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Amoebic infections in goldfishes and granulomatous lesions

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Abstract. Ninety four aquarium fishes were screened for the presence of amoebae in their internal organs. Five specimens of *Carassius auratus* (L.) and one specimen of *Xiphophorus helleri* Heckel were positive. Of the three strains which were isolated from *C. auratus,* successfully cloned and cultivated, one was identified as *Vannella platypodia* (Gläser, 1912) Page, 1976 and two strains as *Rosculus ithacus* Hawes, 1963. Both species are reported for the first time from organs of fish. None of them could be identified with the amoeba-like agent of goldfish granulomas described here.

Severe infections and mass mortalities caused by free-living amoebae in cultured fishes attract attention to these agents. However, only few data exist to date on amoebiasis in aquarium fishes, although it is a host group composed of a wide array of species from many different habitats and pet fish farms. An expansion of cold water ornamental fish farming as well as imports of tropical aquarium fishes make this research quite topical.

Five cases of spontaneous amoebiasis in goldfishes (*Carassius auratus* (L.)) were described from fish hobbyist's and laboratory aquariums by Voelker et al. (1977). Systemic infections of goldfishes caused by amoebae which were briefly described also by Lom and Dyková (1992), and by Steinhagen et al. (1993) had in common granulomatous inflammatory changes in internal organs. Amoebae were postulated to be the causative agents of lesions by means of transmission electron microscopy in all three mentioned publications. However, the genus and species of amoebae remained always unspecified. Only Voelker et al. (1977) suggested the agent might belong to the family Hartmannellidae. Natural intracranial infections caused by *Naegleria* and *Acanthamoeba* spp. were reported recently in goldfishes which manifested erratic swimming movements (Wilson et al. 1995).

Determination of the prevalence of free-living (or amphizoic) amoebae in organs of aquarium fishes, their identification and definition of their potential as agents of diseases may be of fundamental interest because granulomatous inflammatory changes are the most common among the internal lesions diagnosed in aquarium fishes.

Evaluating histopathology in goldfishes experimentally infected while working on another project we faced the problem foretold by Voelker et al. (1977): multiple granulomatous inflammatory lesions have been continuously found in goldfishes used for experiments as well as in control specimens. Tentative light microscopical determination of amoebae in such granulomatous lesions brought us to the detailed study and screening for the presence of amoebae in other species of aquarium fishes.

MATERIALS AND METHODS

Tissue samples of 94 out of 201 specimens of ornamental cold water fishes and tropical aquarium fishes routinely examined with emphasis on protozoan parasites were tested for the presence of amoebae in fresh state (squash preparations), by means of cultivation on agar plates and histologically.

The assemblage of examined fishes included individual specimens of 28 species (46 specimens), and relatively numerous sample of goldfishes, *Carassius auratus* (48 specimens). In total 29 fish species which belonged to 9 families (13 to Cichlidae, 4 to Cyprinidae, 3 to Characidae, 3 to Poeciliidae, 2 to Belontiidae, 1 to Cyprinodontidae, Centropomidae, Lebiasinidae and Helostomatidae) were examined. The majority of goldfishes (fan-tail forms), used for various experimental purposes, were purchased from the same supplier over a period of several years. They were kept for a short summer season in small garden ponds, the rest of the year always in the same green-house tank. The less numerous group of common goldfishes was bred in thermally polluted water and overwintered in a small pond. All other specimens of ornamental fish were obtained live from pet shops and private hobbyists.

In order to complete existing data on histopathology of granulomatous lesions in goldfishes and to determine the agent, the sampling of goldfishes was extended and revision of stored material was done. In addition to 48 specimens which were tested for the presence of amoebae by means of cultivation and histologically, 317 goldfishes from 4 different populations bred in 4 different years (1981, 1982, 1984, 1986) were examined histologically only.

The technique used for isolation of amoebae followed the method of De Jonckheere (1980). Sterile sampling of liver, spleen, kidney, brain and muscles was followed by inoculation on non-nutrient agar surface. Primary isolates were grown on non-nutrient agar seeded with heat-killed *Aerobacter aerogenes* or *Bacillus subtilis* (Page 1967). Attempts were made to establish axenic cultures of isolated amoebae in three basic fluid media SCGYEM, PPG and BCS (Červa 1969, Kalinina and Page 1992) and to test their ability to grow in fish cell cultures (EPC and FHM cell lines). In order to characterize isolated strains morphological criteria by Page and Siemensma (1991) were applied. All strains of amoebae were tested for production of temporary flagellated forms (Page 1967). Nuclear division was characterized using DNA staining with Hoechst 33258 (Gicquaud and Tremblay 1991).

Transmission electron microscopy (TEM) was used to compare the ultrastructure of agent of granulomatous lesions and trophozoites from cultures on agar plates. Tissue samples and cell suspensions were fixed in buffered 2 % osmium tetroxide and embedded routinely in Epon-Araldite. Sections were double stained with saturated aqueous uranyl acetate followed by lead citrate.

RESULTS

a) Gross lesions and light microscopical findings

Granulomatous inflammatory lesions were found in 189 (59 %) out of 317 examined goldfishes, in internal organs as well as among them, in the connective and adipose tissue. Of the parenchymatous organs, trunk kidney was affected regularly (Figs. 1–3); the presence of changes in other internal organs, in the liver, spleen, and pancreas varied according to intensity of infection. Also brain and heart were found to be affected (Fig. 5). All stages of granuloma formation were observed with slight morphological differences in various organs. The comparison of rich material (189 infected goldfishes), which included also early stages of granuloma formation, revealed amoeba-like organisms as agent of lesions. In the light microscope they were detectable either in the very initial stages of their multiplication in organs (Fig. 4), or on the periphery of early but already concentrically arranged stages of granuloma formation (Figs. 2, 6).

In addition to goldfishes, granulomatous inflammatory lesions were found in organs of 13 out of 46 examined specimens of other species of ornamental fishes. There was no light microscopical indication of the presence of amoebae or amoeba-like organisms at all.

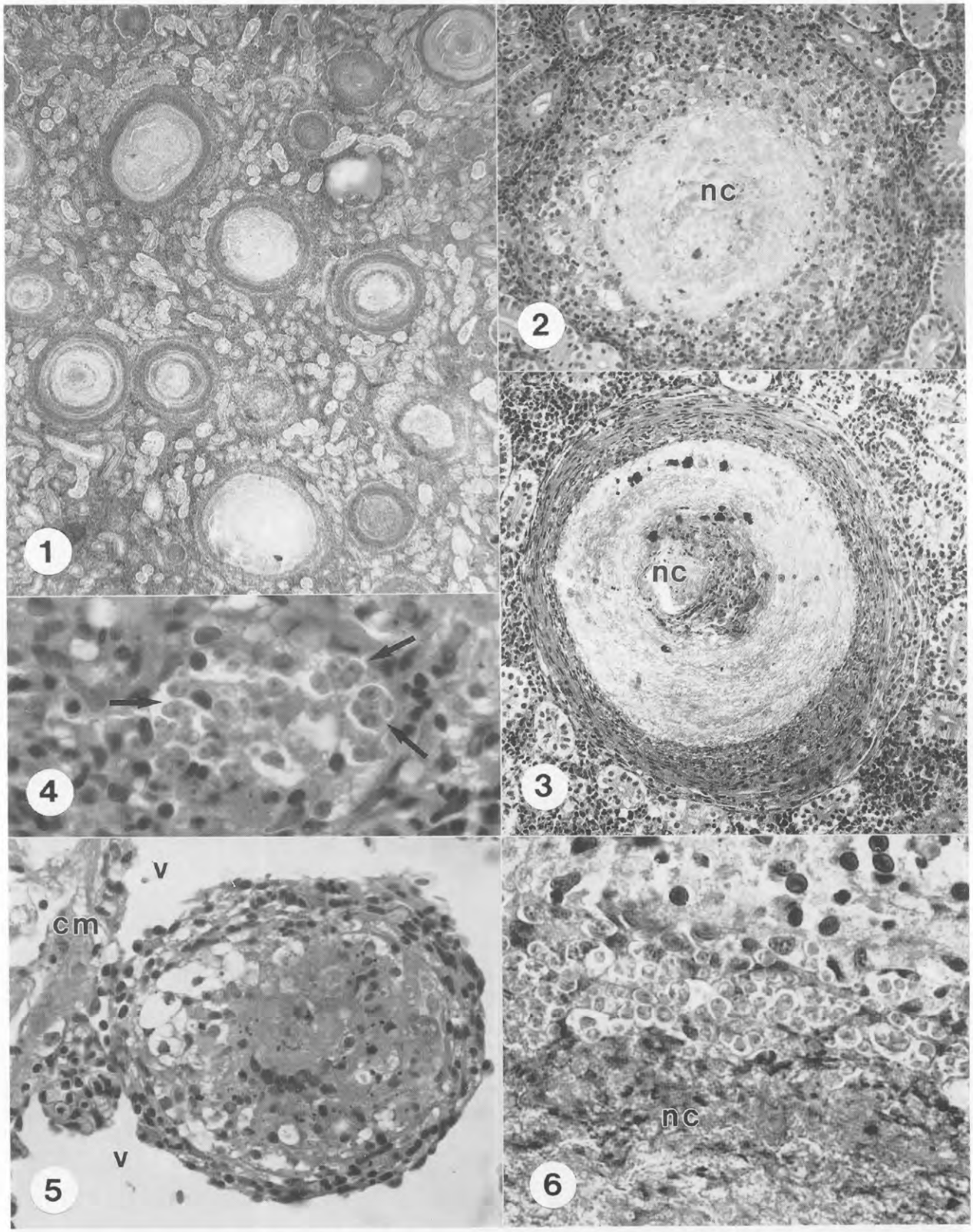
b) Amoebae isolated from internal organs of ornamental fishes

The attempts to establish agar plate cultures of amoebae from goldfishes with grossly visible granulomas as well as from asymptomatic specimens from the same infected stock failed repeatedly. One suspected isolate of this organism in fish cell culture was lost prior to final determination in sub-cultures.

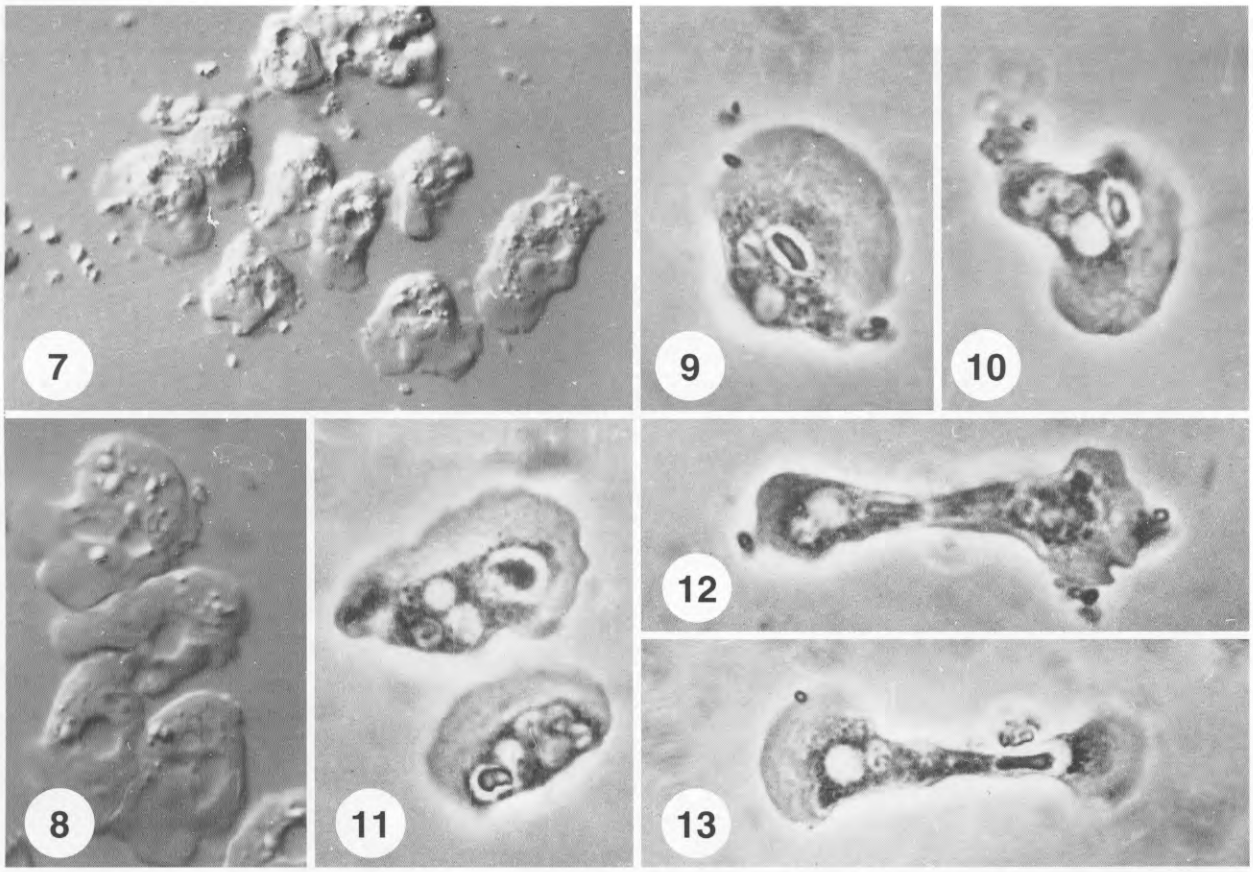
However, during further work, we did isolate amoebae in agar plate cultures from internal organs of five out of 48 goldfishes and from the liver of a single examined specimen of *Xiphophorus helleri* Heckel. Three isolates from *Carassius auratus* (one from common and two from fan-tail goldfish) were successfully cultured and cloned, the strain isolated from *X. helleri* (tentatively assigned to the genus *Acanthamoeba*) was lost.

Strain No. 805 (Figs. 7–21) was isolated from kidney tissue of common goldfish, *Carassius auratus*, 18. 9. 1992. Clone No. 805/XI was studied in detail. Trophozoites were flat, oval or fan-like in shape, the third part or half of locomotion forms was occupied by hyaline ectoplasma (Figs. 7–11). The shape of trophozoites changed rapidly when attaching to the slide. Length (L) of an actively moving amoeba was 19.8 (14–25) μm, breadth (B) 17.8 (12–23) μm, L/B ratio averaged 1.1. Diameter of nucleus was 3.45 (2–5) μm. The rate of amoeba locomotion was 28.8 μm/min. Floating amoebae characterized by 5 to 7 long needle-like pseudopods used to settle very soon. Their pseudopods became shorter and broader and soon disappeared in the hyaloplasm. The strain is maintained by serial sub-culture on agar plates. Axenic culture in liquid medium thus far could not be established, co-cultivation with cell cultures also failed, as did the cultivation at 37°C. Reproduction can be interpreted from Figs. 12–21. From the very beginning of culture no cysts were ever found and no flagellated stages were formed under conditions of flagellation tests. The diagnostic characteristics observed in our strain correspond to the species *Vannella platypodia* (Gläser, 1912) Page, 1976, which was described from fresh water.

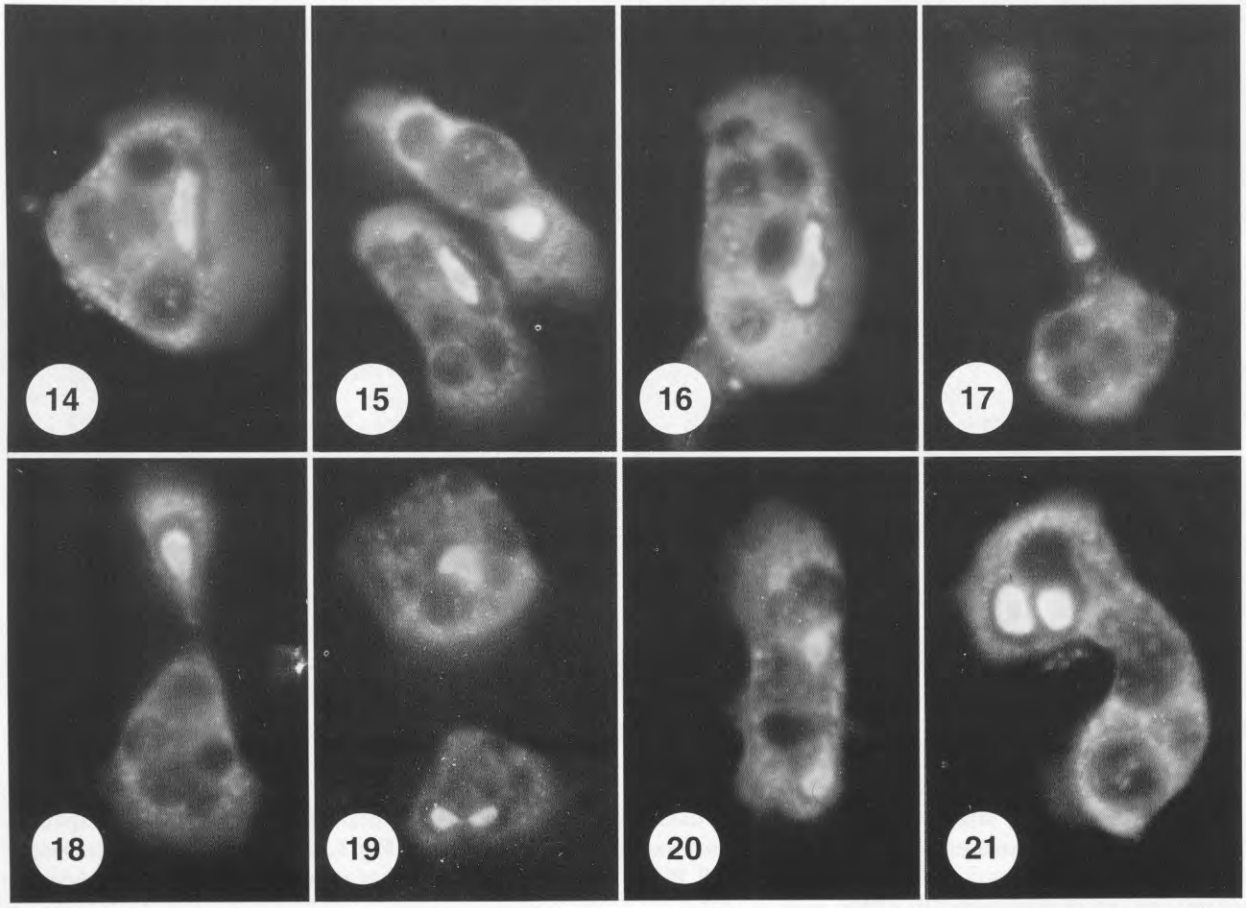
Strain No. 912 (Figs. 22–27) was isolated from the liver of fan-tail goldfish, *Carassius auratus*, 4. 2. 1993. Amoebae in locomotion were flat, oval or fan-like. Their posterior end was narrowed in the digitiform pseudopod. Moving forms changed rapidly their shape



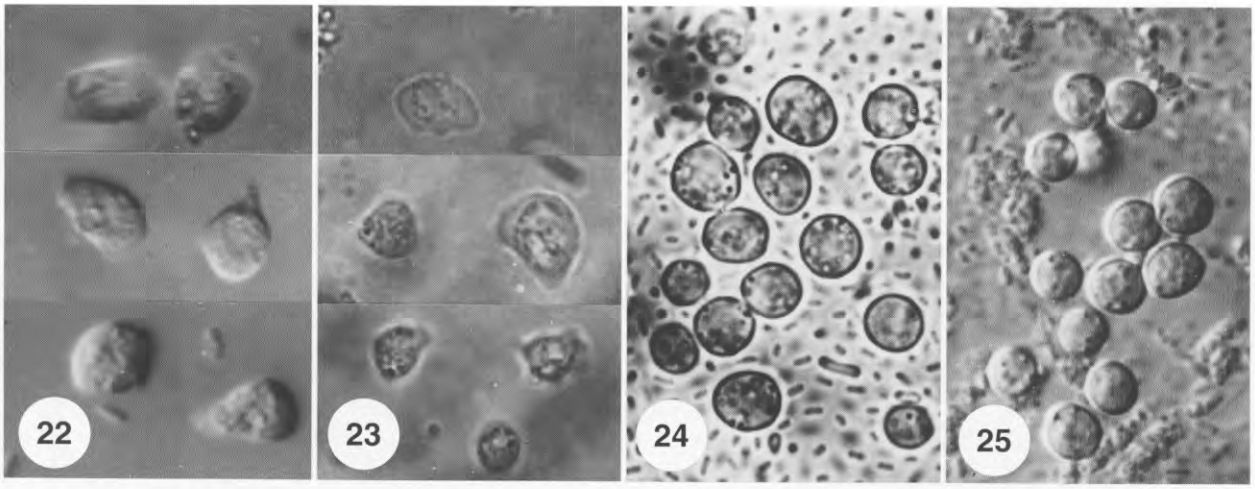
Figs. 1–6. Lesions caused by amoeba-like organisms in organs of goldfishes. **Fig. 1.** Multiple granulomas responsible for considerable extent of damage in the kidney tissue, HE × 45. **Figs. 2, 3.** Two stages of granuloma formation in the kidney tissue. Diameter of the necrotic centre (nc) is large in early stage (Fig. 2, HE × 857) as well as in late stage (Fig. 3, HE × 225) which has a thick concentrically arranged layers of connective tissue on the periphery. **Fig. 4.** Accumulation of amoeba-like organisms (arrows) and slightly developed focal necrosis in an initial stage of infection, HE × 860. **Fig. 5.** Granuloma in the cardiac muscle of goldfish (cm) extending to the ventricle (v), HE × 498. **Fig. 6.** Amoeba-like organisms in a continuous layer surrounding central necrosis (nc) of granuloma, HE × 860.



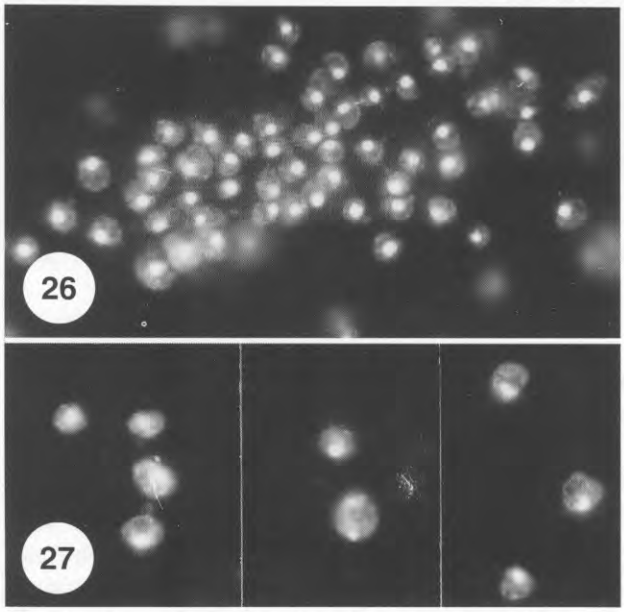
Figs. 7–13. *Vannella platypodia.* **Figs. 7, 8.** Trophozoites observed in Nomarski differential interference contrast, × 1220 (Fig. 7) and × 1345 (Fig. 8). **Figs. 9–13.** Trophozoites stained with Hoechst 33258 and photographed in phase contrast, × 1650.



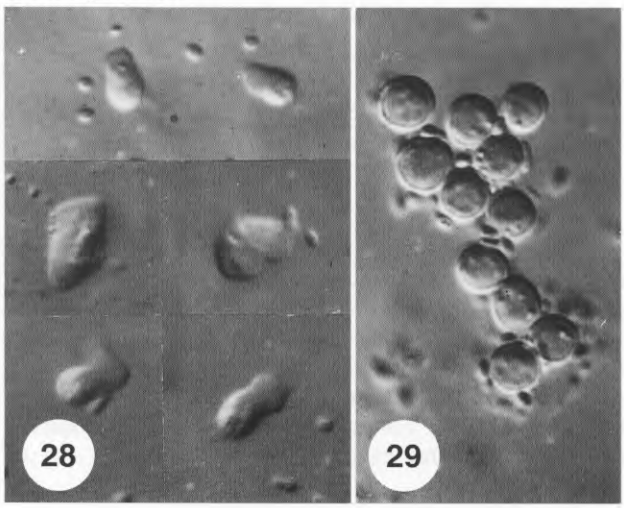
Figs. 14–21. *Vannella platypodia.* Trophozoites stained with Hoechst 33258, observed in epifluorescence microscope, × 1800.



Figs. 22–25. *Rosculus ithacus*, strain No. 912. **Fig. 22.** Trophozoites observed in Nomarski differential interference contrast, × 1580. **Fig. 23.** Trophozoites observed using phase contrast, × 1350. **Figs. 24, 25.** Appearance of cysts in translucent light (Fig. 24, × 2300) and interference contrast image of cysts (Fig. 25, × 2100).



Figs. 26, 27. Trophozoites of *Rosculus ithacus* (strain No. 912) stained with Hoechst 33258 and observed in epifluorescence microscope, × 800.

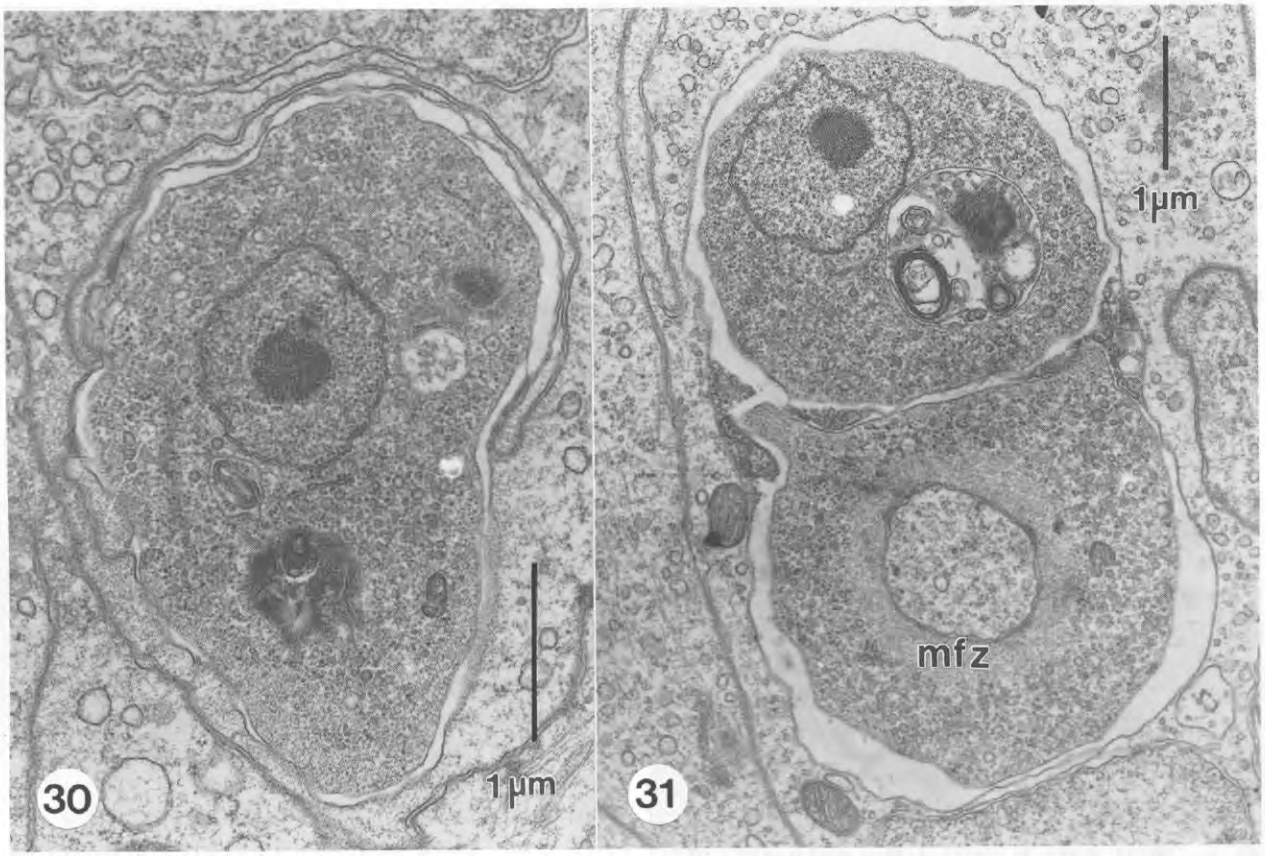


and direction of locomotion, remaining to some extent the eruptive type of trophozoite. Length (L) was 8.23 (5–13) μm, breadth (B) 5.7 (4–8) μm, diameter of nucleus 1.83 (1.5–2) μm, L/B ratio 1.4. The rate of locomotion was 34.6 μm/min. Spherical cysts were smooth-walled (Figs. 24, 25). Diameter of cyst was 3.85 (3–5.5) μm. Flagellation tests were negative, distinctive floating forms were not observed. The incubation at 37°C arrested the growth of trophozoites. The amoeba isolated from goldfish and cultured as a cloned strain No. 912/XII has all morphological and behavioural characteristics of the type species of the genus *Rosculus* Hawes, 1963, *R. ithacus,* established by Hawes (1963), and other strains of the same species described by Page (1974). Transmission electron microscopy of trophozoites washed from the surface of agar plates revealed the presence of mitochondria with discoid cristae (Figs. 34–36) which were almost identical with those documented by Page and Blanton (1985) for Acrasida (*Acrasis rosea* L. S. Olive et Stoianovitch, 1960 and *Pocheina rosea* (Cienkowski, 1873) Loeblich et Tappan, 1961). Cloned strain No. 912/XII is cryopreserved and also maintained by serial sub-culture on agar plates. The attempts to establish axenic culture in liquid media were not successful, inconstant results were obtained thus far in cell culture media.

Strain No. 893 (Figs. 28, 29) isolated from the brain of fan-tail goldfish, *Carassius auratus,* from the Mydlovary fishery (25. 1. 1993) had the characters identical with those of strain No. 912.

Amoebae were also isolated from gills and/or intestine of eleven specimens of ornamental fishes. These

← **Figs. 28, 29.** *Rosculus ithacus* (strain No. 893). Trophozoites (Fig. 28, × 1560) and cysts (Fig. 29, × 1950) observed in Nomarski differential interference contrast.



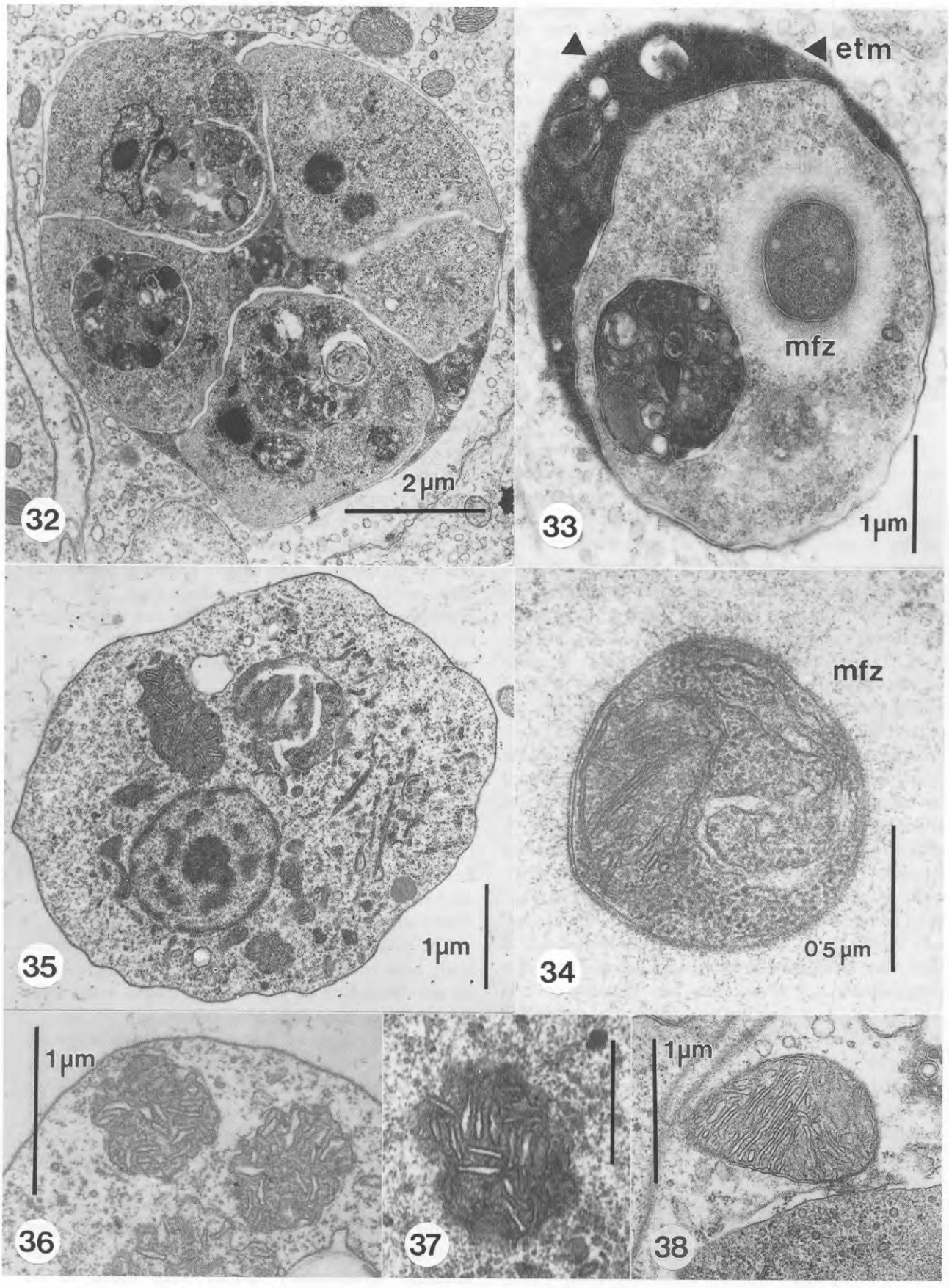
Figs. 30, 31. Ultrastructure of amoeba-like organisms found in granulomatous lesions of goldfishes. **Fig. 30.** Amoeba-like organism with a thin cell membrane located in the parasitophorous vacuole of the host cell contains amorphous electron dense material in the cytoplasm. Bar = 1 µm. **Fig. 31.** Two organisms in the parasitophorous vacuole. One which is sectioned through the nucleus contains a vacuole filled with membrane whorls and myelinated structures, the other one displays endocytotic channel surrounded by a wide microfilamentous zone (mfz). Bar = 1 µm.

findings were not included in the final evaluation because free-living amoebae may be a part of the resident microfauna of gills and intestine and there were no histological signs of multiplication of amoebae in these localizations.

c) **Amoeba-like organisms in goldfish granulomas**

Amoeba-like organisms found in thin sections through the periphery of goldfish granulomas were located intracellularly, in the parasitophorous vacuole (Fig. 30). Sometimes more than two trophozoites were present in one host cell along with expelled trophic material (Figs. 31–33). They were seen to contain more or less spherical nuclei and concentrically located nucleoli, exceptionally small accumulations of material resembling paranucleolar bodies were found on the periphery of irregularly outlined nuclei. Golgi apparatus was present. Vacuoles containing electron dense material, membrane whorls and myelinated structures, obviously of host origin, and vacuole-like structures surrounded by a wide concentric zone of microfilaments, were prominent in the cytoplasm (Figs. 31, 33, 34). The origin of

→ **Figs. 32–34.** Ultrastructure of amoeba-like organisms, agents of goldfish granulomas. **Fig. 32.** Parasitophorous vacuole filled with five amoeboid organisms in a rosette-like arrangement and dense expelled trophic material between them. Bar = 2 µm. **Fig. 33.** Structures characteristic for amoeba-like organisms, vacuole containing electron dense material, endocytotic channel surrounded by a microfilamentous zone (mfz) and expelled trophic material (etm) filling periphery of parasitophorous vacuole. Bar = 1 µm. **Fig. 34.** Endocytotic channel with contents resembling host mitochondrion, zone of microfilaments (mfz). Bar = 0.5 µm. **Fig. 35.** *Rosculus ithacus* trophozoite from the agar plate culture with two mitochondria containing discoid cristae. Bar = 1 µm. **Figs. 36, 37.** Detailed view of mitochondria of *Rosculus ithacus*. Bar = 1 µm for Fig. 36, 0.5 µm for Fig. 37. **Fig. 38.** Host cell mitochondrion. Bar = 1 µm.


2 µm
32
etm
mfz
1µm
33
mfz
0·5 µm
34
1µm
35
1µm
36
37
1µm
38

the latter was recognized due to extensive sectioning in various levels. The same microfilamentous zone was found along the deep invagination with the part of host nucleus incorporated into the cytoplasm of trophozoite, i.e., endocytotic process was observed. Contrary to cultured trophozoites of *Rosculus ithacus* (Figs. 35, 36) we failed to find mitochondria or their remnants in intracellularly located amoeba-like organisms in the goldfish granulomatous lesions. The only discernible remnant of mitochondria was found in endocytotic channel (Fig. 34) and its fine structure corresponded to mitochondria of the host cell (Fig. 38). The size range was the same in both amoeba-like organisms and *R. ithacus* trophozoites.

DISCUSSION

Free-living protozoans that may survive or cause disease in man or animals have been termed "amphizoic" (Page 1974), to distinguish them from obligate parasites. Hawes (1963) proposed the name *Rosculus ithacus* for a small amoeba cultured from the rectum of grass snake, *Natrix natrix* (L.). It was recognized as an amphizoic species because it could be reintroduced into the rectum of the host snake from which it was originally isolated, and then be re-established in laboratory cultures. The taxonomic position of the genus *Rosculus* suggests that it is related to the myxamoebae of slime molds, order Acrasida, rather than to amoebae of the order Euamoebida (Page 1988). Both Hawes (1963), and Page (1974, 1988) noted that these amoebae lost the ability to form cysts and that cultures easily were lost if not subcultured at frequent intervals. Both authors also noted the cannibalism as another characteristic of *R. ithacus*. Similarly, the slime molds *Echinostelium* (Haskins 1968), and *Guttulinopsis* E. W. Olive, 1901, were found to lose ability to form "fruiting bodies" upon continuous cultivation (Olive 1965). Another feature shared by *R. ithacus* and certain slime molds is their very rapid growth when subcultured. Fresh agar plate cultures of *R. ithacus* may be completely covered with amoebae, resembling an epithelial sheet, within 3–4 days. Shipley et al. (1985) reported similar behaviour with myxamoebae of *Physarum polycephalum*. Page and Blanton (1985) studied the ultrastructure of *R. ithacus*, noted that mitochondria were discoid rather than tubular, and placed the amoebae in the slime mold order Acrasida Schröter, 1886, family Guttulinopsidae Olive, 1901. The family Guttulinopsidae was established for spore-forming myxamoebae that were flattened or expanded with a constantly changing shape ranging from triangular to fan-shaped or asymmetrical. Rapid motility by the myxamoebae often rendered them longer than broad in one moment with an almost instantaneous change to broader than long. The rapid change in shape and small size of the myxamoebae belonging to the genus *Guttulinopsis* was shared by *R. ithacus*. Thus Hawes (1963) measured specimens only in the "rounded form", while Page (1974) measured them in terms of their "greatest dimension". Page and Blanton (1985) considered flattened or discoid mitochondria as well as other characteristics to be sufficient justification for including the genus *Rosculus* in the spore-forming order Acrasida. They established the class Heterolobosea to accommodate the order Schizopyrenida Singh, 1952 and the order Acrasida. Well known genera of amoebae such as *Vahlkampfia* Chatton et Lalung-Bonnaire, 1912, *Naegleria* Alexeieff, 1912, *Tetramitus* Perty, 1852, etc. were placed in the Schizopyrenida, and the genera *Acrasis, Pocheina* and *Guttulinopsis*, all spore formers, in the Acrasida. Page (1988) placed the genus *Rosculus* in the Acrasida, commenting that it may be a non spore-forming species of *Guttulinopsis*.

Although the taxonomic position of *Rosculus* is questionable, morphological features of the amoebae, rapid growth in culture, progressive loss of cyst-forming ability and the ease with which cultures are lost, suggest that the organism is more closely related to the acrasid slime molds than to the Euamoebida Page, 1988. The principle difference between *Rosculus* and other acrasid myxamoebae is that the former is an amphizoic organism (Page 1974), while the latter have been known only as free-living organisms.

By contrast, *Vannella platypodia* (Gläser, 1912) has not been found to have any of the characteristics of slime molds, and is a well known free-living amoeba belonging to the order Euamoebida. Bovee (1965) established the genus *Vannella* for small free-living freshwater and marine amoebae that do not form cysts. Page (1988) described the morphological features of 5 well known species of freshwater *Vannella* with *V. platypodia* having the diagnostic characteristics observed in our cloned strain cultured under the No. 805/XI.

The two protozoan species described in the present study have not been reported in previous publications on amoebic infections in fish. Both species warrant future studies on mass and axenic culture.

Causative agents of many diseased conditions are still ignored in ornamental fishes. Pet fish owners usually are not willing to sacrifice fishes unless they are visibly doomed, dying or dead, when an accurate diagnosis and identification of the agent is hardly possible. This applies especially for long-standing granulomatous lesions.

Although our attention was focused mainly on *Carassius auratus* and other species of ornamental fishes constituted only a small part of diagnostic work, granulomatous inflammatory lesions were found to be

common in both groups. The general characteristics of granulomatous lesions may be almost identical in spite of the fact that their etiology differs.

While Voelker et al. (1977), Lom and Dyková (1992), and Steinhagen et al. (1993) suggested that intracellularly located organisms, agents of granulomatous lesions in goldfishes, belong to amoebae, Landsberg and Paperna (1992) were convinced that they found *Dermocystidium*-like organisms which provoked similar, if not identical lesions in goldfishes. Our original assignment of the agent to amoebae (Lom and Dyková 1992) was based mainly on the characteristic presence of vacuoles and endocytotic channels containing material obviously of the host origin, and on the absence of characters unambiguously proving its non -amoebic appurtenance.

Comparison of ultrastructure of *R. ithacus* trophozoites from agar plate culture and intracellularly located amoeba-like organisms found in thin sections through the periphery of goldfish granulomas revealed an important difference. The fact that we failed to find mitochondria or their remnants in amoeba-like organisms located in granulomas raised doubts about the identity of this agent with *R. ithacus* isolated from infected goldfish. *V. platypodia* cannot possibly be the same amoeba-like organism because of the large size of trophozoites. This situation prompts us to reconsider proposed interpretations of etiology and take into account other possible causative agents.

Amoebae which completely lack mitochondria such as *Entamoeba histolytica* Schaudinn, 1903 cannot be taken into consideration because of their obvious morphological difference.

Unfortunately, no ultrastructural details of *Dermocystidium*-like agent of systemic granuloma in goldfish by Landsberg and Paperna (1992) have been published to date to compare them with our findings. *Dermocystidium*-like organisms which were described by Hedrick et al. (1989) in granulomatous lesions in Atlantic salmon, *Salmo salar* L., closely resemble *Dermocystidium macrophagi* Moer, Manier et Bouix, 1986–1987, but differ from amoeba-like organisms reported in this paper among others in having a thick cell wall coated by a fibrogranular layer of host cell origin. The development of thick cell walls was observed during the division by internal cleavage. No endocytosis was observed. The same applies to the agent described from cultured rainbow trout by Nash et al. (1989).

The taxonomic affiliation of amoeba-like organisms described above remains questionable. Further comparative studies on the characteristics of agents and their potential relationships are warranted.

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