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Phylogenetic Position and Ultrastructure of Two *Dermocystidium* Species (Ichthyosporea) from the Common Perch (*Perca fluviatilis*)

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Summary. Sequences of small-subunit rRNA genes were determined for *Dermocystidium percae* and a new *Dermocystidium* species established as *D. fennicum* sp. n. from perch in Finland. On the basis of alignment and phylogenetic analysis both species were placed in the *Dermocystidium-Rhinosporidium* clade within Ichthyosporea, *D. fennicum* as a specific sister taxon to *D. salmonis,* and *D. percae* in a clade different from *D. fennicum*. The ultrastructures of both species well agree with the characteristics approved within Ichthyosporea: walled spores produce uniflagellate zoospores lacking a collar or cortical alveoli. The two *Dermocystidium* species resemble *Rhinosporidium seeberi* (as described by light microscope), a member of the nearest relative genus, but differ in that in *R. seeberi* plasmodia have thousands of nuclei discernible, endospores are discharged through a pore in the wall of the sporangium, and zoospores have not been revealed. The plasmodial stages of both *Dermocystidium* species have a most unusual behaviour of nuclei, although we do not actually know how the nuclei transform during the development. Early stages have an ordinary nucleus with double, fenestrated envelope. In middle-aged plasmodia ordinary nuclei seem to be totally absent or are only seldom discernible until prior to sporogony, when rather numerous nuclei again reappear. Meanwhile single-membrane vacuoles with coarsely granular content, or complicated membranous systems were discernible. Ordinary nuclei may be re-formed within these vacuoles or systems. In *D. percae* small canaliculi and in *D. fennicum* minute vesicles may aid the nucleus-cytoplasm interchange of matter before formation of double-membrane-enveloped nuclei. *Dermocystidium* represents a unique case when a stage of the life cycle of an eukaryote lacks a typical nucleus.

Key words: *Dermocystidium percae*, *D. fennicum* sp. n., development, Ichthyosporea, *Perca fluviatilis*, phylogenetic position, ultrastructure.

INTRODUCTION

The phylogenetic position and composition of the genus *Dermocystidium* Perez, 1908 have long been unclear. Ragan *et al*. (1996) included phylogenetic analysis in their study and assigned the genus in a group called the DRIPs clade (*Dermocystidium*, rosette agent, *Ichthyophonus*, *Psorospermium*), near the dichotomy of animals and fungi. Ragan *et al*. (1996) examined two representatives of *Dermocystidium*, *Dermocystidium* sp. from brook trout and *D. salmonis* Davis, 1947 from chinook salmon in their study. Later, Herr *et al*. (1999), when studying the phylogenetic affinities of *Rhinospo-*

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ridium seeberi, a pathogen of humans and animals, established a new clade called Mesomycetozoa for *Rhinosporidium* and the former DRIPs.

Herr *et al*. (1999) were perhaps unaware of the work of Cavalier-Smith (1998) who in his discussion on probable evolution of Animalia and Fungi from Choanozoa erected for the former DRIPs clade a new class Ichthyosporea, which thus has a priority over Mesomycetozoa, and included it in the subphylum Choanozoa. He chose the name Ichthyosporea for the class because these organisms producing unicellular walled spores infect mostly fish. The walls of the trophic stages lack chitin. If zoospores are present, they are uniflagellate, without a collar or cortical alveoli. The new class included two orders, Dermocystida and Ichthyophonida. Cavalier-Smith (1998) assumed that the members of Dermocystida have flat mitochondrial cristae and those in Ichthyophonida have tubulovesicular cristae. Vacuoles with contents of different density, lipid globules and inclusion bodies are common in Ichthyosporea (see references to description of different species in the Discussion).

Lom and Dyková (1992) defined the description of the genus *Dermocystidium*, dividing the known species into three groups according to their morphology and types of infection. At that time about 20 species were known, mostly thanks to light microscope observation. Knowledge of the life cycle was incomplete and their phylogenetic position was enigmatic. To group 1 (e.g., *D. branchiale* Léger, 1914) were assigned walled multinuclear plasmodia (cysts) formed usually in the skin or gills of fish. These plasmodia divide to form round spores with a solid refractile body. In group 2 (e.g., *D. koi* Hoshina and Sahara, 1950), thick-walled hyphae are formed, and the spores are of variable size. The species in group 3 (*D. macrophagi* van de Moer, Manier and Bouix, 1987) cause visceral infections, a large thick-walled plasmodium is absent and the spores have a large central vacuole instead of an inclusion.

A member of the first group, *D. percae* Reichenbach-Klinke, 1950, described from the skin of the common perch, *Perca fluviatilis* L., has, since its original description in Germany (Reichenbach-Klinke 1950), been found in perch in the Czech Republic (Ergens and Lom 1970), in the former USSR (Pronin 1976) and in Estonia and Finland (Pekkarinen and Lotman 2003). Pronin (1976) and Pekkarinen and Lotman (2003) recorded two morphological types of cysts; Pronin assigned both to the same species *D. percae*, while Pekkarinen and Lotman assigned one type to *D. percae* and the other to an unnamed species *Dermocystidium* sp. The developmental cycles of both vicarious species include walled plasmodium stages, formation of spores from the plasmodium, and ultimately formation of numerous zoospores from each spore (Pekkarinen and Lotman 2003). The two species differ only slightly in morphology from each other in the different shape of the plasmodial cysts and the different thickness of the cyst wall. Moreover, they have different site preferences in the host. The fine structure of the plasmodium, its subsequent developmental stages and nuclear structure remain unknown.

This study presents an outline of the ultrastructure of the sequential stages of both species, and particularly tries to solve the puzzle of the plasmodium nucleus: on the basis of light microscope studies by Pekkarinen and Lotman (2003) it was thought that the nucleus of the young plasmodium later changes to a reticulate structure containing chromatin clumps. The molecular analysis included in this study aims to shed more light on the relationships and position of these organisms in the phylogenetic tree of the Ichthyosporea and to solve the mutual relationship of both *Dermocystidium* species.

MATERIALS AND METHODS

Collecting and management of the fish. The common perch were caught in two lakes in Finland, Lake Hirvijärvi in southern Finland and Lake Juurusvesi in central Finland. Infected fins were removed and refrigerated until preparation. Material from several years was used in the study.

The cysts for 18S rDNA analysis originated from perches collected in Lake Hirvijärvi in the summer of 2000. One large cyst with spores of *Dermocystidium* sp. was used, and several cysts of *D. percae* from a few individual perch were pooled for the sample. These latter cysts also contained pre-spore stages. The cysts were purified from host tissue, put in a very small amount of dilute NaCl, and frozen. The samples were packed with dry ice for the courier transportation to Canada (Institute for Marine Biosciences, Halifax, Nova Scotia). The samples did not thaw during the transport.

In order to find early stages of *D. percae*, an experimental infection was carried out in August 2001. 0-group perch were collected from inshore areas of lake Hirvijärvi and acclimated to aquarium conditions (tap water 20-23°C) for a few days. The fish were fed with aquarium fish food (chironomid and mosquito larvae), and most of the water in both test and control aquaria was replaced with aged tap water at least once a day. Cysts of *D. percae* were removed from perch fins in early August, put in tap water in small Petri dishes (at about 22-25°C) for 5 days (only two cysts produced zoospores). In mid-August the contents of the already ruptured cysts were mixed with the food offered to the 0-group test fish. The zoospore culture was repeated with new cysts (for 2 days), and the products were

mixed in part with the fish food and in part with the water in the test aquarium on August 18. After two weeks the test and control fishes were put into flowing, charcoal-filtered tap water (14-17°C) (the same feeding was continued). The experiment ended on October 23, 2001. For zoospore studies lower incubation temperatures were also used (10 and 15-17°C).

Amplification and sequencing of 18S rDNAs. Spores of *Dermocystidium* sp. and *D. percae* were collected by centrifugation, and DNA was extracted using the Y-DER yeast extraction reagent kit (Pierce). The gene encoding nuclear small-subunit (18S) ribosomal RNA was amplified using universal primers GO1 and GO7 (Saunders and Kraft) and the rTth DNA Polymerase XL kit (Perkin Elmer). Amplification conditions were: hot start (AmpliWax PCR Gem50 beads: (Perkin Elmer) at 94°C for 2 min; 16 cycles of denaturation at 94°C for 15 s, reannealing at 65°C for 4 min, then 12 cycles with the same denaturation conditions but extending the reannealing step by 15 s per cycle; finally 72°C for 10 min before cooling to 4°C.

A 1-µl aliquot was removed and placed into a new amplification reaction under the same conditions, except that the reannealing step was conducted at 52°C. Gel electrophoresis of the products showed a single clean amplicon in each case. Primers and reagents were removed on a Centricon-100 column, and amplicons were sequenced directly on an ABI 377 (Applied Biosystems) using the ABI Prism Big Dye Terminator Cycle Sequencing Ready reaction kit and standard 18S rDNA primers (Saunders and Kraft 1994).

The amplicon from *Dermocystidium* sp. sequenced smoothly, but that from *D. percae* showed overlaid signals indicative of a heterogeneous product. Therefore the amplicon was cloned into PCR 2.1 vector (Invitrogen) and colonies for individual DNA preparation and sequencing were selected.

Alignment and phylogenetic analyses. rDNA sequences were aligned visually with others from the Ichthyophonales (see legend to Fig. 1), taking secondary structure (Gutell 2002) into account as necessary. Ambiguous nucleotides (N, R, Y) at positions where all other nucleotides at that position in an alignment were identical were reassigned to agree with the others. A small number of positions (columns) that could not be securely aligned, or were mostly empty (e.g. extreme 5' and 3' termini), were removed prior to phylogenetic analysis, yielding a 25 species x 1811 position matrix. Bayesian trees were inferred using MRBAYES 2.0 (Huelsenbeck and Ronquist 2001, Huelsenbeck *et al*. 2001) under a general time-reversible model (Yang 1994), using a discrete (8-category) approximation to a continuous Γ model of nucleotide substitution rates across sites (Yang 1993) and Markov chain temperature initialized at 0.2000. In most analyses eight Markov chains were propagated for 50000 Markov-chain generations; burnin was conservatively set at 1000 generations. For other phylogenetic methods we used programs in PHYLIP 3.6a2.1 (Felsenstein 2002).

Sequences. For explanation, see legend to Fig. 1.

Electron microscopy. The parasites were fixed either *in situ* in fish fins or separately, or embedded in nonfish tissue or gelatin. The pre-fixatives used were 2.5-3 % phosphate-buffered (0.1M, pH 7.2) glutaraldehyde (usually), 4% buffered formaldehyde, or formaldehyde-glutaraldehyde or formaldehyde-glutaraldehyde-picric acid mixtures. The 1% buffered osmium tetroxide fixation was sometimes used alone. The excess pre-fixative was washed off from the samples with the phosphate buffer, and the samples were treated with 1% OsO_4 in the phosphate buffer, dehydrated with an ethanol series and embedded in Epoxy resin. Ultrathin sections from the samples were stained with uranyl acetate and lead citrate. The subsequent osmification-embedding protocol, final sectioning and the subsequent staining of the sections were made with the help of the staff of the EM unit, Institute of Biotechnology, University of Helsinki. The sections were examined and photographed in the same unit with a Jeol 1200EX electron microscope. Some prefixed or already embedded samples were examined with a Jeol 1010 electron microscope in the Institute of Parasitology of the Academy of Sciences of the Czech Republic in České Budějovice.

RESULTS

18S rRNA genes of the new *Dermocystidium* sp. and *D. percae*

Sequence determination. Amplified rDNAs of lengths 1812 (*Dermocystidium* sp.) and 1857-1865 (*D. percae*) were recovered and sequenced. One clone of *D. percae* had to be re-amplified (using inset primers) prior to sequencing, yielding only 1792 nucleotides. The additional length in the *D. percae* sequences results from 19 insertions of 1 to 13 nucleotides each. Accession numbers, gi numbers and other information for these ten new sequences are presented in Table 1. No introns were found.

The 9 sequences recovered from the bacterial clones expressing 18S rDNA of *D. percae* differ among themselves in 52 positions (~2.8%), with the greatest pairwise difference being 33 positions (1.77%). By fitting the *D. percae* SSU-rDNA to an established secondary structure model (Gutell 2002) we identified the type of structural region in which these differences occur. Among the 24 differences occurring in only one of these nine sequences, 6 are internal to a stem region; 4 expand a

Table 1. Nuclear small-subunit ribosomal RNA genes as recovered in *Dermocystidium.*

Species or clone	Length (nt)	Accession no.	gi number
D. percae 1	1853	AF533941	28629202
D. percae 4	1865	AF533942	28629203
D. percae 5	1863	AF533943	28629204
D. percae 6	1861	AF533944	28629205
D. percae 9	1792	AF533945	28629206
D. percae 33	1857	AF533946	28629207
D. percae 34	1863	AF533947	28629208
D. percae 35	1863	AF533948	28629209
D. percae 52	1862	AF533949	28629210
D. fennicum sp. n.	1812	AF533950	28629211

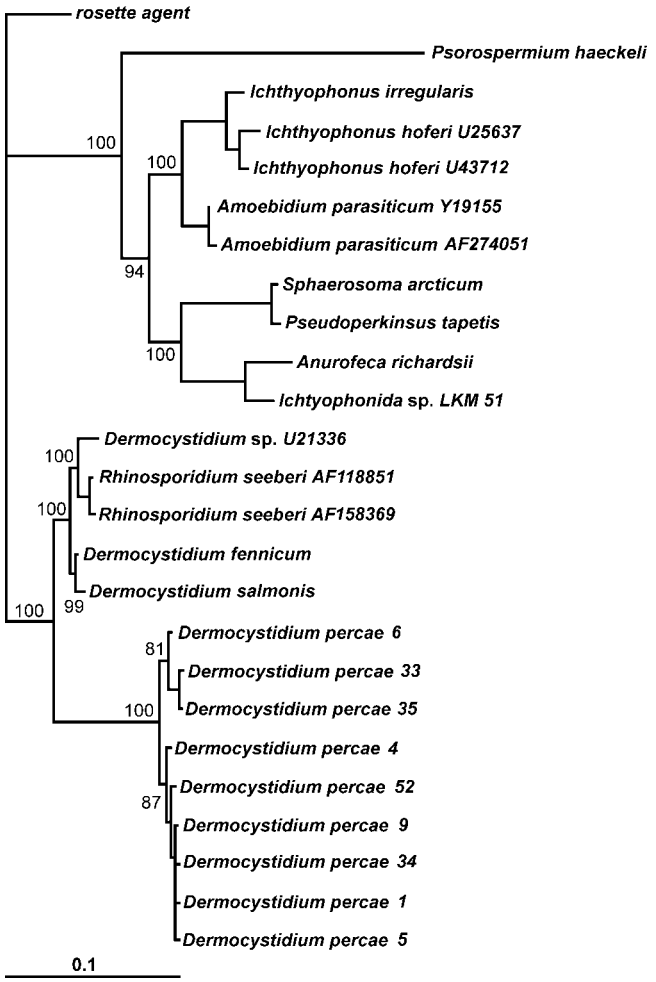


Fig. 1. Bayesian phylogenetic tree relating SSU rDNA sequences of *Dermocystidium fennicum*, *D. percae* and other members of Ichthyosporea (*Amoebidium parasiticum* Y19155, gi:10432427 and AF274051, gi: 9964066; *Anurofeca richardsi* AF070445, gi:4322029; *Dermocystidium percae* clones 1, 4, 5, 6, 9, 33, 34, 35 and 52, this paper; *Dermocystidium salmonis* U21337, gi:857457; *Dermocystidium fennicum*, this paper; *Dermocystidium* sp. U21336, gi:857456; *Ichthyophonida* sp. LKM51, AJ130859, gi:3894141; *Ichthyophonus hoferi* U25637, gi:1002422 and U43712, gi:1542965; *Ichthyophonus irregularis* AF232303, gi:7769642; *Pseudoperkinsus tapetis* AF192386, gi:6288975; *Psorospermium haeckeli* U33180, gi:1143192; *Rhinosporidium seeberi* AF118851, gi:6538786 and AF158369, gi:7110146; rosette agent of chinook salmon L29455, gi:1000847; *Sphaerosoma arcticum* Y16260, gi:4995786).

loop or bulge at the end of a stem; 9 occur in a loop, bulge, sequence end or other unpaired region; and 5 are in the poorly structured region between positions *ca* 654 and 913. Of the 28 differences common to two or more of the *D. percae* clones, 13 are internal to a stem; 1 expands a bulge; 11 occur in a loop or bulge; and 3 are in the above-mentioned poorly structured region. Transitions and transversions are almost equally balanced.

The variations internal to stem regions are of particular note. Among the 9 unpaired variations internal to a stem, only 2 would obviously weaken the between-strands interaction, and hence might be candidates for PCR errors; 3 seem likely to strengthen the interaction (taking the other *Dermocystidium* species as outgroups). Of the 13 shared variations, 8 exist in co-varying pairs; 3 pairs maintain a Watson-Crick or other energetically favourable base pair, while the other avoids an energetically favourable base pair. Co-variation in a highly structured region strongly implies that the corresponding SSU rRNA is being maintained under selection. These co-variations, plus 12 other shared differences, tend to divide the 9 clones into two groups (clones 6, 33 and 35 *vs* the others).

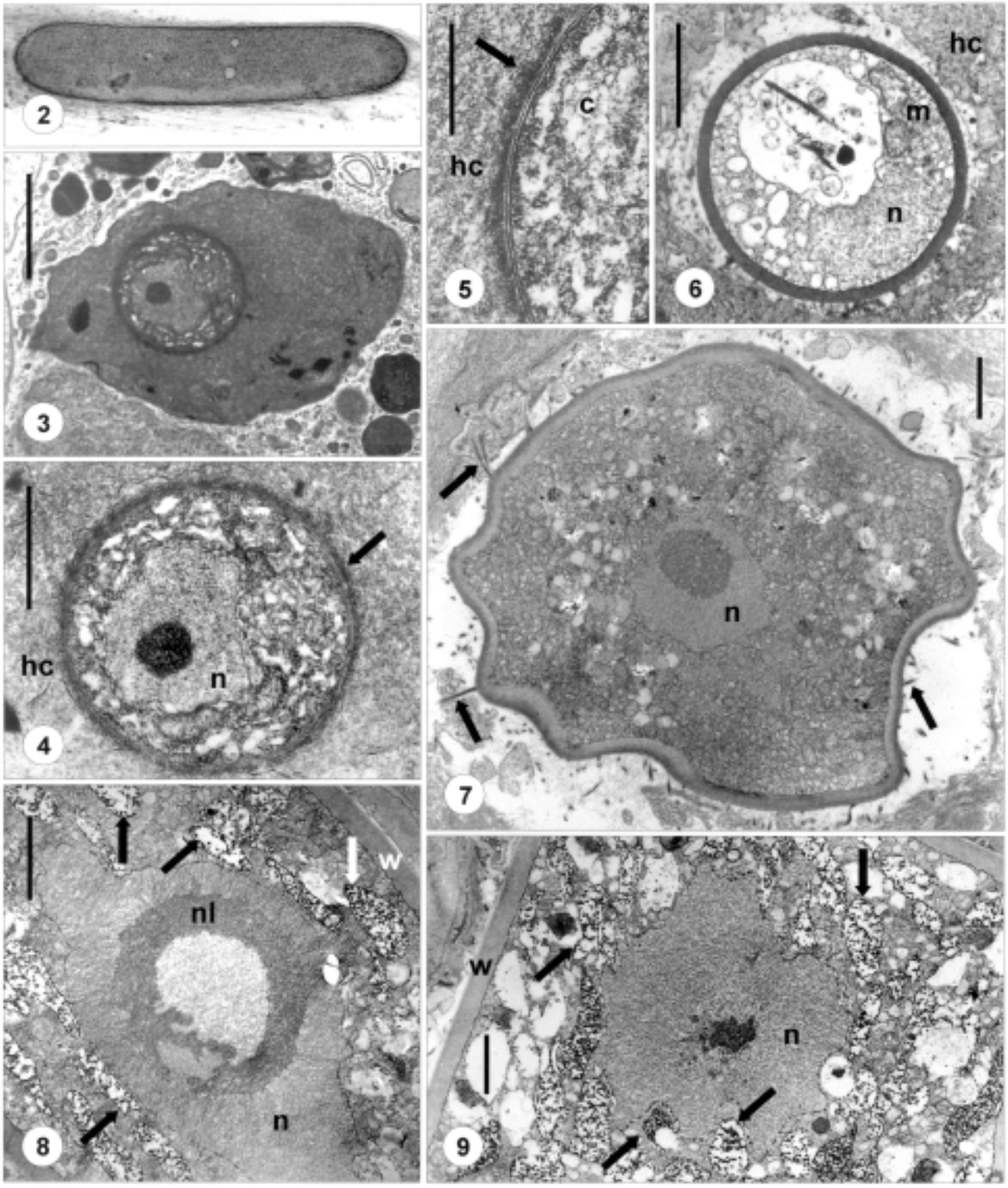
Alignment and phylogenetic analysis. The unrooted tree recovered Bayesian analysis of our 25 x 1811-alignment matrix is shown in Fig. 1. Trees very similar in all significant respects were recovered following neighbour-joining and parsimony analyses (results not shown).

Dermocystidium percae ultrastructure

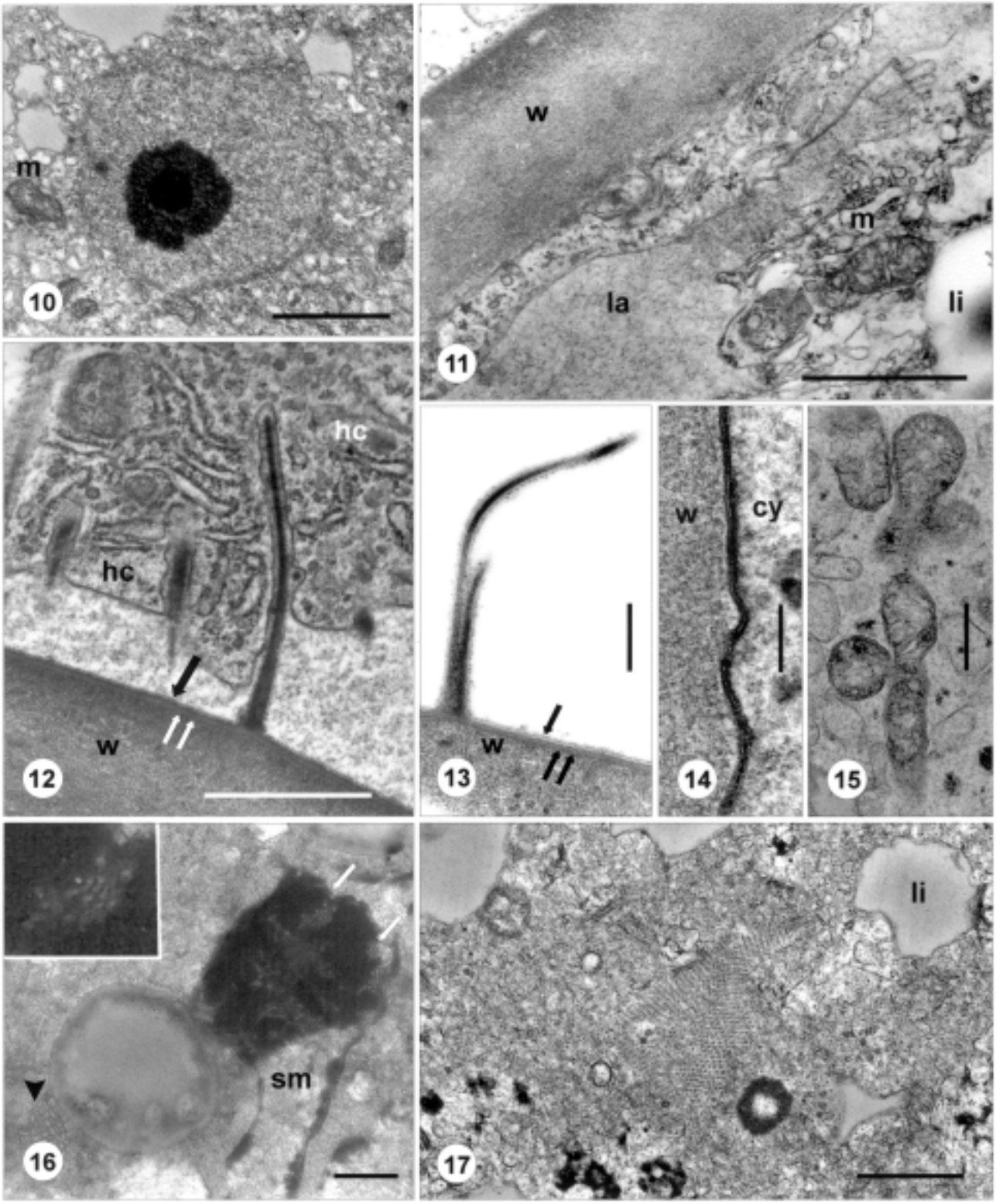
Figure 2 shows the typical elongated cyst shape of *Dermocystidium percae* observed in perch fins.

Early stages. The earliest stages of *D. percae* observed were intracellular (Fig. 3) and circular in transverse section with a diameter of 2.4 μm (Fig. 4). They had vacuolated cytoplasm with numerous ribosomes and a large, slightly eccentric nucleus with a nucleolus and double-membrane envelope. The parasite cell was bounded with a trilaminar envelope about 20 nm thick covered with a fuzzy cell coat about 80 nm thick (Fig. 5). What can be interpreted as a slightly more advanced stage, about 2.7 μm in diameter, had an electron dense wall about 170 nm thick; the trilaminar envelope could no longer be discerned. The cytoplasm showed RER cisternae, and in addition to small vacuoles, there were also larger ones with granular and formed inclusions. In stages of about 5.2 μm in diameter, the dense wall, about 220 nm thick, seemed very compact and the parasite was surrounded by an empty space (Fig. 6).

In stages in transition to a larger plasmodium, about 15 μm in diameter, the cyst wall was about 0.5 to 1.0 μm in thickness, already with numerous fine outer projections (Fig. 7). The wall was differentiated into a denser outer layer and a less dense, thicker inner layer. These



Figs 2-9. *Dermocystidium percae*. **2 -** a cyst from perch fin, actual length is 580 μm; **3** - intracellularly located early stage; **4** - the stage from Fig. 3, enlarged, arrow indicates the cell envelope; **5** - cross section of the cell envelope of the preceding stage; arrow indicates the fuzzy cell coat; **6** - a slightly more advanced stage encased with a thick wall; **7** - growing young plasmodium; arrows indicates the fine projections of the wall; **8** - part of a growing plasmodium with a huge nucleus and a hollow, large nucleolus, arrows indicates vacuoles with dense granules; **9** - part of a growing plasmodium with a nucleus with short projections; some of the vacuoles with dense granules (arrows) are lodged between them. c - parasite cytoplasm, hc - host cell cytoplasm, m - mitochondria, n - nucleus, nl - nucleolus, w - plasmodium wall. Scale bars - 0.5 μm (5); 1 μm (4); 2 μm (3, 6-9).



Figs 10-17. *Dermocystidium percae*. **10** - a normal nucleus in a young plasmodium; **11** - a branched, single membrane-bound lacuna with finely granular content; **12** - plasmodium wall with its homogeneous outer layer (white double arrows) covered by fine surface coat (arrow); **13** - a villus branched near the surface of the wall; arrow - surface coat, double arrow - outer homogeneous layer of the wall ; **14** - the dense lamina lining the inner face of the plasmodium wall; **15** - mitochondria in the cytoplasm; **16** - smooth-membrane envelope (sm) containing dense substance with small tubules (arrows, enlarged in the inset); at left, a multivesicular body-like structure (arrowhead); **17** - bundle of small fibrils in the plasmodia. cy - plasmodium cytoplasm, hc - host cell, la - membrane-bound lacuna, li - lipid inclusion, m - mitochondrion, w - plasmodium wall. Scale bars - 100 nm (14); 0.4 μm (13), 0.5 μm (15, 16); 1 μm (11, 12, 17); 2 μm (10).

stages had a large nucleus with finely granular nucleoplasm and a sizeable eccentric nucleolus. The cytoplasm was replete with small vacuoles; also small lipid vacuoles appeared, absent in previous early stages and constituting later a common feature of more developed plasmodia.

In apparently more advanced plasmodia, there were vacuoles with coarsely granular, dark, chromatin-like material (Figs 8, 9). It is difficult to put observed plasmodia in sequential developmental order; referring to the wall thickness, the plasmodia in Figs 8 and 9 may perhaps be stages following that in Fig. 7. In these plasmodia the central nucleus is quite large (8 to 12 μm) with centrally situated, sometimes hollow large nucleolus (5.5 μm) such as in Fig. 7; fine chromatin strands run radially from this central darker area to the nuclear envelope (Fig. 8). The nuclear envelope is normal, equipped with pores. The vacuoles with dark granules, which become quite numerous, are slightly reminiscent of prophase nuclei were it not for their small size and their single-membrane envelope. In some of these bodies, the dense particles adhered to the membrane, as chromatin particles would do in a nucleus. The vacuoles were sometimes situated between short extensions of the nucleus (Fig. 9). Later on, only occasionally could small normal nuclei be observed in the plasmodia (Fig. 10). Similar vacuoles with coarsely granular matter could also be found in plasmodia without any nuclei. We also found plasmodia where even the coarse granule-containing vacuoles were absent. Instead, branched membranous lacunae with finely granular content could sometimes be seen (Fig. 11).

Developing and developed plasmodia. The cyst wall of the middle-aged plasmodium was a compact, finely granulo-fibrillar structure ranging between 1.5 to 4 μm (rarely 6 μm) in thickness (Figs 11, 12). The outer face of the wall formed a homogeneous layer about 30 nm thick. This was covered by a fine granular surface coat, which also covered the villi-like protrusions or spikes, which reached a length of about 2.5 μm (Fig. 12). The villi were sunk into the surrounding host cells, the cell membrane of which provided an additional membrane for the villi. Some of the villi branched near their base, forming a bifurcate structure (Fig. 13). The inner face of the wall was lined with a dense lamina approximately 10 μm thick, with the structure of a unit membrane (Fig. 14). The plasmodium abutted on the wall; there were no villi or projection extending towards the wall.

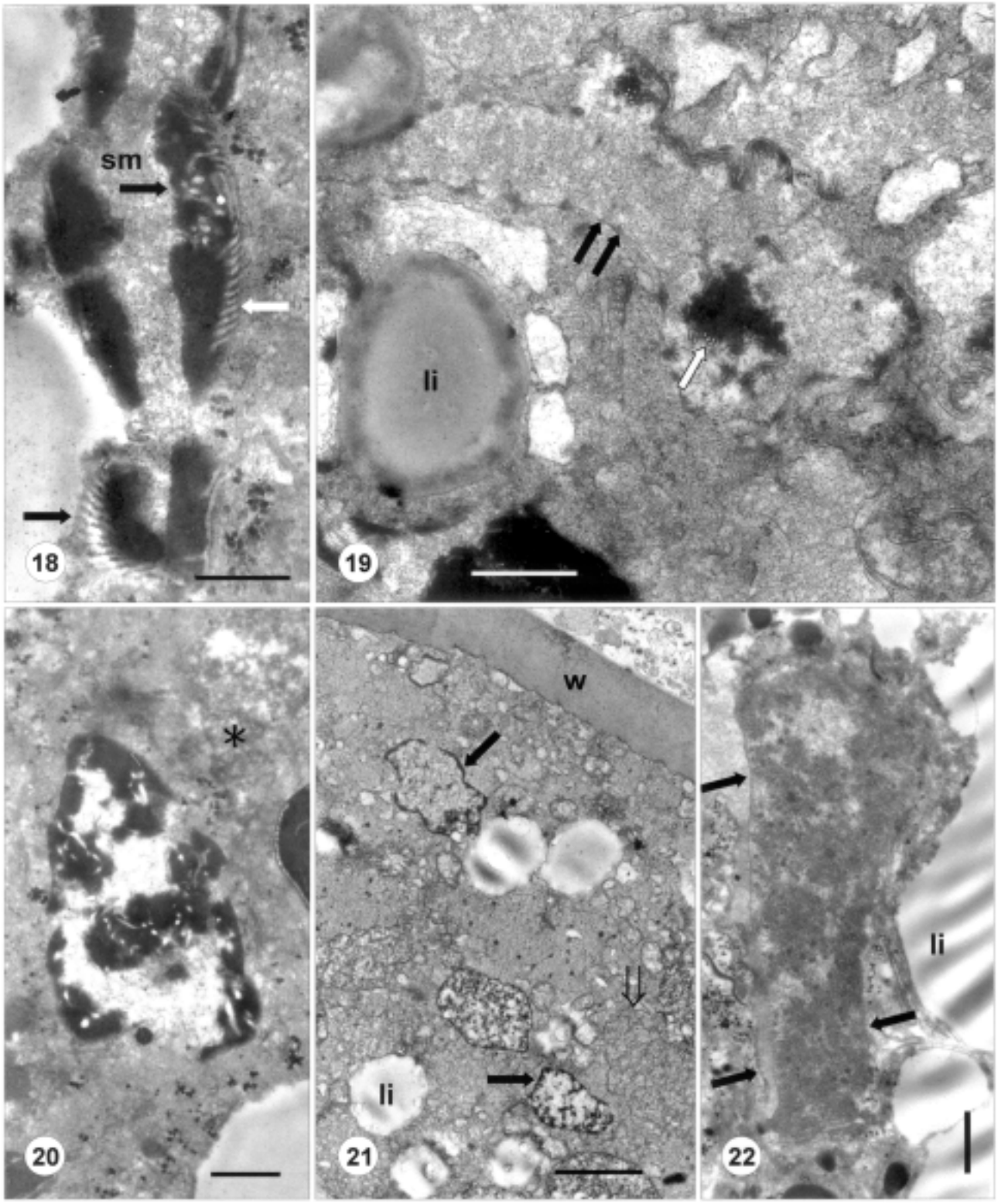
The diversity and variety of plasmodium structure can hardly be described in full. What was most striking, however, is that there was no trace of a regular nucleus. The nuclei were easily visible only at the early stage of development and then at the stage preceding sporoblast formation, when numerous small nuclei reappeared.

According to the stage of its development, the organelles within the plasmodium were mitochondria (Fig. 15), Golgi bodies comprising several cisternae only, most varied vesicles with lucent or finely granular content, ribosomes, cisternae of endoplasmic reticulum, glycogen granules arranged in rosettes and numerous lipid vacuoles; these organelles varied in size and number. Faintly dense lipid vacuoles were always present, of different size and number. Mitochondria had flat, plate-like cristae, in some the cristae were less conspicuous but there was a central dense body or a few granules. Structures reminiscent of multivesicular bodies were sometimes observed (Fig. 16). Bundles of fibrils or crystal-like structures in the plasmodia were rarely observed (Fig. 17).

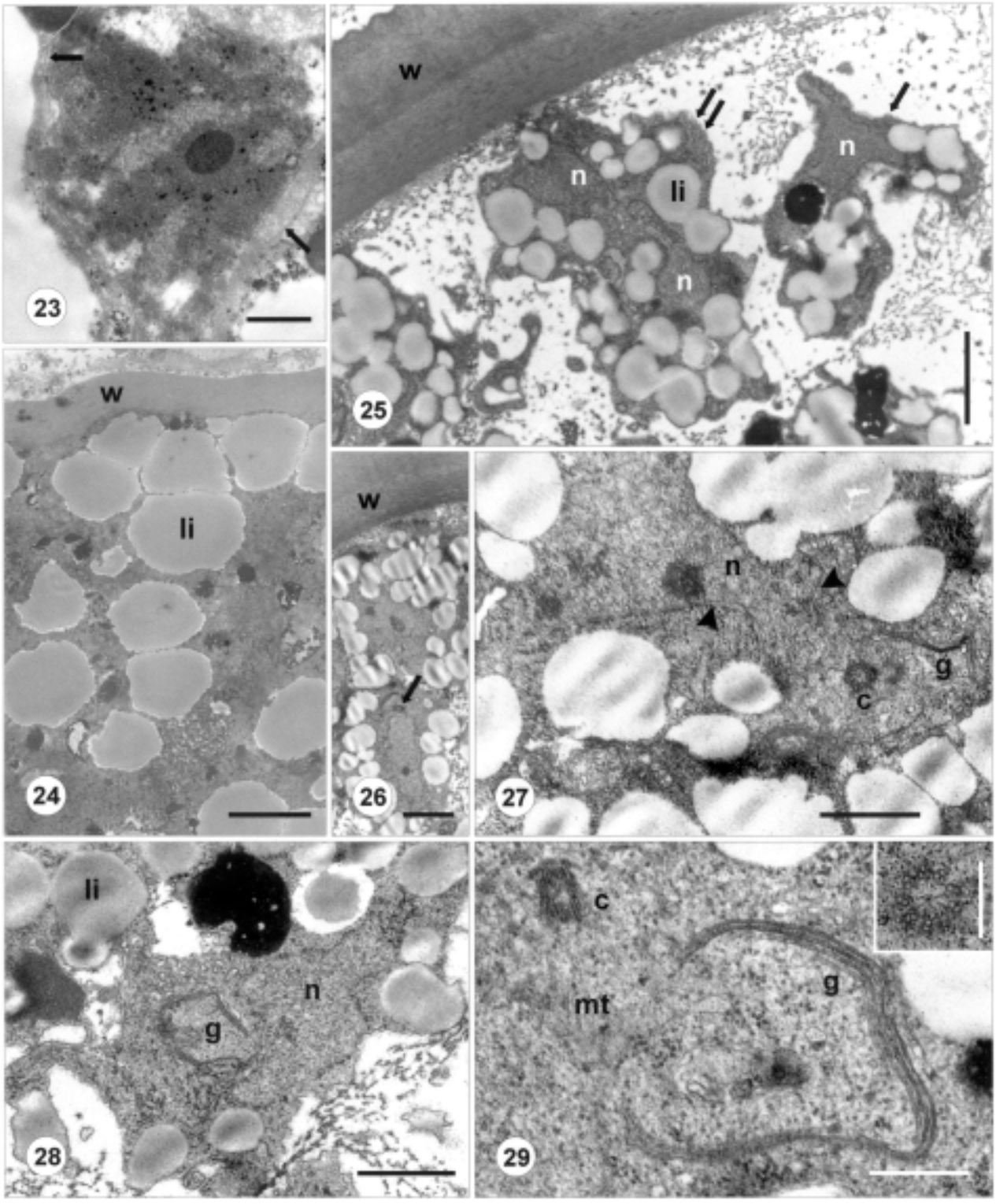
In the cytoplasm of plasmodia without detectable nuclei there were smooth membranes, sometimes folded and convoluted in a complicated way. A similar membranous system was observed to form a single-membrane envelope containing chromatin-like material (Fig. 16). Dark matter formed spindle-shaped or irregular concentrations along the inner sides of the membranes (Figs 16, 18-20). At the ends, and sometimes at the centres, of the membranous extensions there were also concentrations of dark matter (Figs 16, 19). Interestingly, there were small tubules (about 30 to 40 nm in diameter) in the dark matter or in the finely granular matrix in the extensions (Figs 16, 18-20).

Before the onset of sporogenesis, occasional nuclei could be seen. In some plasmodia, small, single-membrane vacuoles with granular matter (as in younger plasmodia, see Figs 8, 9) were observed. In some vacuoles the granular matter was condensing, or alternatively the dark matter of the extensions (described previously) may have been dispersing (Figs 20, 21). The previously mentioned tubules were still visible among the dark matter. It is tempting to believe that the typical heterochromatin was then composed from the dark or granular matter (Figs 22, 23). Membranes from the cytoplasm may form the outer layer of the envelope of the re-organised nucleus.

The lipid droplets were largest before sporogenesis, about 4 μm in diameter; later the droplets began to



Figs 18-22. *Dermocystidium percae*, grown plasmodia. **18** - dense matter within a space delimited by a smooth membrane with small tubules (arrows); **19, 20** - smooth membrane envelopes (double arrow in Fig. 19) containing dense, chromatin-like material with small tubuli (white arrow in Fig. 19) in a lucent or finely granular matrix, asterisk - granular matter; **21** - single membrane vacuoles with coarsely (arrows) or finely granular substance (hollow arrow); **22** - a single membrane-bound envelope, probably the precursor of the new nucleus, filled with moderately dense granulation; arrows indicates the envelope wall. li - lipid inclusion, sm - matter within a space, w - plasmodium wall. Scale bars - 0.4 μm (18); 0.5 μm (19, 20, 22); 2 μm (21).



Figs. 23-29. *Dermocystidium percae*, mature and presporogonic plasmodia. **23** - a single membrane-bound structure (arrows indicate the envelope) with granular content of variable density; a possible precursor of the new nucleus; **24** - part of the periphery of the presporogonic plasmodium with a mass of large lipid droplets; **25** - the plasmodium fragmenting to form sporoblast mother cells with two nuclei (double arrow) which divide to produce sporoblasts (arrow); the wall is now two-layered; **26** - part of the periphery of the fragmenting plasmodium which is seen in more detail in Fig. 27; arrow points at the centriole; **27** - formation of sporoblasts, with a new nucleus with envelope marked by arrowheads; **28** - sporoblast formation with nucleus and Golgi; **29** - cells forming sporoblasts: centriole next to Golgi; inset, upper right - transverse section through a centriole, bar - 0.2 µm. c - centriole, g - Golgi, li - lipid droplets, mt - microtubules, n - nucleus, w - plasmodium wall. Scale bars - 0.5 µm (23, 29); 1 µm (27, 28); 2 µm (25, 26); 4 µm (24).

divide. They were sometimes seen to group together (Fig. 24). Small ruptures as signs of future plasmotomy were seen in the plasmodium.

Sporogenesis. At about the time when sporoblasts were being formed within the plasmodium, the wall differentiated into two layers, the outer slightly more lucent and the inner more dense with interspersed dense, comma-like structures (Fig. 25).

The plasmodium fragmented into smaller parts (Figs 25, 26). The nuclei formed in the late stage of the plasmodium did not represent the final number of the sporoblasts, since the segmentation of the plasmodia produced sporoblast mother cells which divided to form sporoblasts (Fig. 25). The nuclei of the sporoblast mother cells ("sporonts") and sporoblasts were irregular in shape, with a small nucleolus and hardly visible chromatin; they became centres for a small amount of cytoplasm, surrounded or even penetrated by lipid droplets. These small lipid vacuoles originated from the preceding large plasmodial vacuoles and still continued dividing to be ultimately about 0.5 to 1 μm in diameter, sometimes appearing quite lucent.

During the division producing sporoblasts, the nuclear envelope, or at least most of it, persisted. Centrioles were often visible at this stage (Figs 26, 27), and a prominent, cup-shaped Golgi body was seen in the vicinity of the centriole (Fig. 29). Microtubules were found near the centriole and near the nuclear envelope. Numerous lucent vesicles were seen at the cis-side of many Golgi bodies, and it seemed as if the vesicles and some cisternae were in the vicinity of electron dense concretions (Fig. 28).

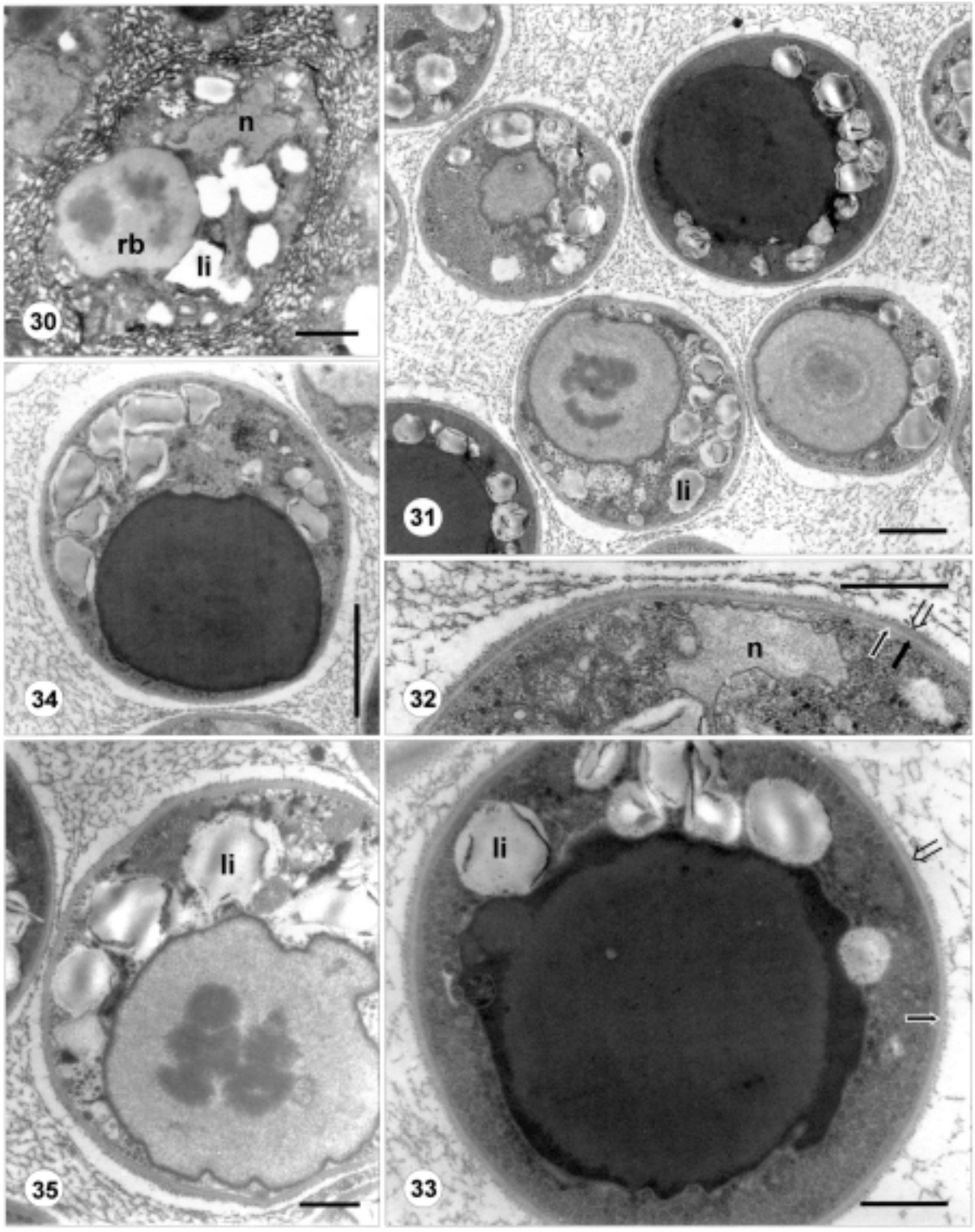
Sporoblasts, 4 to 6 μm in diameter, were separated from each other by a foamy matrix (Fig. 30). The outside of the nuclear membrane in sporoblasts was covered by ribosomes and there were some RER cisternae in the cytoplasm. There were indistinct mitochondria and scarce glycogen granules. Roundish bodies of dark or lighter matter were distributed to the sporoblasts possibly from the plasmodium. An apparently primordial stage of the typical large spore inclusion could be observed in some nascent spores (Fig. 31). The growing inclusion in sporoblasts was light when observed with the electron microscope, sometimes with darker flecks or concentric rings at the centre. As the spore matures, the inclusion becomes denser.

Spores. In young spores the nucleus was indistinct and irregular in contour (Fig. 32), in older spores it was roundish and more easily visible and had a distinct nucleolus. The cytoplasm was laden with glycogen rosettes. The slightly eccentrically located inclusion body, about 4 to 7.5 μm in diameter had a moderately dense matrix and a much darker, irregular rim (Fig. 33). The dark rim apparently engulfed the glycogen and some bulges of the inclusion could be seen. Cisternae and vesicles of the Golgi body or endoplasmic reticulum may be involved in modification of the central inclusion body.

The size of mature spores was approximately 6 to 8 μm. Spore age and mode of fixation affect the size of spores; spores kept in water sometimes increased in size from 6-7 μm to more than 10 μm. The spores (Fig. 34) had an indistinctly visible cell membrane, covered by a rather electron lucent wall of fibrous or lamellar appearance, approximately 40 nm thick, and a surface coat made up of rough granules (Figs 32, 33). The inclusion was moderately dense, sometimes, probably in what were older spores, with up to 6 faintly dense concentric layers (Fig. 36). Lipid vacuoles at the periphery of the spore, next to the inclusion, were 0.5 to 1 μm in diameter; some, even in young spores, displayed crumpled lamellae and may have lost some of their content (Fig. 35). The number of lipid vacuoles may decrease in older spores. The cytoplasm contained scarce mitochondria and cisternae probably of Golgi nature. In older spores glycogen rosettes were scarcer but there were numerous ribosomes.

Zoosporogenesis. The spores, when incubated in water, divided to form zoospores. The process began with an increase in size of the nucleus (Fig. 37). The inclusion became lighter showing concentric rings or flecks, its size was reduced until it almost disappeared. The nucleus moved to the centre of the cell and became irregular in shape (Fig. 38). Many small electron dense globules appeared in the cytoplasm, some with an electron lucent centre; the globules may arise from fragmentation of the central inclusion. Sometimes small tubuli surrounded a globule (Fig. 39). There were ribosomes, small vacuoles and/or lipid droplets and small mitochondria in the cytoplasm.

As the nucleus moved to the cell centre, the cytoplasm begun to detach from the spore wall (Fig. 38). The margin of the cell showed small villi. The cell then divided into two halves, then into four cells (Fig. 40) and the division proceeded until a cell group (a rosette) of developing zoospores was formed (Figs 41, 42). Dark inclusions were then scarce but lipid-like droplets were



Figs 30-35. *Dermocystidium percae*, sporogenesis. **30** - sporoblast separated from the others by a foamy matrix; rounded body, possibly a precursor of the large spore inclusion; **31** - a group of one mature (upper right) and several young spores with the large central inclusions; all are embedded in a foamy matrix; **32** - section through the periphery of a young spore; black arrow marks the cell membrane, white-lined arrow the lucent spore wall, hollow arrow marks the surface coat. **33** - spore, note the irregular rim of the central inclusion, denser than the inclusion itself; white-lined arrow points at the wall of the spore, hollow arrow at the surface coat; **34** - another mature spore with distinctly eccentric inclusion; **35** - young spore with immature inclusion, reminiscent of the rounded body of Fig. 30, with lipid vacuoles displaying crumpled lamellae. The mitochondrion at left of the lipid inclusion is exceptional in having tubular cristae. n - nucleus, li - lipid vacuole or inclusion, rb - rounded body. Scale bars - 1 µm (30-33, 35); 2 µm (31); 3 µm (34).

present. The zoospore cells had irregular contours, nuclei, and a few mitochondria with flat cristae (Fig. 42). An inclusion which appeared grey, with a denser core, developed in each cell (Figs 41, 42). The flagellum developed before the zoospores were separated.

Mature zoospores. The inclusion body - possibly proteinaceous in nature (Figs 43, 44, 47) - usually had a dense core, and one side of the inclusion was also denser; this side consisted of thin lamellae, possibly of crystallised matter. The single, posterior flagellum (Fig. 44) of the zoospore originated in the vicinity of the inclusion body (Fig. 43). The nucleus was in intimate contact with the single, large mitochondrion. The latter had basically plate-like cristae - sometimes reminiscent of annulate lamellae (Figs 45, 46) and a fairly dense matrix with some granules. At the cell margin beside the inclusion there was a rumposome-like structure (Figs 44, 47).

The basal body of the single posterior flagellum was located near the inclusion body. Below this basal body, and at a right angle to it, there was a non-functional centriole (Fig. 48). At least one microtubule extended anteriorly below the cell membrane from the vicinity of the basal body and the non-functional centriole. A rhizoplast with transverse striae ran from the vicinity of the non-functional centriole and its associated dark bands between the cell margin and the inclusion body (Figs 49, 50, 62).

Zoospores then escaped from the spore envelope, which was left empty behind.

Dermocystidium fennicum sp. n. ultrastructure

Plasmodium stage. The plasmodium cysts (Fig. 51) were roundish, oval or dumb-bell-shaped. The cyst wall was about 4 to 7 μm thick; the outer, homogeneous layer was less electron dense (Fig. 52). The villi on the surface did not exceed 1.5 μm in length.

A common feature of the plasmodia was the abundance of more or less lucent lipid vacuoles. There were numerous smooth and rough membranes and free ribosomes and glycogen rosettes. Mitochondria with plate-like or somewhat swollen cristae were sometimes seen.

As in *D. percae*, in most plasmodia no nucleus could be detected. Small nuclei with a distinct nucleolus were only occasionally seen. Spaces delimited by single membranes with finely granular content found in some plasmodia might possibly represent nuclear structures. In some, possibly older plasmodia similar spaces also included larger and smaller concentrations of dense matter (Figs 53, 54). Prior to plasmotomy, nuclei may be formed at the sites of such concentrations. Nuclei possibly arose first by condensation of a nucleolus and then the chromatin matter around it (Fig. 55).

The developing nucleus had a single membrane with irregular chromatin concentrations on its inner side. Nucleolus-like structures could often be seen (Fig. 53). Small vesicles (50 to 150 nm) were seen near the condensing chromatin (Fig. 54). The nuclear envelope then acquired an outer membrane (Fig. 56); in this figure the double envelope at the right side of the nucleus is clearly seen.

Sporogony and spores. Sporoblasts were formed as the result of plasmotomy, and were almost identical to those of *D. percae*: the glycogen rosettes were perhaps more numerous. The transformation of sporoblasts to spores was also similar. In young spores a darker rim of the central inclusion seemed to engulf glycogen. In the cytoplasm of mature spores of *D. fennicum* glycogen appeared more plentiful (Fig. 57) than in mature spores of *D. percae*.

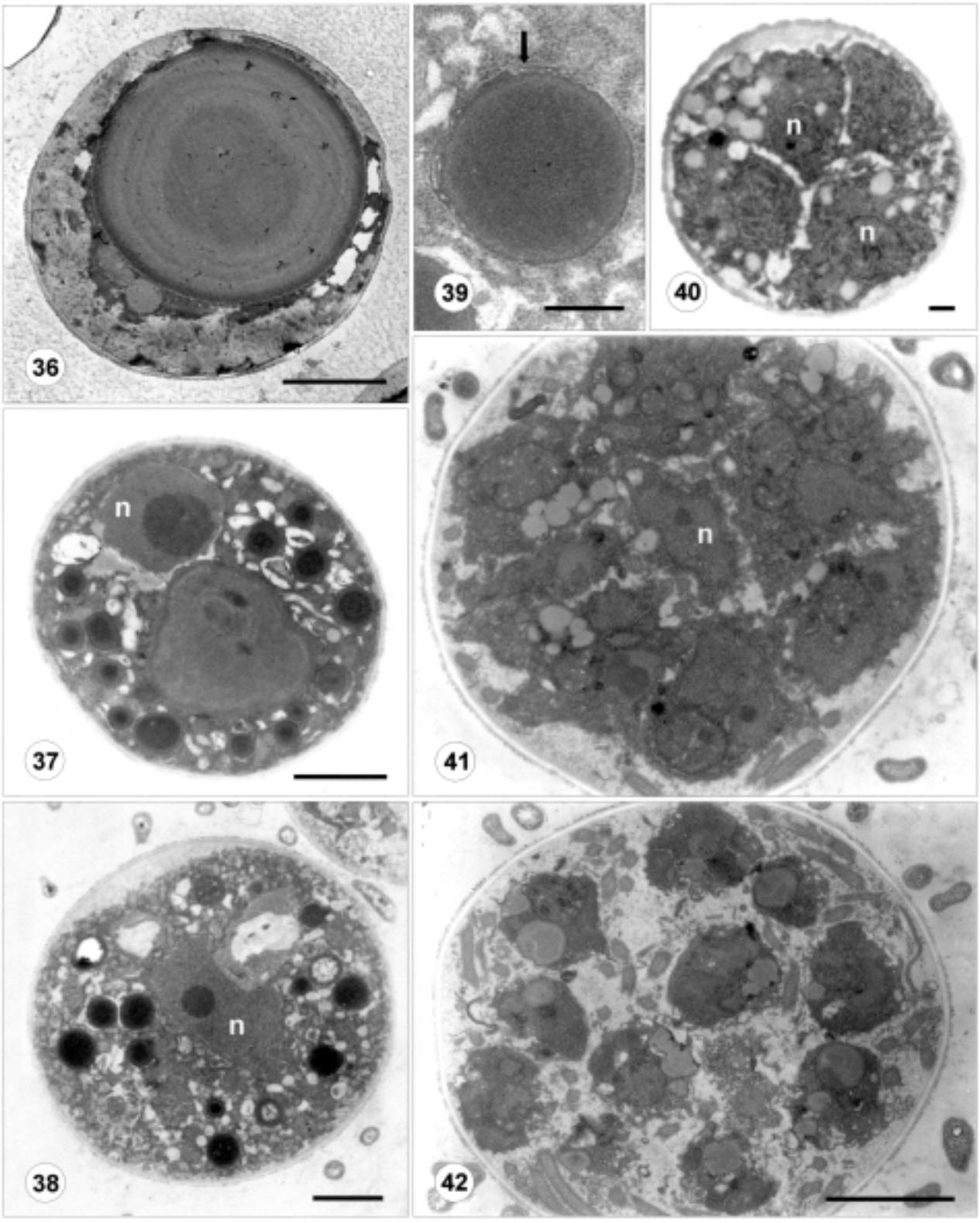
Zoosporogony and zoospores. The pre-division and division processes of the *D. fennicum* spore did not differ from those of *D. percae* (Fig. 58). The zoospore stage had a large mitochondrion with fairly dense matrix, which contained many dense granules. When the cristae could be discerned, they were lamellar in structure (Fig. 59). The nucleus partially enveloped the mitochondrion. The zoospores had a large glycogen reserve, a lipid droplet and sometimes, in the organisms freed from spore envelopes, a groove or pit could be seen (Fig. 60).

The flagellated zoospores were possibly of short duration, at least when incubated at higher temperatures (23-25°C); flagella were never found still attached to the bodies. Remains of an inclusion were seen being discharged from the body (Fig. 61).

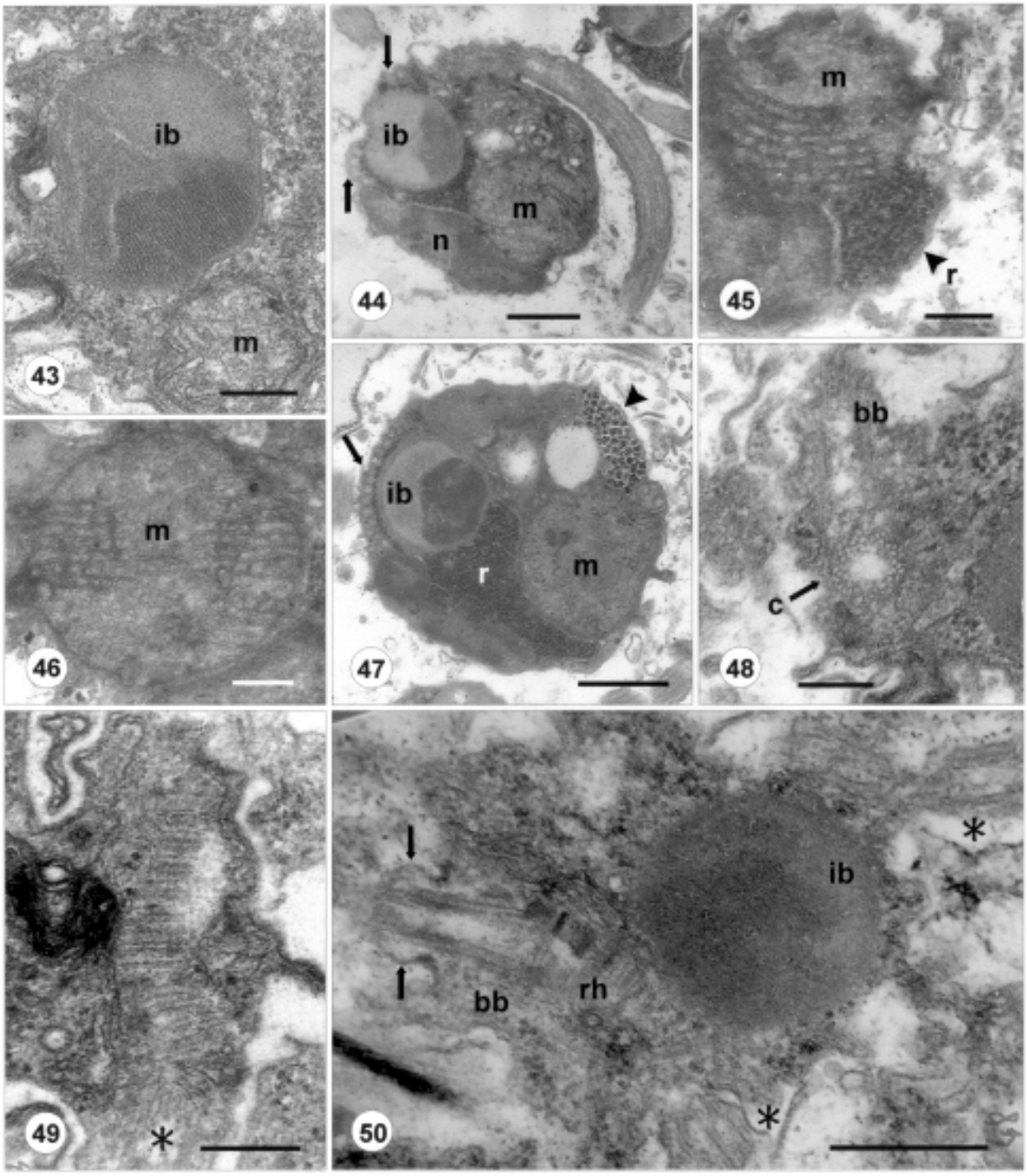
DISCUSSION

Phylogenetic position of *Dermocystidium fennicum* and *D. percae* within Ichthyosporea

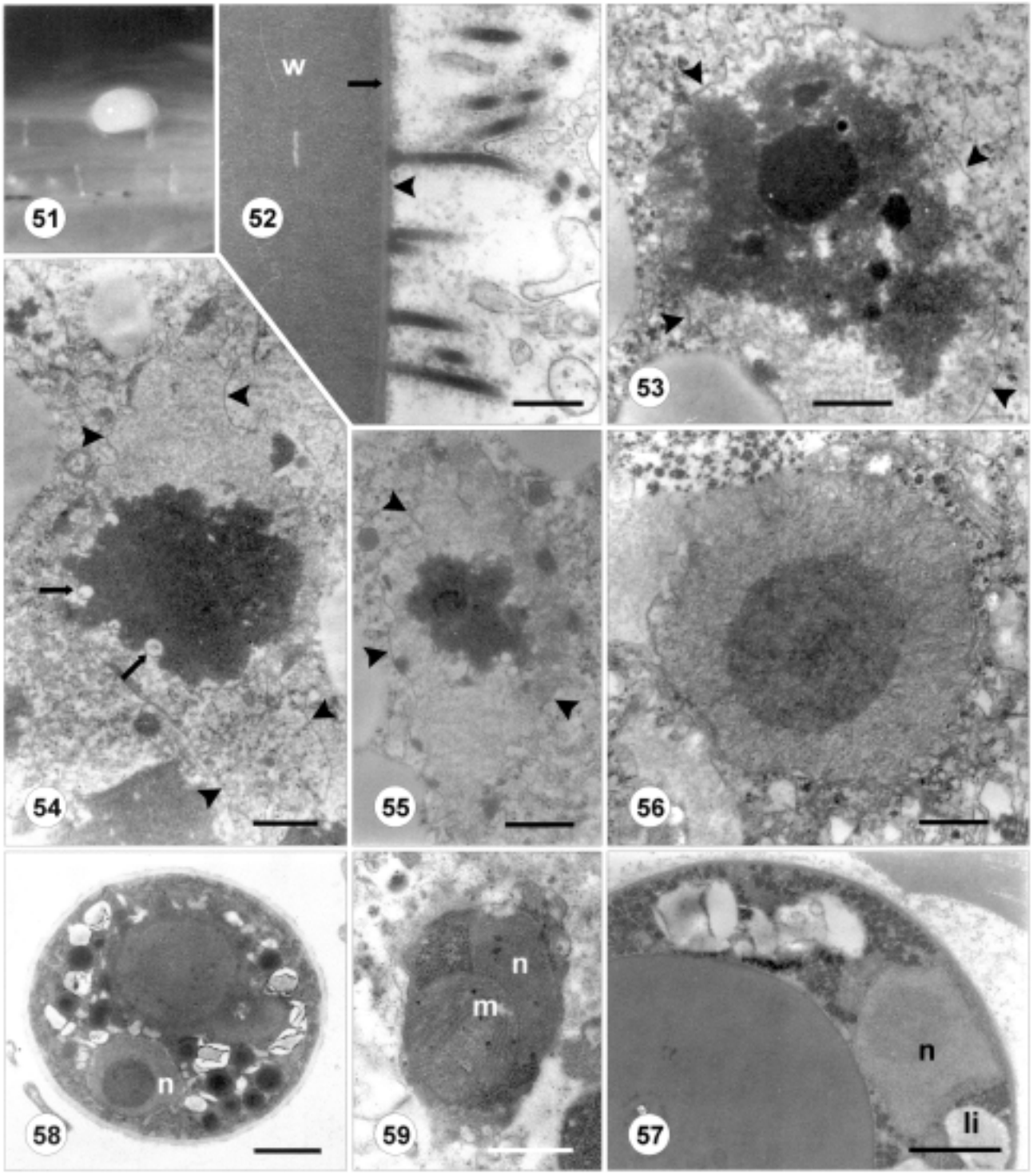
In the unrooted phylogenetic tree shown in Fig. 1 the rosette agent is displayed as the outgroup, it is suggested that for ease of interpretation, a root should be visualised joining at the midpoint of the long left-hand vertical edge. The 18S rDNA of rosette agent can be resolved, with high bootstrap support, as the most-basal branch within the Ichthyosporea. Our Bayesian phylogenetic analysis resolves two groups with 100% posterior probability



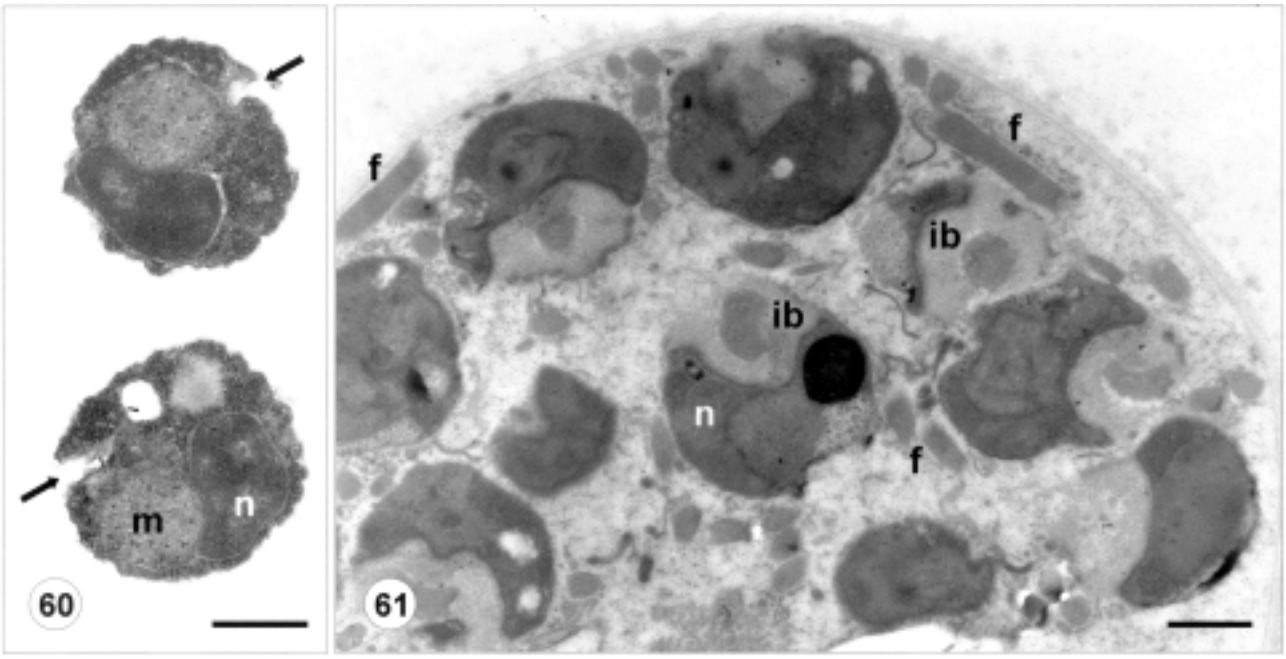
Figs 36-42. *Dermocystidium percae*, spores and zoosporogenesis. **36** - an old spore in which lipid and glycogen reserves became scarce, with distinct concentric layers in the inclusion; **37** - first phase of zoosporogenesis with enlarged nucleus of the spore; note the appearance of dense globules in the cytoplasm; **38** - next step of sporogenesis; the central inclusion has almost vanished; **39** - one of the dense globules from Fig. 38 enlarged; arrow points at the tubuli at the periphery of the globule; **40** - the spore has divided into four cells; **41** - a rosette of daughter cells within the old spore wall; **42** - old spore wall with developing zoospores inside it. n - nucleus. Scale bars - 0.5 μm (39, 40); 1 μm (38); 2 μm (36, 37, 41, 42).



Figs 43-50. Details of zoospore structures in *Dermocystidium percae*. **43** - inclusion body of the zoospore, part of it revealing a densely striated structure; **44** - the zoospore with its single, posteriorly curved flagellum; arrows point at the rumposome-like structure; **45, 46** - mitochondria with diverse structures of cristae. **47** - section through the zoospore; next to the inclusion there is the rumposome-like structure (arrow); arrowhead points at glycogen rosettes. **48** - basal body of the zoospore flagellum; beneath it is the barren (non-functional) centriole; **49** - transversely striated rhizoplast, associated with the basal body of the flagellum (asterisk); **50** - basal body with the flagellum extending through the cell membrane (arrows) and its associated rhizoplast; asterisks - flagella of neighbouring zoospores. bb - basal body, c - centriole, ib - inclusion body, m - mitochondrion, n - nucleus, r - ribosomes, rh - rhizoplast. Scale bars - 0.2 µm (43, 45, 46, 49); 0.4 µm (44, 48, 50); 0.5 µm (47).



Figs 51-59. *Dermocystidium fennicum* sp. n. **51** - a cyst on the surface of the gills, actual size is 360 μm; **52** - the cyst wall and the villi; arrow points at the outer homogeneous layer of the wall; arrowhead points at the surface coat; **53-55** - single membrane-bound structures with (chromatin-like) concentrates of dense matter, possible precursors of (presporogonic) nuclei; arrowheads point at the delimiting membranes, arrows at vesicles wedged in the margin of the dense substance; **56** - a presporogonic nucleus with double envelope. **57** - a sector of mature spore with the inclusion, mass of glycogen, nucleus and lipid inclusion; **58** - spore in the early phase of zoosporogenesis with reduced inclusion, dense globules, lipid globules and growing nucleus; **59** - a mitochondrion of the preceding stage. li - lipid inclusion, m - mitochondrion, n - nucleus, w - cyst wall. Scale bars - 0.4 μm (54); 0.5 μm (52, 53, 55, 59); 1 μm (57, 58); 2 μm (58).



Figs 60, 61. *Dermocystidium fennicum* sp. n. **60** - zoospores escaped from spore walls with a pit (arrow) in their cell; **61** - zoospores discharging their inclusion bodies. ib - inclusion bodies, f - flagellum, m - mitochondrion, n - nucleus. Scale bars - 0.5 μm (60, 61).

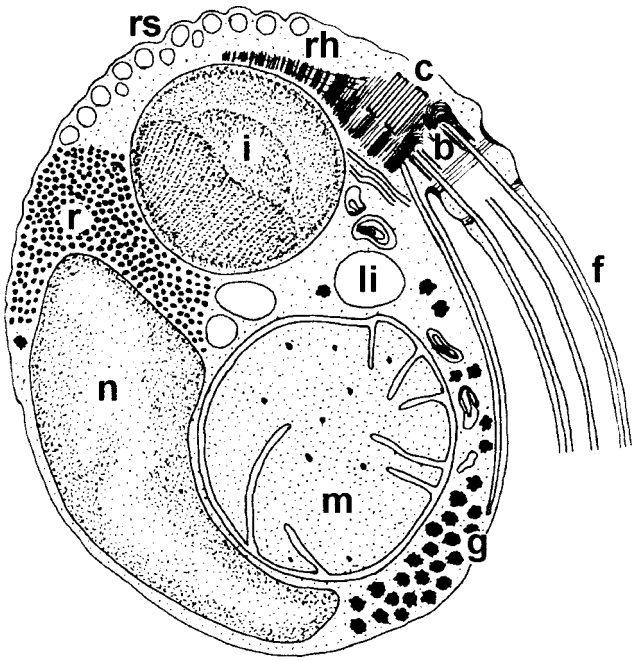


Fig. 62. A drawing of the site of the flagellar basal body and associated structures in *Dermocystidium percae* zoospore. b - basal body, c - non-functional centriole, f - flagellum, g - glycogen rosettes, i - inclusion body, li - lipid inclusion, m - mitochondrium, n - nucleus, r -ribosomes, rh - rhizoplast, rs - rumposome.

within the Ichthyosporea. In trees that also contain sequences from organisms other than Ichthyosporea (e.g., Benny and O'Donnell 2000, Figueras *et al*. 2000, Rand *et al*. 2000, Ustinova *et al*. 2000), ichthyosporean SSU rDNAs are resolved into two clades, one consisting of rDNAs of *Dermocystidium* species and *Rhinosporidium seeberi*, the other with rDNAs of *Amoebidium parasiticum*, *Anurofeca richardsi*, *Ichthyophonida* sp. LKM51, *Ichthyophonus* sp., *Pseudoperkinsus tapetis*, *Psorospermium haeckeli* and *Sphaerosoma arcticum*.

Sequences from both *Dermocystidium* isolates reported here group securely within the *Dermocystidium-Rhinosporidium* clade. The SSU rDNA of the new *Dermocystidium fennicum* described here is resolved, with 99% posterior probability, as a specific sister-group with that of *D. salmonis*. Based on SSU rDNA sequences, our new *D. fennicum* is not specifically related to the *Dermocystidium* sp. studied by Ragan *et al*. (1996), which by contrast groups with 100% posterior probability with several isolates of *R. seeberi*.

Sequences from the nine *D. percae* clones are resolved into a single group with 100% posterior probability. Within *D. percae*, clones 6, 33 and 35 are resolved separately (see above - Fig. 1). To answer the

possibility whether different sequences of clones of *D. percae* might reflect expression in different life history stages, one could carry out RT-PCR studies which will demonstrate whether the different sequences reflect pseudogenes or are really expressed. This could, however, raise enormous technical challenges. First, one would have to separate different life-history stages in amounts that allow RT-PCR. RT-PCR is typically done from poly(A)-mRNA, but the primary transcript that contains SSU-rDNA is a large RNA and (even assuming that it is polyadenylated in the first place) standard RT-PCR could not read far enough in from the 5' end to reach the SSU coding region. Special PCR techniques might help (or might not). This is why the close sequence analysis of variants has been included. Most variants show evidence of being under selection, which would not be the case if they were pseudogenes. Thus these are probably not pseudogenes.

Given our experimental design, we cannot distinguish whether these results indicate that the rDNAs of *D. percae* are microheterogeneous, or that our biomass contained very closely related organismal variants (e.g. subspecies). Several cysts from a few host fish individuals were included in the sample. If these sequences reflect microheterogeneity within a unitary species, it would be interesting to examine whether the different sequences are differentially expressed throughout the life history of *D. percae.*

The possible occurrence of subspecies in *D. percae* will be addressed in future.

Classification of *Dermocystidium* species from the perch

Pekkarinen and Lotman (2003) suggested that two different *Dermocystidium* species occurred in perch in Finland. Both developed independently. In the same light microscope study, the species differed in their cyst shape, thickness of their cyst wall and in microhabitat preference of the cysts on the fish surface. At the ultrastructural level, the two species do not differ much. Restructuring of the plasmodial nuclei may, however, be slightly different. Summary of all observed differences, including those in the SSU rDNAs, justifies considering the species as separate. We propose therefore to establish a new species, *Dermocystidium fennicum* sp. n. with the following differences from *D. percae*:

- the cyst formed by the plasmodium of *D. fennicum* is roundish, more rarely oval or dumb-bell shaped compared to the elongate-cylindrical shape of *D. percae*;

- the cysts of *D. fennicum* are situated on the skin of the head region and bases of the fins, while *D. percae* forms cysts in the skin of the fins. *D. fennicum* favours the dorsal fin, while *D. percae* does not. (Only in O-group fish may cysts of *D. percae* be formed anywhere in the skin).

- the wall of *D. fennicum* is 4 to 7 μm thick, compared with 1.5 to 4, rarely 6 μm in *D. percae*.

- within the *Rhinosporidium - Dermocystidium* clade, *D. fennicum* is a sister taxon with *D. salmonis*, while *D. percae* forms a sub-clade of its own.

Specimens of *D. fennicum* are deposited in the collection of the Institute of Parasitology in České Budějovice under the numbers H-PH-073, 074, 075, 076.

Comparison with other *Dermocystidium* species and ichthyosporeans

Throughout this paper, we have used the traditional term "cyst" for the *Dermocystidium* plasmodium with its thick wall, although the term "sporocyst" as used by Vogt and Rug (1999) for the same stage in *Psorospermium* would be more convenient.

Until now, three cyst-forming *Dermocystidium* species have been studied with the electron microscope. Wootten and McVicar (1982) presented a few electron micrographs of the cyst wall and a micrograph of the spore of *D. anguillae* Spangenberg, 1978. Olson *et al*. (1991) reported the formation of uniflagellated zoospores within discharged spores of *D. salmonis* Davis, 1947. Lotman *et al*. (2000) investigated the fine structure of *D. cyprini* Červinka and Lom, 1974. All are principally gill parasites, and *D. salmonis* and *D. cyprini* have been described to produce infective uniflagellate zoospores. In *D. cyprini* zoosporulation may take place in compartments and in *D. salmonis* it takes place in the spore envelope. Thus *D. fennicum* and *D. percae* resemble *D. salmonis* in this respect. The cyst development of *D. salmonis* is, however, very rapid; Olson *et al*. (1991) did not describe any plasmodial stages, and the immature spores developed in an experimental infection did not resemble the sporoblasts described in this study. The ultrastructure of *D. salmonis* needs further study.

The cyst-forming dermocystidia are quite varied morphologically, reflecting the diversity of members of the whole group of Ichthyosporea; this applies especially to the cyst wall. In *D. cyprini* it differs from that in perch dermocystidia; there is an only 0.1 μm thick dense layer, from which thin tubular villi extend and which is subtended by a layer with dense granules and a

granulofibrillar layer, the whole being about 3 µm across. In *D. salmonis* the cyst wall (as calculated from the photomicrograph) is about 0.3 µm thick, whilst in *D. granulosum*, according to light microscope observations of Sterba and Naumann (1970), it is multilayered and 4 to 5 µm thick. Only in *D. cyprini* has the cyst cytoplasm been investigated (Lotman *et al*. 2000), but it includes normal nuclei and lacks the unusual vacuoles with dense material, found in perch dermocystidia.

Flagellated cells exist both among choanoflagellates and chytrids and in having them, dermocystidia reflect their position near the early branching of the animal and fungal lines. The *Dermocystidium* zoospores show structures of Chytridiomycetes; they have a single, posterior flagellum. The zoospores of *D. percae* even seem to have a rumposome-like structure. The rumposome is a honeycomb-like structure of unknown function usually near a lipid globule in some Chytridiomycetes (Barr and Hartmann 1976, Lange and Olson 1979). In *D. percae* the structure was, however, associated with the possibly proteinaceous inclusion. Some Chytridiomycetes also have a striated fibrous rhizoplast projecting inside the zoospore body from the basal body of the flagellum; striated flagellar roots or rootlets, rhizoplasts, are also known in animal cilia and flagella, too (e.g., Tyler 1984).

The rhizoplast of the *D. percae* zoospore is different from that of *D. cyprini* zoospores in having a less dense striation. In both *D. percae* and *D. cyprini* zoospores the non-functional centriole seems to be at a right angle to the functional kinetosome. The flagellar roots of *D. fennicum* and *D. salmonis* have not been described.

The shape of mitochondrial cristae has often been thought to serve as a guide to the phylogenetic position of organisms. Animals and true fungi generally have flat mitochondrial cristae, while the Mesozoa and what is now considered to be the Protozoa have tubulovesicular cristae. The DRIPs clade introduced by Ragan *et al*. (1996) and widened later to Ichthyosporea by Cavalier-Smith (1998), forms a basal branch near animals and fungi. It was thought that within the Ichthyosporea *Ichthyophonus* and other representatives of the group have tubulovesicular cristae, and the members of the *Dermocystidium-Rhinosporidium* group have flat cristae. The hypothesis seems to be true among the *Dermocystidium* species studied: i.e., *Dermocystidium* sp. from brook trout (Ragan *et al.* 1996), *D. cyprini* from carp (Lotman *et al*. 2000) and *D. percae* and *D. fennicum* from perch (this study). The mitochondrial cristae of *Rhinosporidium seeberi* were first described as flat (Herr *et al*. 1999) and later as tubulovesicular (Fredricks *et al*. 2000). Fredricks *et al*.(2000) explain this discrepancy by possibly different methods of preparation or by different developmental stages of the parasite. In the *Ichthyophonus-Psorospermium* clade, *Ichthyophonus hoferi* has tubulovesicular cristae (Spanggaard *et al*. 1996, Rahimian 1998), and *Pseudoperkinsus tapetis* has vesicular cristae (J. Lom, unpublished observation), whereas *Amoebidium parasiticum* has mostly flat cristae (Whistler and Fuller 1968). The mitochondrial cristae in Ichthyosporea evidently do not unambiguously follow the phylogenetic grouping. The rosette agent, in the most basal branch of Ichthyosporea in our study, has plate-like mitochondrial cristae (according to micrographs presented by Arkush *et al*. (1998) and Harrell *et al*. (1986)).

The crayfish parasite *Psorospermiun haeckeli*, and the ectosymbiont of arthropod *Amoebidium parasiticum* very clearly differ in morphology from *Dermocystidium* species. *P. haeckeli* and its genetically diverse forms (Rug and Vogt 1995, Bangyeekhun *et al*. 2001) are best known at the oval sporocyst stage. The wall of the organism is composed of plates (Nylund *et al*. 1983), which distinguishes it form the other ichthyosporeans. *A. parasiticum* reproduces either by nonmotile sporangiospores or by amoebae (Ustinova *et al*. 2000). Uniquely, thalli of *A. parasiticum* are attached externally to a host by an acellular holdfast (Benny and O'Donnell 2000). Figueras *et al*. (2000) included *Perkinsus atlanticus*-like organisms, with the proposed name *Pseudoperkinsus tapetis*, from carpet shell clams in northwestern Spain to the DRIPs clade. It is not certain that the *Perkinsus atlanticus* described by Azevedo (1989) from Portugal is an identical organism. Two flagella and an ultrastructure typical of apicomplexans formed in the zoospores of *Perkinsus atlanticus* sensu Azevedo refer to the Perkinsea in the Alveolata.

Anurofeca richardsi, parasitic in the gut of tadpoles, described by Beebee (1991), Wong and Beebee (1994) and Baker *et al*. (1999), and the intracellular, cell cluster-forming parasites in salmonids, *viz.*, rosette agent, described e.g., by Harrell *et al*. (1986) and Arkush *et al*. (1998), may not differ very much from *Dermocystidium* species. The daughter cells in dividing forms of *A. richardsi* and of the rosette agent are walled in contrast to developing zoospores of *D. percae*, which, at least in experimental culture, we have found to be irregular in contour and naked.

Ichthyophonus hoferi, occurring in many fish species, includes, as is typical of ichthyosporeans, vacuoles of varying density and also lipid-like bodies (Paperna 1986, Spanggaard *et al*. 1996, Rand 1994). Active spores of *I. hoferi* described by Rahimian (1998) show some similarity to the plasmodial cysts of *D. percae*. Viewed at a low magnification, the ground cytoplasm of the spore (plasmodium) has a network of fibre-like cords, and dense bodies (cf. Fig. 10 in Rahimian 1998 and Fig. 2h in Pekkarinen and Lotman (2003)). Nuclei were, however, easily seen in *I. hoferi* and could be a few or several hundred in number. The organism was divided into units containing from one to a few nuclei accompanied by cytoplasmic material, just as was the case in *D. percae*, and *D. fennicum*. No flagella were described in *I. hoferi*. Hyphae-like structures of *I. hoferi* (Ruggieri *et al*. 1970) and thalli of *I. irregularis* (Rand 1994) suggest a closeness to fungi. There is one report where aseptate hyphae of a *Dermocystidium* species have been described (Dyková and Lom 1992): in koi carp, spores of *D. koi* developed in subcutaneous hyphae. Production of hyphae-like structures thus occurs rarely in both groups of the Ichthyosporea (*Ichthyophonus* group and *Dermocystidium-Rhinosporidium* group).

There have been a few cases of visceral and systemic infections in salmonid fish by an intracellular parasite identified as a *Dermocystidium* species. It was found mainly in the visceral cavity (McVicar and Wootten 1980), or in melanomacrophages (Hedrick *et al*. 1989); or it induced systemic infections (van de Moer *et al.* 1987, Nash *et al*. 1989). These organisms and the rosette agent are fairly similar in structure in that the daughter individuals are produced by segmentation within the common wall. The infection by rosette agent can ultimately result in host cell death (Arkush *et al*. 1998). The agent can cause limited nodules in different organs or systemic dispersion of the organisms with minimal host inflammatory cell response.

Rhinosporidium seeberi produces thick-walled spherical structures containing protein spherules and lipid globules (see Ashworth (1923) for references). At variance with our *Dermocystidium* species, *R. seeberi* clearly acquires numerous nuclei during its development. A pore appears in the wall of the 'sporangium' and the organism then discharges 12000 to 16000 endospores (Ashworth 1923, Herr *et al*. 2000), which in the host tissue mature again into endosporulating sporangia. Flagellated zoospores of *R. seeberi* have not been discovered. However, there has been a debate about discharge of 'sporules' or 'sporozoites' from the spores (see Ashworth (1923) for references). The parasite causes tumor-like growths in the nose or nasopharynx of humans and animals, mostly in the tropics. Fredricks *et al*. (2000) hypothesise that *R. seeberi*´s natural hosts are fish or other aquatic animals, and humans acquire infection when they come into contact with water containing these fish and their parasites.

Nuclear cycle of *Dermocystidium*

The young cell of *D. percae* had an ordinary, fairly large nucleus, with a large nucleolus and double, fenestrated envelope. As the cell grew in size, the nucleus sent out short extensions, but these nuclei later disappeared. Possible mitotic events in young stages remained obscure. The growing cell soon became intercellular and ended up as a plasmodium 1 to 2 mm in length (Pekkarinen and Lotman 2003). Strangely, in medium-size plasmodia, odd enough, a typical nucleus could not be seen. The branched lacunae in the medium-sized plasmodia of *D. percae* and *D. fennicum* could be suggested as a vicarious, nucleus-like structure. In a related species of the *Dermocystidium*-clade, *Rhinosporidium seeberi,* Ashworth (1923) summarized the structures of the earliest stages as evident from the previous studies by Seeber, Minchin, Fantham, Beattie, and others (see Ashworth (1923) for references): "The youngest parasite is oval, round, irregular, or even amoeboid, with vesicular nucleus containing nucleolus. It soon becomes a multinucleate plasmodium, but the mode of division of the nucleus has not been ascertained". The first nuclear spindle was visible in plasmodia 50 to 60 μm in diameter (Ashworth 1923). Later (mitotic) nuclear divisions were thought to be synchronous and rapid, 'for the nuclei being found in the "resting" condition in the stages described between two to one hundred and thirty-two nuclei'. No actual mitoses were seen from the telophase of the first division until after the seventh division. In our *Dermocystidium* species, numerous nuclei did not reappear until before division of the advanced plasmodium. Mitoses occurred in the prespore stages. Centrioles were frequently seen outside the nuclei and at least most of the nuclear envelope seemed to persist. In *R. seeberi* the centrioles of the later nuclear spindles were intranuclear and the nuclear envelopes disappeared during the mitosis (Ashworth 1923). In *D. percae,* Golgi bodies were prominent and were sometimes associated with the centriole and microtubules.

The events of the nuclear cycle of *D. percae* and *D. fennicum* are open to speculation. The chromosomal DNA obviously goes through a period of cryptic existence, after which it is reorganised in typical eukaryotic nuclei. During the cryptic stage it is most probably associated with the dark substance seen in single-membrane bound vacuoles or spaces.

We failed to find similar cases among protists or other lower eukaryotes. Among the protists revealing striking changes in their nuclear cycle are many radiolarians in which the nuclei show cyclic polyploidy, undergoing polyploidization at some stages and depolyploidization at other stages of life cycle (Raikov 1982). The polyploid chromatin may not stain with usual DNA-stains. Radiolarians such as *Thalassicolla spumida* possess a very large polyploid nucleus (called primary nucleus) which is apparently unable to divide in two and they therefore reproduce by sporogenesis. The primary nucleus first breaks up into large fragments that continue to divide and initiate many secondary nuclei. The secondary nuclei form zoospore nuclei after more mitotic divisions. The presence of numerous secondary nuclei is of short duration in this case (Raikov 1982). A figure (Fig. 68b) in Raikov (1982, after Hollande and Enjumet (1953)) depicting fragments originating in *T. spumida* after its nucleus was broken into pieces resembles to some extent the vacuoles and lacunae with dark material in growing plasmodia of *D. percae.* In a related radiolarian, *Thalassophyta sanguinolenta,* the structure of the primary nucleus changes before the beginning of sporogenesis. A homogeneous intranuclear centrosphere appears in the centre of the nucleus and all the chromosomes radially converge on this sphere. Nucleolar ribonucleoprotein is extruded into cytoplasm as sacculiform outpockets and breaks down before the beginning of the sporogenesis (Raikov 1982). A large nucleolus (centrosphere?) with lighter core was seen in this paper in a young stage of *D. percae*. Nevertheless, the similarities between nuclear cycle stages of radiolarians and dermocystidia may be just superficial since none of these stages was proven to lack a true nuclear envelope.

The chemical nature of the granular contents of certain vacuoles (Figs 9, 21) in *Dermocystidium* plasmodia is not known (chromatin, ribonucleoprotein?). Some of the vacuoles with dark material could be the origins of true secondary nuclei. The vacuoles have small canaliculi among the electron dense matter, which may serve to increase the exchange of materials with cytoplasm. In the nuclei of *D. fennicum* in the present study the vesicles released from the condensing centre of the future nucleus may also perform the same function. The large concretions in the growing *D. percae* plasmodium (Pekkarinen and Lotman 2003) may be fragments of the nucleolus or centrosphere of the primary nucleus.

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