Ultrastructural justification for the transfer of *Pleistophora anguillarum* Hoshina, 1959 to the genus *Heterosporis* Schubert, 1969

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**ABSTRACT:** This study presents the ultrastructure of the microsporidian infecting the trunk musculature of *Anguilla japonica* and originally described as *Pleistophora anguillarum* Hoshina, 1959. All stages develop within a special structure, the sporophorocyst (SPC), which is equipped with a thick dense wall. This wall grows along with the growth of the parasites within it. Meronts are uni- to binucleate, which divide and steadily give rise to sporonts. During transition to sporonts the cell coat of the meronts increases its thickness, temporarily featuring thick irregular projections. Eventually a uniformly thick sporont wall is formed, then the sporont cells detach themselves from the wall (= future wall of the sporophorous vesicle, SPV) and start a series of divisions to produce sporoblasts. The SPV wall is compact, has no pores and consists of 2 layers. The presence of the SPC justifies the transfer of the species into the genus *Heterosporis*. Spores from disrupted SPCs are ingested by macrophages and within them are spread into various body tissues including the outermost layers of the epidermis. From here, they can easily be released to the outside and can contaminate the environment while the host is still alive.

**KEY WORDS:** Microsporidia · Ultrastructure · *Heterosporis anguillarum* · *Pleistophora* · *Anguilla japonica*

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**INTRODUCTION**

Microsporidia which infect sarcocytes of the trunk musculature of fish are represented by the genera *Pleistophora* Gurley, 1893, *Kabatana* Lom, Dyková & Tonguthai, 2000 and *Heterosporis* Schubert, 1969. The type species of the latter, *H. finki* Schubert, 1969 originally described from connective tissue of *Pterophysillum scalare* from a pet shop in Germany (Schubert 1969a,b), was later found to produce massive infection of the myocytes of the same host (Michel et al. 1989). Another muscle-infecting species, *Heterosporis schuberti* Lom, Dyková, Körting & Klinger 1989, was described from ornamental fish *Ancistrus cirrhosus* and *Pseudocrenilabrus multicolor*. Later, an unnamed species, *Heterosporis* sp. of Lom, Dyková & Tonguthai, 1993 was recorded from *Betta splendens*. The key feature of the genus *Heterosporis* is the presence of a sporophorocyst, which is a dense, rather solid wall enclosing all developmental stages of the parasite, i.e., meronts, sporonts and sporophorous vesicles with sporoblasts and spores. Comparing our own findings on *Heterosporis* with data in the literature, we came to the conclusion that *Pleistophora anguillarum* Hoshina, 1959 is a species of the genus *Heterosporis*. In view of the economic importance of this species, several papers have dealt with this species, e.g., Hashimoto et al. (1976, 1979), Hashimoto & Takinami (1976), Kano & Fukui (1982) and Kano et al. (1982). The paper by T’sui & Wang (1988) showed 5 electron micrographs of this species, but the authors still treated it as a *Pleistophora* species.
Since an overall description of this species can be found in T'sui et al. (1988), we have concentrated in this paper on ultrastructural features confirming beyond doubt its assignment to the genus *Heterosporis*. The species deserves attention since it has the pathogenic potential to endanger Southeast Asian eel cultures and it presents a possible threat to eel cultures everywhere. A parallel can be seen in the example of the nematode *Anguillicola crassus* introduced to Europe.

**MATERIAL AND METHODS**

Material for this study was obtained from experimental infections and the following steps were taken:

**Purification of spores for experimental infection.**

Eels showing the 'curved body', physical symptoms of 'beko disease', were immobilized by ice and the infected muscle was removed and digested in 0.05% trypsin in phosphate buffer solution (PBS) at pH 8.0 for 3 to 4 h with constant stirring. The digests were then filtered through 5 layers of gauze, and the filtrate was centrifuged at 800 g for 10 min. The precipitate was discarded and the supernatant was centrifuged again at 3000 × g for 30 min. The precipitate was resuspended in PBS to a final spore concentration of 10⁹ ml⁻¹. Spore purification was done by Percoll gradient centrifugation as described in T'sui et al. (1988). For experimental infection, the spore concentration was adjusted to 10⁷ ml⁻¹.

**Experimental infection.** The eels (0.15 g each) were immersed in 100 ml of spore suspension solution (10⁷ ml⁻¹) at 25°C for 12 h and then cultured in aquaria in the laboratory and fed with *Tubifex* sp. for 20 to 23 d. Most of the infected elvers showed the obvious 'curved body' physical symptoms of 'beko disease'.

**Electron microscopy.** The elvers were sacrificed and the infected tissues were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, at 4°C for 3 h and then postfixed in 1% osmium tetroxide in the same buffer at 4°C for 2 h. After dehydration in an alcohol gradient series, the infected tissues were embedded in 0.1 M phosphate buffer, pH 7.2, at 4°C for 3 h and almost touch it with their frayed ends (Fig. 12). There are only a few meronts in the SPC, but these divide to produce additional meronts and steadily give rise to new sporonts.

During the transition of uni- or binucleate meronts to sporonts, the dense substance at the surface of the meronts gradually increases in volume (Fig. 4) and the newly accrued substance forms conspicuous, thick, irregular projections (Fig. 5). Later, the tops of the projections flatten and the plasmalemma bears a coat of uniformly thick, amorphous matter, the actual SPV wall. The next step is detachment of the sporont plasmalemma from the SPV wall (Fig. 6). The space thus formed is filled with a fine granular substance. The sporont which has stacks of endoplasmic reticulum in the cytoplasm, its nuclei having divided (Fig. 8), segments into separate sporoblasts (Figs. 7 & 9). The SPV wall may undergo further differentiation (Fig. 10); beneath an amorphous dense lamella 30 to 40 nm thick there is a layer of less dense material 90 to 210 nm thick, delimited towards the outer lamella by a rippled boundary.

The dense wall of the sporophorocyst has rather fuzzy surfaces and on the outer side it is contiguous with the layer of disintegrated sarcoplasm. The muscle fibres may run parallel to the SPC wall (Fig. 11), or their microfibrils are positioned perpendicularly to it and almost touch it with their frayed ends (Fig. 12). The immature spore contains 2 large dense globules (about 1.5 to 2 µm in size) in the space of the future posterior vacuole (Fig. 13); these globules are considered to be identical with the secretion granules associated with the Golgi which are also found in other microsporidian spores. Mature spores have a huge posterior vacuole filled with dense floccular material and encircled by turns of the coiled polar tube. There are 33 to 46 (mean 40, n = 30) turns (in what can be termed macrospores) arranged in a single layer at midspore length and in 3 irregular superimposed layers in the posterior half of the spore (Fig. 15). The anchoring disc of the polar tube is situated terminally and has a typical appearance, with 7 alternating dense and lucent layers at the bottom above the thick shaft of the...
tube. The substance of the disc forms a cylinder which extends downward for some distance in between the lucent walls of the proximal end of the tube (Fig. 14). Flat polaroplast lamellae, consisting of 2 layers, are densely spaced around the proximal straight end of the tube. Posteriorly, the lamellae are looser and undulated. In the cytoplasm between the posterior vacuole and the polaroplast a single nucleus and a few polyribosomal strand lie sidewise. The endospore averages 180 nm in thickness at mid-body length and about 70 nm at the apex (Fig. 15).

The possibility of autoinfection must be considered since occasionally one can encounter extruded polar tubes discharged into the neighbouring muscle fibres (Fig. 17).

In the heavily invaded muscle tissue the SPCs are eventually disrupted and macrophages ingest the released spores and are virtually full of them. They migrate into various body tissues including the skin, which they pervade, extending up to the outermost layers of epidermis (Fig. 16). There they can easily break open and the spores are thus released into the environment while the host is still alive.

**DISCUSSION**

Following incomplete observations on spores of *Pleistophora anguillarum*, as the parasite was called at that time, by Hashimoto & Takinami (1976), a more thorough study of the parasite was undertaken by T’sui & Wang (1988). Using a light microscope, they were able to describe early stages which they called uninucleate schizonts and uninucleate sporonts. The early stages produced an external cyst wall and underwent sporogony. Sporonts gave rise to multinucleate plasmodium and these structures were observed to some extent with the electron microscope (T’sui & Wang 1988). Inside the cyst, they observed sporophorous vesicles (‘pansporoblasts’ in their terminology) in different stages of development. No details on the developing sporonts and ultrastructure of the cyst...
Further split into at least 3 genera: *Pleistophora*, *Heterosporis* and another genus for the ‘pleistophoras’ infecting gonads such as *Pleistophora mirandellae* (M. Pekkarinen & J.L. unpubl. obs.).

Lom et al. (1989) came to the conclusion that the SPC wall around the small early meronts of *Heterosporis schuberti* was of parasite origin and the fact that the later stages were completely identical with those observed in the present study constitutes the evidence that the same origin of the SPC wall may be presumed in *H. anguillarum*. The actual genesis of the wall in the earliest stages of infection will be the topic of the next study.

Hung et al. (1998) compared sequence data of SSU rRNA of *Heterosporis anguillarum* with those of several other microsporidia and found that the sequences were most similar to *Vavraia oncoperae* and cautioned that the low mean distance values between the sequences of *V. oncoperae* and *Trachipleistophora hominis* should be satisfactorily explained before *H. anguillarum* can be ultimately assigned to the genus *Heterosporis*. To explain the proximity of *V. oncoperae* and *T. hominis*, Cheney et al. (2000) offer the possibility that both species may perhaps be congeneric: a *Vavraia* species from a haematophagous insect feeding on man may be able to initiate a human *Trachipleistophora* infection. Cheney et al. (2000) also report that the *Pleistophora typicalis* clade is only distantly related to *Heterosporis* (‘*Pleistophora*’) *typicalis*, the sequence divergence being >11.5%. Nilsen (2000) also tested microsporidian affinities using the SSU rRNA sequence data of a number of genera and species. He found that with the exception of *Nucleospora*, all fish microsporidia occurred in the same group and concluded that *Heterosporis anguillarum* does not belong to the genus *Pleistophora*. Nilsen’s (2000) results support the earlier proposed transfer (Lom et al. 1989) into the genus *Heterosporis*. It is a sister taxon to *P. mirandellae* (probably also not a true *Pleistophora*), and they are both separate from the clade comprising *P. typicalis* and related pleistophorans. Hence, both morphological and molecular evidence coincide and support the assignment of the former *Pleistophora anguillarum* into the genus *Heterosporis*. 

**Figs. 2 to 9. Heterosporis anguillarum.** Figs. 2 & 3. Meronts of *H. anguillarum* (M) with 1 or 2 nuclei (n) wedged between sporophorous vesicles (SV), sporonts (Ss) and sporophorocyst wall (arrowhead). Fig. 4. The dense substance (arrows) on the plasmalemma of nascent sporonts (Ss); arrowhead: sporophorocyst wall (W). W: wall of the sporophorous vesicle. Fig. 5. Projections of dense substance on the surface of the sporont-to-be (Ss); arrowhead: sporophorocyst wall. Fig. 6. Plasmalemma (small arrows) of the sporont detached (asterisk) from the sporophorous vesicle wall (W). Fig. 7. A young sporophorous vesicle with developing sporoblasts (Sb). Fig. 8. A binucleate sporont with a stack of endoplasmic reticulum (arrow). Fig. 9. Sporophorous vesicle with 3 cells destined to divide further into sporoblasts. Scale bars = 2 μm, except in Fig. 4 (scale bar = 0.5 μm) and Fig. 7 (scale bar = 5 μm).
Figs 10 to 17. *Heterosporis anguillarum*. Fig. 10. Two apposed sporophorous vesicle walls each with a rippled boundary (arrows) beneath the outer dense lamella (arrowheads). E: endospore of the adhering spore (S). Scale bar = 0.5 µm. Fig. 11. Sporophorocyst wall (arrow) adhering to parallel muscle fibrils (F). Scale bar = 2 µm. Fig. 12. Sporophorocyst wall (arrow) with muscle fibrils (F) attached perpendicularly. Arrowheads: wall of sporophorous vesicle. Scale bar = 0.1 µm. Fig. 13. Dense globules (asterisk) inside an immature spore. Scale bar = 2 µm. Fig. 14. Anchoring disc at the spore apex; asterisk: lucent layer of the shaft of the polar tube. R: polaroplast, E: endospore, arrows point at exospore. Scale bar = 0.1 µm. Fig. 15. Longitudinal section through a mature spore; asterisk: posterior vacuole. Scale bar = 1 µm. Fig. 16. Macrophages packed full with spores (arrows) on their way to and through the epidermis (ES) surface. Q: corium. Scale bar = 50 µm. Fig. 17. Extruded polar tube extending through the muscle fibre. Scale bar = 0.5 µm.

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