosed in subsequent divisions a sporogonial plasmodium with two, four and eight nuclei. The individual nuclei were located at the outer wall of the plasmodium and protruded as a finger - like buds forming a rosette (Figs. 2, 8). The buds separated and formed „crumpled“ sporoblasts with poor differentiation of the interior where the polar filament and columns of ribosomes were formed. During this stage minute tubules of secretions were formed in the episporontal space between sporoblasts and wall of the sporophorous vesicle. The formation of the polysomes with long spiral coils of ribosomes (9 in each turn) during spore formation was observed (Fig. 9).

With the deposition of the thin electron - lucent endospore the crumpled wall of the sporoblast was smoothened and the internal structures of the spore were formed. In young spores (Fig. 11) the polaroplast was formed from two parts: the anterior lamellar part (P) and the vacuolated central part (V) with broad chambers. The lamellar part enclosed the vacuolated part again at the posterior part of the polaroplast (Fig. 14). One single elongate nucleus was located in the central part. The anisofilar polar filament was apically fixed in a flat anchoring disc (a) and posteriorly coiled in 9 - 10 turns with 5 - 6 turns of larger diameter and 3 - 4 narrower turns. The filament was connected with the tubules of the Golgi system of the posterosome. The large electron - dense granule is the surplus product of the Golgi and can be identified as the red stained metachromatic granule of young spores in Giemsa smears.

In the mature spore (Fig. 12) the anchoring disc ended in a circular hinge, fixed in position by a broad thin umbrella. The polar filament with well differentiated internal structures was fixed in the anchoring disc with an apical calyx and it crossed the polaroplast which filled the first half of the spore. The mature polar filament was coiled in 5 - 6 broader turns (Fig. 12) and 4 - 5 narrower turns. The Golgi complex filled the posterosome, especially its vacuole. The spore wall was composed of a thin electron - dense exospore (Fig. 14) and a rather thick electron - lucent endospore (E) which was attenuated at the apical pole.

Some sporophorous vesicles contained shorter spores measuring 4 x 2.5 - 3 µm. In ultrathin sections they were rare (Fig. 13), more constricted at their apical end. Their endospore was thicker, but indistinct and the polar filament was coiled in 7 - 8 turns. Its anisofilar arrangement was less distinct, the first 4 turns were broader. Some disporous vesicles contained spores of irregular shape, eventually teratospores.

DISCUSSION

Infected host

Only one male in the inspected locality was infected with symptoms of reduced transparency of its body and orange colour of the spore masses. The orange colour of an infected organ in rather transparent larvae is known also from infections with *Caudospora* Weiser, 1946 in blackflies where the membranes on the surface of the infected fat body have a brown to red pigmentation (Weiser 1961). This staining is manifested in one and absent in another host and locality. The infection was not in its final phase, there were developmental stages in all inspected parts mixed together with mature spores within the persistent octosporous sporophorous vesicles. The male gonads are the site of infection, microsporidian invaded epithelial cover cells of the testes and some nutritive cells in the germarium and the epididymis, respectively. Other tissues were not found infected.

General life cycle

The prominent symptom of this infection is a rich representation of vegetative stages, mainly the merogonial sequences. Analogous to merogony we propose the term meiogony for the meiotic sequence and the term meionts

for individual stages involved. It is a series most prominent in *Amblyospora* Hazard and Oldacre, 1975 and *Parathelohania* Codreanu, 1966 and was studied mainly by Debaisieuxi and Gastaldi (1919) and Kudo (1924), recently by Becnel and Andreadis (1999).

The vegetative part of the life cycle in the host was represented in our material by one morphological series with two types of spores. The invasive primary cycle which enables the spread of the microsporidian from its port of entry in the midgut to its tissue of destination is provided by primary (early) spores (Weiser *et al*. 1998, Maddox *et al.* 1999) characterized by a large posterior vacuole when fresh and by differences in ultrastructure of the polar filament. In our material the primary spores are shorter and differ in the number of turns of the polar filament. There is no clear evidence of the anisofilarity except that the four first cross sections are more regular and separate of the rest. The sequence ending with disporous vesicles can be explained as a teratological type of the development. In some cases primary spores may be present as empty spores in the invaded tissue (Weiser *et al.* 1999). In our case an analogous situation is in some sections (Fig. 2) where a sporophorous vesicle with two spores is cut transversally. The spore is too dark to find any details defining it as a primary spore except the constricted apical pole.

Merogony of the secondary type

The distribution of germ cells, gamonts, from empty spores may not be very rich in the invasion of the target tissue and such cells analogous to gamets presented in *Edhazardia aedis* (Kudo, 1930), Becnel, Sprague and Fukuda 1989 (Becnel and Andreadis 1999) can be recognized in light microscope with difficulties (cells indicated as g in Fig. 1a). Also the first merogonial series with round stages with dense cytoplasm and minor compact nuclei is not very rich and it is not the series which provides maximum reproduction of the parasite, as it is in *Nosema* Nägeli, 1857 or *Vairimorpha* Pilley, 1976 species. This is well shown in the review of stages of the cycle in the counted 2000 organisms.

The series of meiotic stages (meiogony), typical for *Amblyosporidae* Weiser, 1977 is characterized by the large nuclei and does not participate intensively in the multiplication of the pathogen. But it is a series where karyogamy proceeds rather slowly and therefore it is so common in all smears. During the sporogony 8 spores are produced from each single sporont. The sporophorous vesicle is formed around each sporont as soon as its plasmalemma is thickened. Its origin could not be identified in our material. A step which can be identified in each microsporidian is the stage of the crumpled sporoblast which indicates the short period of first formation of the electron - lucent endospore, when the wall is impermeable for fixation and this causes compression of the content of the sporoblast. The formation of the thickened wall of the sporont begins during the last meiotic changes of the nucleus (Fig. 6). During the crumpled stage there are tubules of electron - dense material released from the forming sporoblast and are resorbed back during the early spore stage.

The reason and fate of the multiple smooth lamellae is not clear. They may eventually be connected with the preparation of the polar sac connected with the active Golgi system and formation of the polar filament. In a rather teratological sporoblast (Fig. 10) it is evident that the parts of the filament are supported by the production of the material in the tubules of the Golgi system. In other section the system of membranes is located close to the nucleus of the bud of the rosette.

The secretions are not very rich in this microsporidian. The spore formation is connected with the formation of masses of ribosomes which are fixed together in columns of polysomes, usually with 9 ribosomes in one circle. The bipartite polaroplast of the mature spore is composed of the anterior lamellar part and a centrally located system of broad chambers. This arrangement does not fit precisely to any type offered in the study of Larsson (l986) but may eventually represent the early step in formation of the helicoidal polaroplast of nosemospores in *Parathelohania* species. The polar filament is anisofilar from its first formation. The electron - dense central chord is in its axis and is fed by material from the Golgi system with evidently connected tubules.

The mature polar filament is organized in 4 principal layers (Vávra 1976, Vávra and Larsson 1999). In the well fixed filament in transversal sections we find four distinct layers and three interspaces. The surface layer l is the membrane of the former polar sac, tightly adhering to the filament. The layer 2 is the electron - dense smooth cover which is the transport channel after inversion of the polar tube. In the layer 3 we find the electron - lucent layer, usually amorphous. In well fixed material a longitudinal string of electron - lucent spherical or oval granules forming 12 longitudinal microcylinders was found by Liu and Davies (1973) and 18 subunits were described by Canning and Nicholas (1974). The number

of the longitudinal columns of electron - lucent granules in our microsporidian varied from 22 to 24 and the number of granules is reduced in the narrow turns.

The metachromatic red granule in Giemsa stained smears characterizing the posterosome (Weiser and Žižka l975) in immature spores is the surplus of product of the Golgi system (the electron - dense mass connected with Golgi tubules).

The microsporidia known from European water Heteroptera do not have any morphologically rich sequence of meiogony. The spores of *Toxoglugea gerridis* and *T. mercieri* have horseshoe -like shape (Poisson 1941, Jírovec 1936) and *Chapmanium nepae* has minute spores 2 - 3 x 1.4 - 1.8 µm in navicular sporophorous vesicles (Hazard and Oldacre 1975). Lipa (1966) mentioned only scarce vegetative stages. *Thelohania veliae* had rather long oval spores (4 x 9 - 11 µm) or broad spores (5.5 - 7 x 7 µm) within octosporous sporophorous vesicles, without distinct merogonial stages (Poisson 1928).

Among microsporidia infecting freshwater insects prominent series of meiotic stages, anisofilar polar filaments and alveolate polaroplasts are characteristics of the members of the family *Amblyosporidae* (Sprague *et al.*,1992). As polymorphic microsporidia they have prominent meiogonial series and anisofilar polar filament in uninucleate thickwalled octospores in larval mosquitoes and the alveolate or helicoidal polaroplast in the thinwalled binucleate spores in adult mosquitoes. Octospores of the microsporidian in *S. lateralis* are thinwalled and uninucleate and the vegetative stages include distinct meiotic series. Therefore we range *B. sigarae* in *Amblyosporidae* but broad chambers we propose new genus *Becnelia* with following characteristics:

***Becnelia* gen. n.**

Microsporidian infecting freshwater arthropods. Schizogony with uninucleate meronts and prominent meiogony with diplokaryotic stages. Sporonts closed in sporophorous vesicles form octosporous plasmodia dividing in a rosette into 8 sporoblasts and spores. Octosporous persistent sporophorous vesicles contain thinwalled elongate or oval uninucleate spores. Polar filament anisofilar. Polaroplast with centrally located broad chambers enclosed in the lamellar parts. Early spores more rounded, with shorter polar filament. Parasites of gonads of aquatic insects.

***Becnelia sigarae* sp. n.**

With characteristics of the genus. End of meiogony and first part of sporogony with thickened plasmatic membrane forming in sporogony a persistent sporophorous vesicle. Minute secretion tubules are resorbed during spore formation. Spores elongate or oval, with rounded ends, slightly curved, 5 ± 0.5 x 2.5 ± 0.5 µm, with anisofilar filament coiled in 9 - 11 turns: 5 - 6 turns thicker, 4 - 5 turns narrower. Early spores 4 x 2.5 - 3 µm, more constricted at the apical pole, filament coiled in 7 - 8 turns.

Site of infection: testes in male.

Host and locality: water boatmen, *Sigara lateralis* Leach, 1817 (Heteroptera: Corixidae), temporary pool near Bavorov, South Bohemia, Czech Republic.

Type material: collection of authors.

Etymology: the generic name is dedicated to J. J. Becnel specialized in research on *Amblyosporidae*.

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