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Ultrastructure and Development of *Ceratomyxa protopsettae* Fujita, 1923 (Myxosporea) in the Gallbladder of Cultured Olive Flounder, *Paralichthys olivaceus*

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Summary. Spore morphology and sporogenesis process of *Ceratomyxa protopsettae* Fujita, 1923 found in the gallbladder of cultured olive flounder, *Paralichthys olivaceus* from southern Korea were studied by light- and transmission electron microscopy. Crescent-shaped mature spores were 11.64 ± 0.95 µm in length, 46.63 ± 5.8 µm in width (in sutural view). Two equal sized polar capsules were spherical, 4.15 ± 0.34 µm in diameter and each contained a polar filament with five to six turns and an opening at the anterior end. A binucleate sporoplasm was distributed unequally in the spore cavity. There were numerous pinocytotic invaginations, and pseudopodia or rhizoid-like projections at the peripheral portion of trophozoites. Plasmodium was proliferated by endo- and exogenous budding. Asynchronous divisions of generative cells without pansporoblast formation give arise to two or more than spores within the trophozoite. Capsulogenic cells in the sporoblast had large amounts of rough endoplasmic reticulum, external tubules, and capsular primordia. Histologically, vacuolization and hyperplasia of the epithelium were seen in sections of the gallbladder of parasitized fishes.

Key words: *Ceratomyxa protopsettae*, Myxosporea, sporogenesis.

INTRODUCTION

The myxosporean parasite *Ceratomyxa protopsettae* was described from the gallbladder of 10 wild flatfish species including olive flounder, *Paralichthys olivaceus*, in Japan (Fujita 1923). However, the original description of *C. protopsettae* lacks adequate data regarding the development, and only line drawings of the spore using light microscopy are available.

Olive flounder is a successfully cultured, commercially valuable species in Korea. Recently, we found a high prevalence of *C. protopsettae* in the gallbladder of cultured olive flounder in Korea. In the present study, we investigated the sporogenesis and histopathology of *C. protopsettae* in the gallbladder of olive flounder using light and transmission electron microscopy (TEM).

MATERIALS AND METHODS

One hundred juvenile olive flounder, *Paralichthys olivaceus* (10-15 cm in body length) were obtained from a commercial farm in southern Korea. From each fish, a drop of bile fluid from the gallblad-

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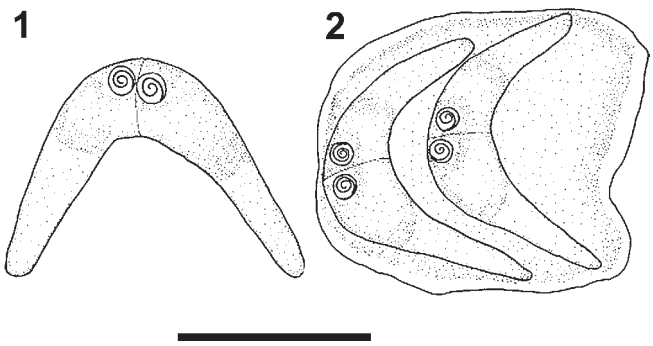
der was smeared on a slide, air-dried and stained with Diff-Quik (International Reagents Co., Japan). Mature spores were observed under a differential-interference-contrast (DIC) microscope and measured using an ocular micrometer and image analysis software (ImageTool ver 2.0, UTHSCSA, USA) according to Lom and Arthur's (1989) criteria. Mean and standard deviations of each spore dimension were obtained from 150 fresh mature spores. Developmental stages were drawn from Diff-Quik stained specimens using a camera lucida.

For histological study, semithin sections were obtained from small pieces of gallbladder fixed in 2% glutaraldehyde, embedded in Spurr resin and stained with toluidine blue.

For TEM study, a small portion of the gallbladder tissue was fixed in 2% v/v glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C overnight and postfixed in 1% w/v cacodylic OsO_4 for 2 h. The specimens were dehydrated, embedded in epoxy resin (Spurr) and ultrathin-sectioned, stained with uranyl acetate and lead citrate, and examined in a JEOL JEM1200 transmission electron microscope (JEOL LTD., Japan).



Figs 1, 2. *Ceratomyxa protopsettae* from frontal view. **1 -** mature spore; **2 -** disporic trophozoite. Scale bar 20 µm.

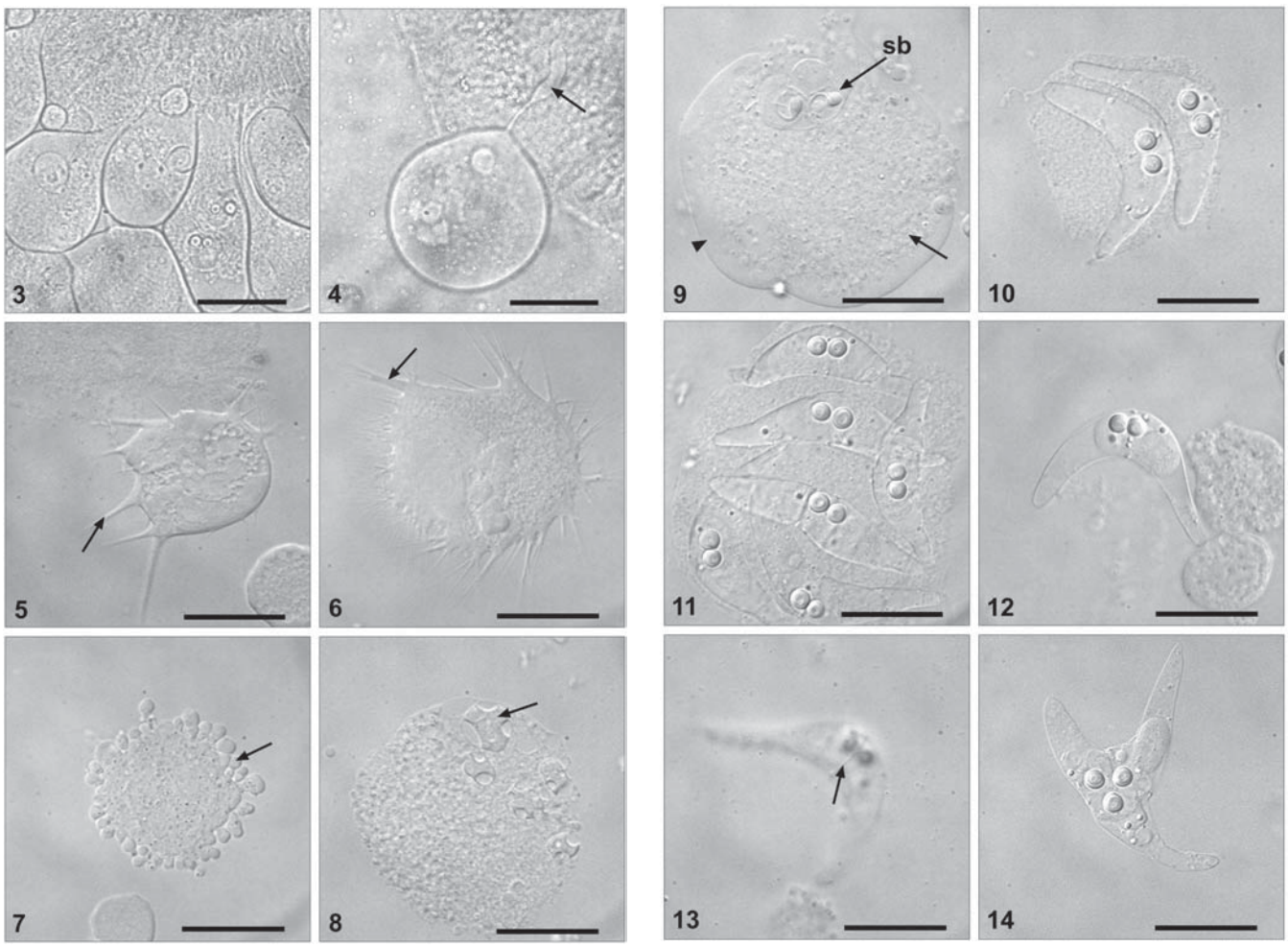
RESULTS

Spore. Mature spores (Figs 1, 12) were crescent-shaped with round or blunt ends, extremely elongated to the sutural line, and measuring 11.64 ± 0.95 µm in length, 46.63 ± 5.8 µm in width in sutural view. Two smooth valves were highly flexible and unequal in size, adhering together along the sutural line of the spore (Fig. 13). Two polar capsules were spherical and almost equal in size (4.15 ± 0.34 µm in diameter), apposed near the suture line, and each contained a polar filament with 5-6 coils, and an apical opening was present at the anterior end (Fig. 45). A binucleate sporoplasm filled the spore cavity and was generally distributed asymmetrically (Fig. 12). Occasionally, aberrant spores with 3 polar capsules and 3 valves were found (Fig. 14). Immature spores in disporic (Figs 2, 10, 24) or polysporic trophozoite (Fig. 11) were surrounded by remnants of the envelope.

Vegetative form. Trophozoites were freely floating in bile or lodged on the epithelium of the gallbladder. Trophozoites shape varied and included amoeboid or rounded forms (Figs 3-9). They had numerous pseudopodia at their peripheral portion, and a great variety of morphology in their pseudopodia was observed (Figs 5, 6). In attached trophozoites, short or long finger-like projections penetrated between the gallbladder epithelial cells (Figs 3, 4, 48). Enlarged trophozoites had finely granular endoplasm and transparent exoplasm (Figs 9, 18, 19, 23). Plasmodia were proliferated by endogenous or exogenous budding without destroying the integrity of mother plasmodia (Figs 7, 8, 28).

Presporogonic phase. Based on fresh (Figs 9-12), Diff-Quick stained (Figs 15-30) and TEM (Figs 31-46) observations, a hypothetical sporogenesis process was constructed (Fig. 47). The earliest stage found was amoeboid or spherical, with the primary cell containing a nucleus (Figs 31, 47a). The nucleus divided into a vegetative nucleus and a generative one (Figs 15, 34, 47b). The vegetative nucleus was situated freely in the cytoplasm of the primary cell, whereas the generative nucleus was surrounded by its own cytoplasm and appeared as an independent cell (secondary cell). In the ultrastructure, cytoplasmic extensions and long mitochondria were well developed at the peripheral portion of these cells (Figs 36, 38). In later developmental stages, the nucleus and cytoplasm of the secondary cell were enlarged, and the generative cell divided into 2 secondary cells (Figs 16, 17, 47c, d). After a generative cell divided once, the resulting 2 secondary cells developed directly into a di-sporogonic phase (Figs 19-24, 47g-l) or subsequently divided into further generative ones and developed into a poly-sporogonic phase (Figs 25-30, 47g'-j'). As a result of internal cleavage of the secondary cells, one or two tertiary daughter cells were produced in each secondary cell (Figs 39, 40). In TEM observations (Figs 34-36, 39, 40), the cytoplasm of inner generative cells (secondary or tertiary cell) was more electron dense than those in the mother cell (primary cell).

Sporogonic phase. Two or 3 secondary or generative cells were closely associated with each other (Figs 35, 37). These cells were surrounded by a common membrane, without surrounding pericyte or vacuole (Fig. 38). These cell aggregates give rise to sporoblast



Figs 3-14. Light micrographs of fresh preparations of *Ceratomyxa protopsettae*; **3, 4 -** attached trophozoite on the wall of gall bladder, note pseudopodial projections (arrow); **5, 6 -** irregular form with numerous pseudopodia (arrow); **7 -** exogenous budding (arrow); **8 -** endogenous budding (arrow); **9 -** a sporoblast (sb) in trophozoite (arrow - finely granulated endoplasm, arrowhead - transparent ectoplasm); **10 -** disporic trophozoite; **11 -** polysporic trophozoite; **12, 13 -** mature spore (arrow - suture line); **14 -** abnormal spore with 3 valves and 3 polar capsules. Scale bar 20 µm.

for spore formation (Figs 9, 21-23, 29). Maturation of spores in a primary cell was asynchronous (Fig. 21). In the disporic phase, there are two sporoblasts, each consisting of two valvogenic cells, one binucleate sporoplasmic cell, and two capsulogenic cells (Figs 41-44, 47i-k). The polysporic phase followed the similar pattern to that described for disporic ones (Figs 25, 26, 29, 47g'-j'). The cytoplasm of capsulogenic cells contained a capsular primordium or several external tubules as well as numerous ribosomes and rough ER (Figs 41-43), and often had cytoplasmic invaginations closely associated with sporoplasmic extensions (Fig. 43). Three differential layers in electron density were present in the capsule (Fig. 44). Almost fully matured polar capsules were subspherical and contained 5 to 6 turns of a polar filament with a globular apical opening for filament discharge (Fig. 45). Two valvogenic cells completely enveloped both capsulogenic and sporoplasmic cells and became flattened as the spore matured (Figs 41-44).

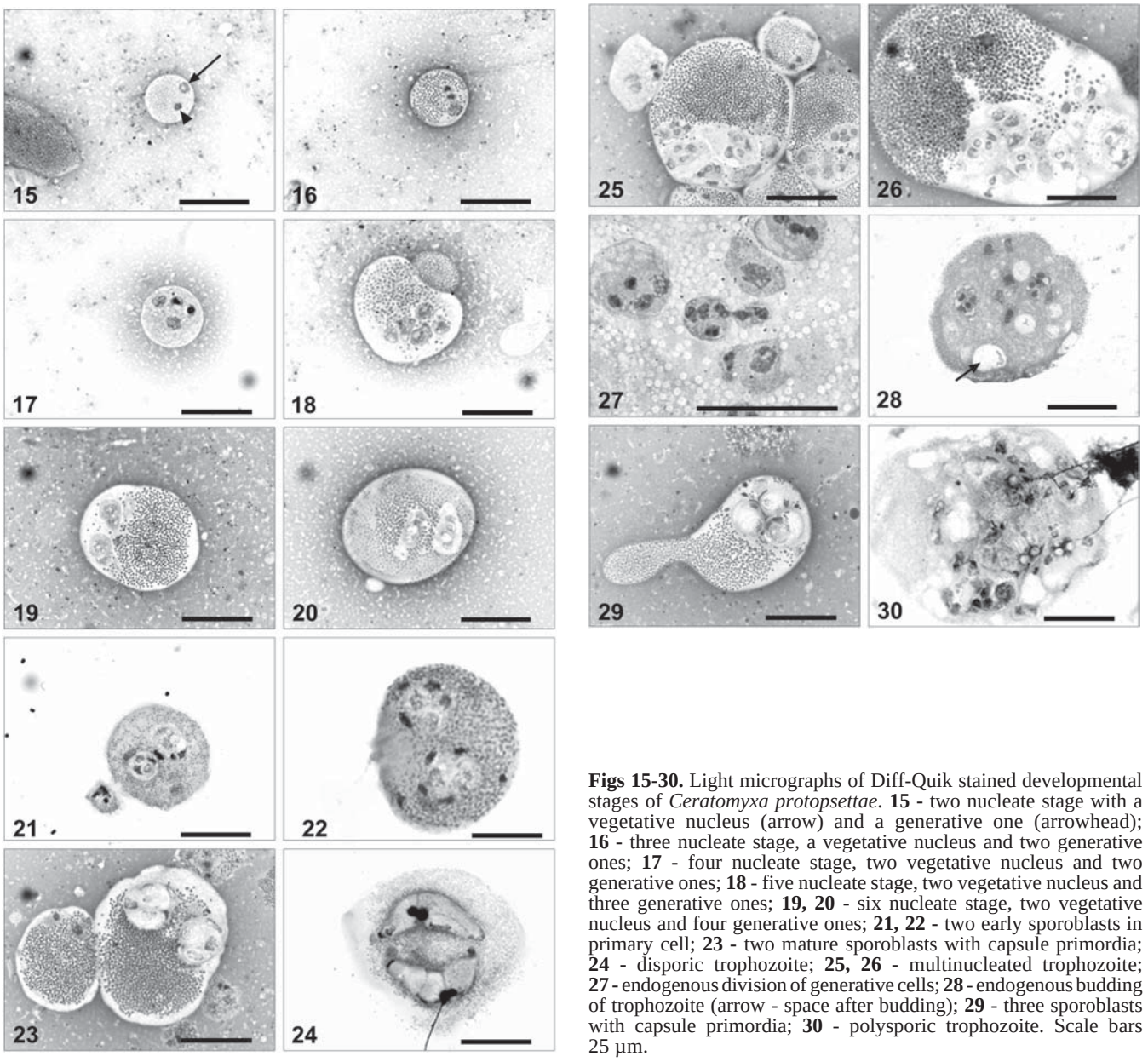
Histology. Various developmental stages were found on the epithelium of the gallbladder. In heavily infected fish, various vegetative stages of the parasite covered the entire surface of the wall of the gallbladder, inducing hyperplasia and vacuolization of the base of epithelial cells (Figs 48, 49).

Host: Olive flounder, *Paralichthys olivaceus.*

Locality: Kampo, Kyongsangbuk-Do, South Korea.

Site of infection: Lumen and wall of the gallbladder.

Prevalence: 100% (100 fish infected/100 fish examined).

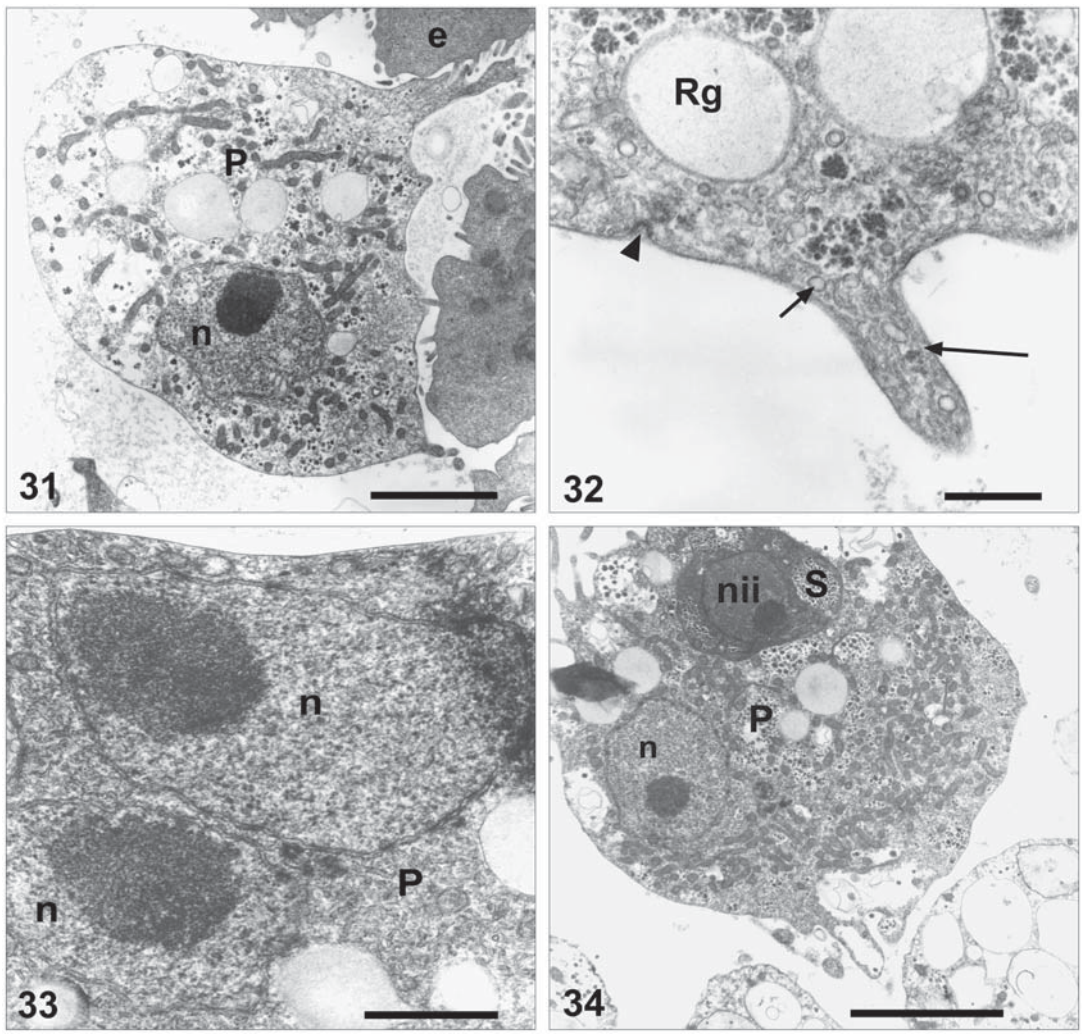


Figs 15-30. Light micrographs of Diff-Quik stained developmental stages of *Ceratomyxa protopsettae*. **15 -** two nucleate stage with a vegetative nucleus (arrow) and a generative one (arrowhead); **16 -** three nucleate stage, a vegetative nucleus and two generative ones; **17** - four nucleate stage, two vegetative nucleus and two generative ones; **18 -** five nucleate stage, two vegetative nucleus and three generative ones; **19, 20 -** six nucleate stage, two vegetative nucleus and four generative ones; **21, 22 -** two early sporoblasts in primary cell; **23 -** two mature sporoblasts with capsule primordia; **24 -** disporic trophozoite; **25, 26 -** multinucleated trophozoite; **27 -** endogenous division of generative cells; **28 -** endogenous budding of trophozoite (arrow - space after budding); **29** - three sporoblasts with capsule primordia; **30** - polysporic trophozoite. Scale bars 25 µm.

Materials deposited: Diff-Quik stained slides; H&E stained histological sections; 90% alcohol-fixed spores. Laboratory of Fish and Shellfish Parasitology, Department of Aquatic Life Medicine, Pukyong National University, South Korea. Accession number PKNU-Pmy-200212.

DISCUSSION

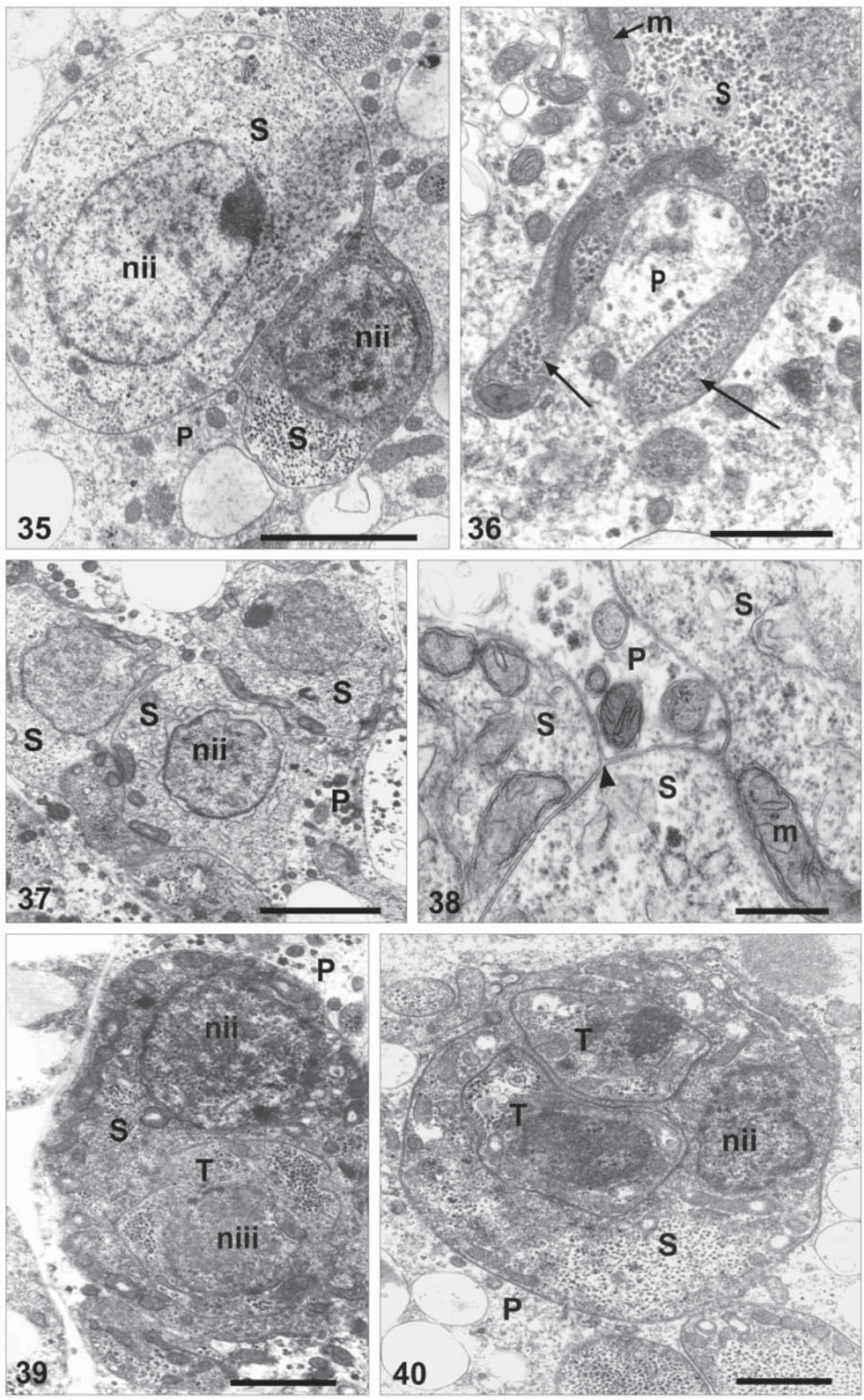
Although the polar capsule size of the present species was somewhat smaller than that of *Ceratomyxa protopsettae* Fujita, 1923 (Table 1), we identified it as *C. protopsettae* on the basis of the same host species and geographical distribution (Lom and Dyková 1992). The general morphology of the *C. protopsettae* spore was typical of the genus *Ceratomyxa*, but aberrant spores with 3 valves and 3 polar capsules were observed occasionally. Sitjà-Bobadilla and Alvarez-Pellitero (1993c) also reported tri-capsular spores of *C. labracis* and *C. diplodae* from wild and cultured sea bass, *Dicentrarchus labrax*. The binucleated sporoplasm has also been reported in other *Ceratomyxa* species - e.g. *Ceratomyxa shasta* (see Yamamoto and Sanders 1979),



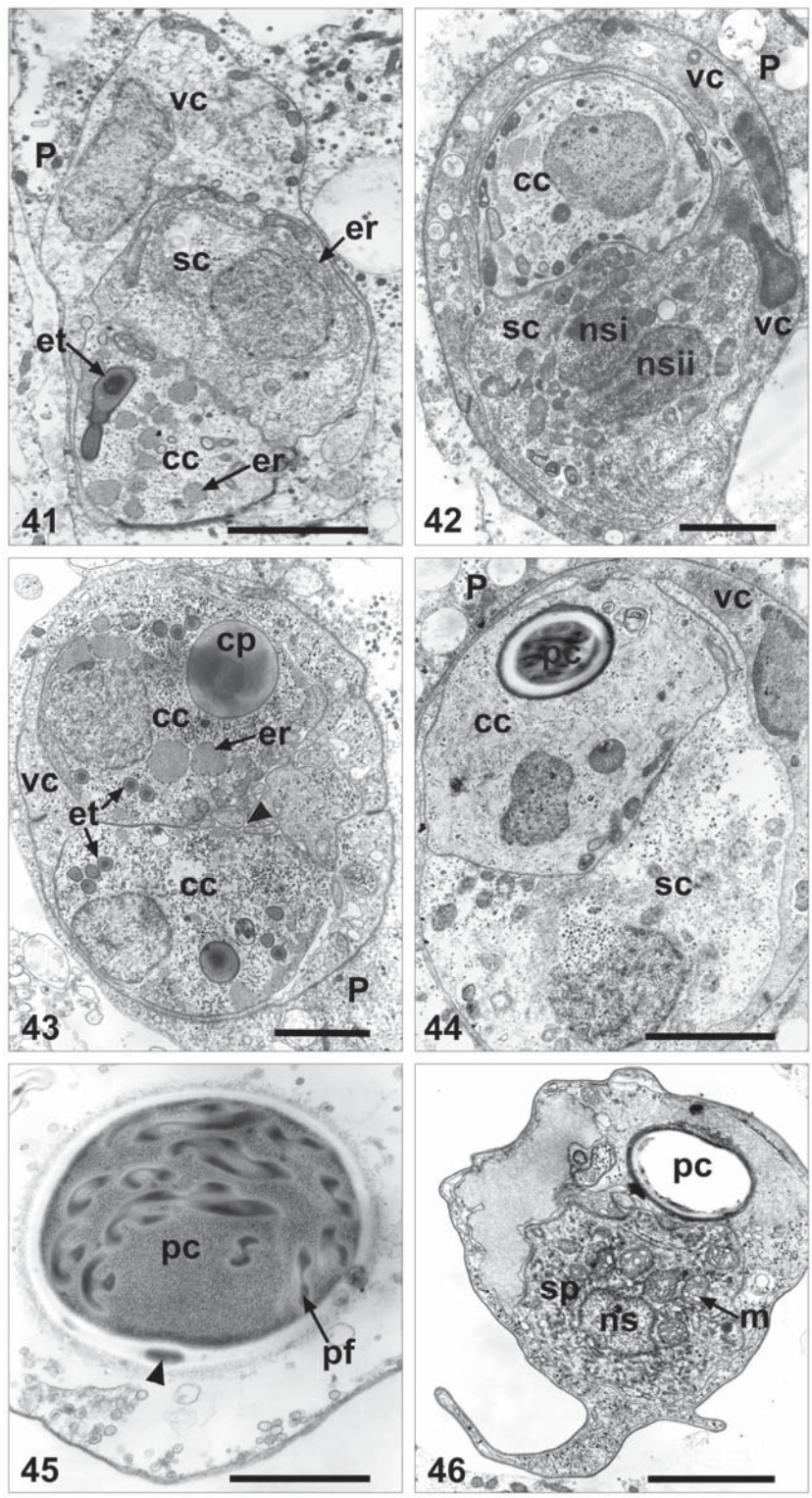
Figs 31-34. Electron-micrographs of early stages of *Ceratomyxa protopsettae*. **31 -** primary cell (P), n - nucleus of primary cell; **32 -** refractive granules (Rg) in endoplasm and, surface projection (long arrow), pinocytotic invagination (arrow head) or pinocytotic vesicle (short arrow) on the surface of trophozoite; **33 -** two vegetative nucleus of primary cell. Note prominent eccentric nucleolus; **34 -** secondary cell (S) within primary cell, nii- nucleus of secondary cell. Scale bars 400 nm (32); 750 nm (33); 1 µm (31); 2 µm (34).

Table 1. Comparison of spore characteristics between original description of Fujita (1923) and the present specimens.

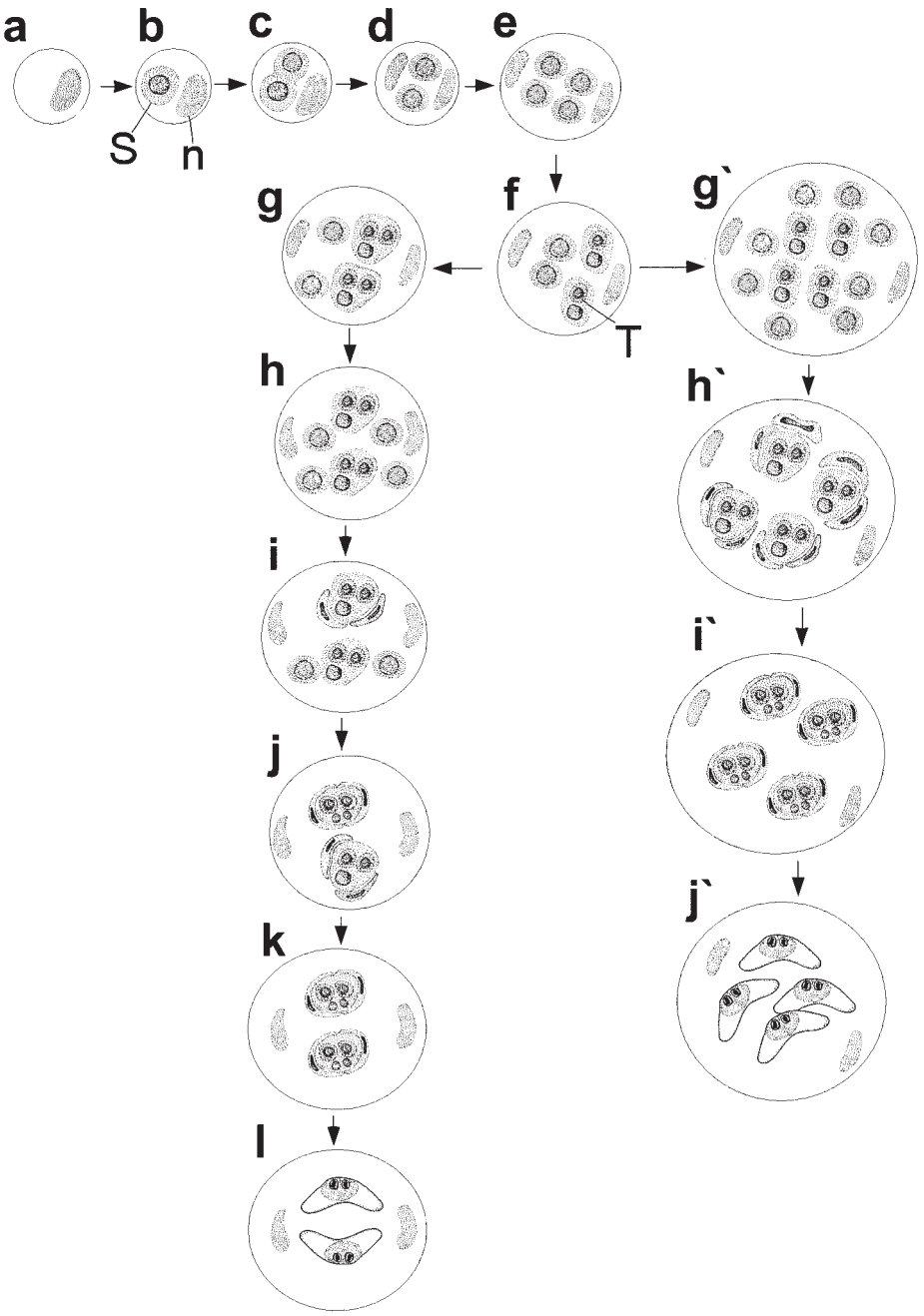
	Fujita, 1923	Present
Spore	unequal valve and variation in curvature of the shell	
Length (µm)	10 ~ 12	11.64 ± 0.95
Width (µm)	12 ~ 13	-
Thickness (µm)	50 ~ 65	46.63 ± 5.8
Polar capsule	two large and ovate	two subspherical
Length (µm)	6	4.15 ± 0.34
Breadth (µm)	4	-
Polar filament	-	5 ~ 6 coiled
Sporoplasm	asymmetrically distributed and binucleated	
Host	*Paralichthys olivaceus*	
Organ	gallbladder	
Geographical location	Hokkaido, Japan	East Sea, South Korea



Figs 35-40. Electron-micrographs of presporogonic stages of *Ceratomyxa protopsettae*. **35 -** secondary cell (S) associated with other one, nii - nucleus of secondary cell, P- primary cell or plasmodium; **36** - cytoplasmic extensions (arrow) of secondary cell, m- mitochondria; **37 -** association of three secondary cells; **38 -** magnification of three secondary cell surrounded by common membrane (arrowhead). There were no surrounding vacuoles around generative cells; **39 -** secondary cell containing a tertiary cell (T); **40 -** secondary cell containing 2 tertiary cells. Scale bars 400 nm (38); 500 nm (35); 800 nm (36); 1 µm (39, 40); 2 µm (37).



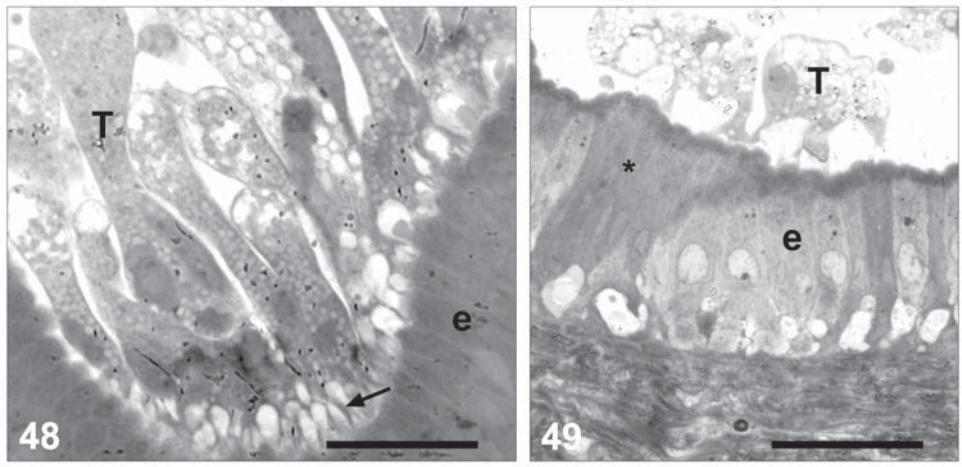
Figs 41-46. Electron-micrographs of sporogonic stages of *Ceratomyxa protopsettae*. **41 -** early sporoblast consists of capsulogenic cell (cc), sporoplasmic cell (sc) and valvogenic cell (vc), et - external tubule, er - endoplasmic reticulum, p - primary cell, sc - sporoplasmic cell, vc - valvogenic cell; **42 -** two valvogenic cell completely surrounding a binucleate sporoplasmic cell and capsulogenic cell. nsi, nsii - nucleus of sporoplasmic cell; **43 -** asynchronous maturation of capsulogenic cells, cp - capsular primordia; **44 -** formation of mature polar capsule (pc) within capsulogenic cell. **45 -** apical pore (arrowhead) and polar filaments (pf) of polar capsule of mature spore; **46 -** sporoplasm (sp) of mature spore. Note numerous mitochondria (m) and nucleus (ns). Scale bars 800 nm (45); 1 µm (44); 2 µm, (41-43, 46).



Figs 47a-f. Hypothetical sporogenesis of *Ceratomyxa protopsettae* from gallbladder of olive flounder, *Paralichthys olivaceus*. **a** - earliest stage with a nucleus; **b** - two nucleate stage, S - generative cell or secondary cell, n - vegetative nucleus or nucleus of the primary cell, **c** - three nucleate stage; **d** - four nucleate stage; **e** - six nucleate stage; **f** - eight nucleate stage. Secondary cell harboring inner tertiary cell (T). From subfigure **g** to **l**, each generative cell with inner 2 tertiary cells gives rise to a sporoblast in a primary cell and consequently forming disporic trophozoite (l). In other case (see subfigures **g'-j'**), multiple division of generative cells results in polysporic trophozoite.

C. globulifera (see Desportes and Théodoridès 1982), *C. diplodae* and *C. labracis* (see Sitjà-Bobadilla and Alvarez-Pellitero 1993c).

Free floating trophozoites with long or short needle-like pseudopodia showed sluggish amoeba-like movement, whereas attached trophozoites had rhizoid-like



Figs 48-49. Histological sections of gallbladder of olive flounder, *Paralichthys olivaceus* infected by trophozoites of *Ceratomyxa protopsettae*. Semi-thin sectioned, toluidine blue stained. **48** - trophozoites with numerous rhizoid-like pseudopodia (arrow) firmly attached on the epithelial cell; **49** - heavily infected gallbladder wall with trophozoites showing hyperplasia (asterisk) and vacuolization of epithelial cells. T - trophozoite, e - epithelial cell. Scale bars 25 µm.

projections at one side to strengthen attachment to the gallbladder epithelium. These holdfast projections have also been reported in ultrastructural descriptions of other coelozoic species (Lom *et al*. 1986, Paperna *et al*. 1987, Alvarez-Pellitero and Sitjà-Bobadilla 1993a, Sitjà-Bobadilla and Alvarez-Pellitero 1993b, El-Matbouli and Hoffmann 1994). Multiplication of the trophozoite by endo- or exogenous budding have also been described in trophozoites of *Myxidium liberkuhni* by Cohn (1895), *Sinuolinea dimorpha* by Davis (1916), and *Ceratomyxa blennius* by Noble (1941).

Concerning sporogenesis, *C. protopsettae* showed either a disporic or polysporic pattern. Disporous development has been commonly reported from *Ceratomyxa* species (Fujita 1923, Kalavati and MacKenzie 1999, Yokoyama and Fukuda 2001), whereas disporous to polysporous development was reported also in *C. recurvata* by Davis (1917). Unequal division of generative cells and asynchronous spore formation in *C. protopsettae* were in agreement with those of other myxosporeans (Desser *et al*. 1983; Dyková *et al*. 1990; Sitjà-Bobadilla and Alvarez-Pellitero 1992, 1993a).

Myxosporeans that form polysporic trophozoites (i.e. *Myxobolus, Henneguya, Thelohanellus, Sphaeromyxa, Zschokkella, Myxidium,* and *Hoferellus*) produce spores in a pansporoblast. Pansporoblast formations have been frequently reported in light microscopical descriptions of developmental stages of other *Ceratomyxa* species (Averintsev 1908, 1909; Mavor 1916; Georgévitch 1929; Noble 1941). In the present *C. protopsettae*, internal cleavages of a single generative cell rather than the association of two generative cells gave rise to the sporoblast, indicating no pansporoblast formation. In pansporoblast formation, the membrane of two generative cells persists and the sporogonic cell is enclosed in a tightly fitting vacuole in the pericyte (Lom and Dyková 1992). In the present ultrastructure of generative cells of *C. protopsettae*, reminiscent of a pericyte was not observed. Recently, Sitjà-Bobadilla *et al*. (1995) also reported no pansporoblast formation in *C. sparusaurati*. TEM observations of capsulogenic cells in early sporoblasts of *C. protopsettae* did not reveal the presence of a Golgi apparatus, but high amounts of smooth and rough ER were observed. Therefore, as in previous reports of capsulogenesis (Schubert 1968, Lom 1969), smooth or rough ER seems to be involved in the formation of capsular primordia of *C. protopsettae*.

The parasite induced response in the host was characterized by vacuolization and hyperplastic reaction of epithelial cells. These changes of the epithelial cells resembled that in other gallbladder myxosporean infections (Desportes and Théodoridès 1982; Alvarez-Pellitero and Sitjà-Bobadilla 1993b; Sitjà-Bobadilla and Alvarez-Pellitero 1993b, c).

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