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Aphanomyces infection in juvenile soft-shelled turtle, *Pelodiscus sinensis*, imported from Singapore

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***Aphanomyces* sp. was isolated from the carapaces of two juvenile soft-shelled turtles with fungal infections imported from Singapore. Their sizes were 2.9-3.5 cm in carapace length. Lesions with integumental necrosis and ulceration looked like white cotton. The fungus exhibited slow growth, hyphae were 7.5-15 μm in diam, coarse, and abundantly branched. Zoosporangia observed in the isolate were complex, its entire thallus being converted into zoosporangial units, with short or long lateral evacuation tubes, and isodiametric, 100-500 μm in length. Clusters of zoospores were also produced at the terminals of hyphae. The production of the primary zoospores was achlyoid. The primary encysted zoospores were spherical, 10-15 μm in diam. No sexual stages were observed on a hemp seed incubated in sterile tap water. The optimal temperature for the fungus was 30°C.**

Key Words——*Aphanomyces* sp.; histopathology; morphology; *Pelodiscus sinensis*; soft-shelled turtle.

The importance of reptiles is widely recognized today. Many play a significant ecological role in the wild. Large numbers are kept in captivity - as pets, as exhibits, for food and for research - and there is increasing concern over their health, welfare and conservation (Beynon et al., 1992). The role of disease in the decline of wild reptiles is not fully understood and much research is needed on this subject. Nevertheless, it seems likely that both infectious and noninfectious diseases may, under certain circumstances, contribute to a decline in populations or limit their ability to spread. Pathogens, at any rate, may perhaps be considered as predators and competitors. Poor husbandry, usually through ignorance, is, either directly or indirectly, the major cause of disease in captive reptiles (Frye, 1991). The care of reptiles must, therefore, be based on a knowledge of the basic biological and environmental requirements of the animal. Fungal diseases are commonly encountered in reptiles, particularly in cutaneous or subcutaneous lesions in lizards and snakes (Jacobson et al., 1980). However, there are few reports on fungal diseases, especially due to lower fungi, in turtles.

In May 1995, *Aphanomyces* infection was found in soft-shelled turtles, *Pelodiscus sinensis* (Wiegmann), imported from Singapore to Japan as pets. This was the first time such an infection had been found in Japan, although some species of *Aphanomyces* are well known as common fungal pathogens of fish and shellfish (Hatai, 1989).

This report mainly describes biological characteristics of the fungus isolated from the lesions of the infected soft-shelled turtles.

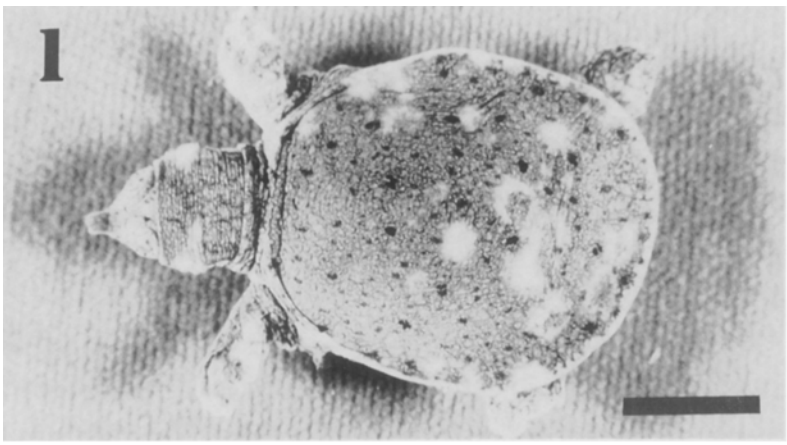
Materials and Methods

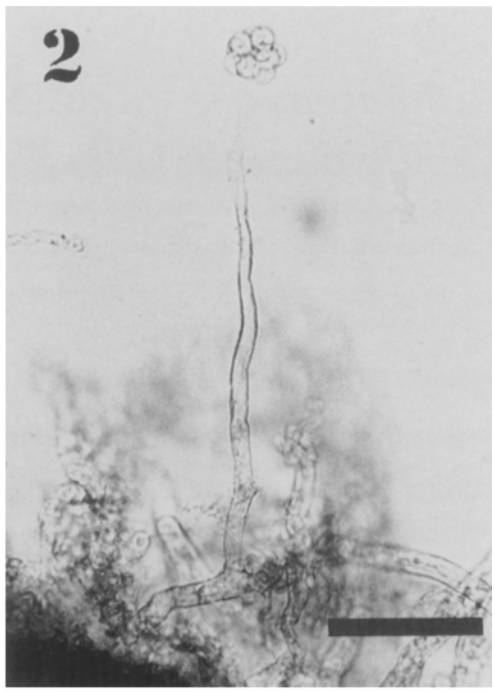
Diseased turtles Two juvenile soft-shelled turtles with fungal infections imported from Singapore were collected from a private farm in Tokyo in May 1995. After external examination of the turtles, the infecting fungi were isolated and diseased tissue was fixed for histopathological examination.

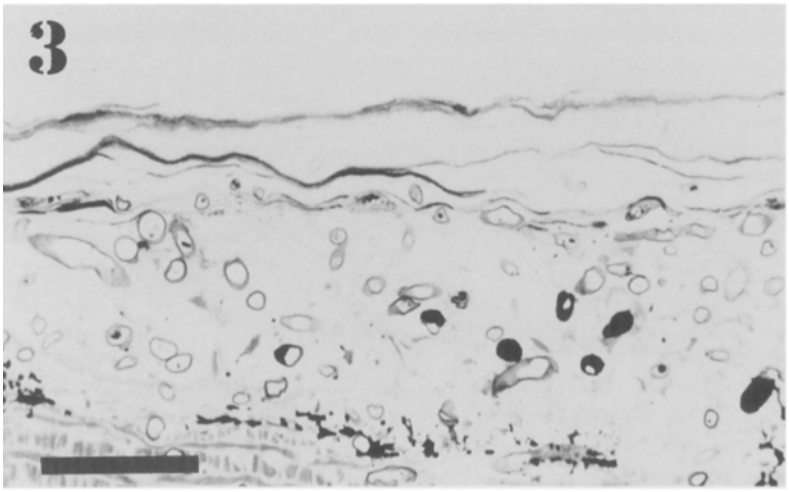
Isolation Fungi were isolated from the lesions by inoculating a piece approximately 2 mm in diam onto glucose-yeast agar (GY agar) (Hatai and Egusa, 1979) and incubating at 25°C. For inhibition of bacterial growth, addition of 500 μg each of ampicillin and streptomycin into the medium was required. Growing fungal colonies were subcultured onto GY agar, and transferred to fresh GY agar once every 15 d for maintenance.

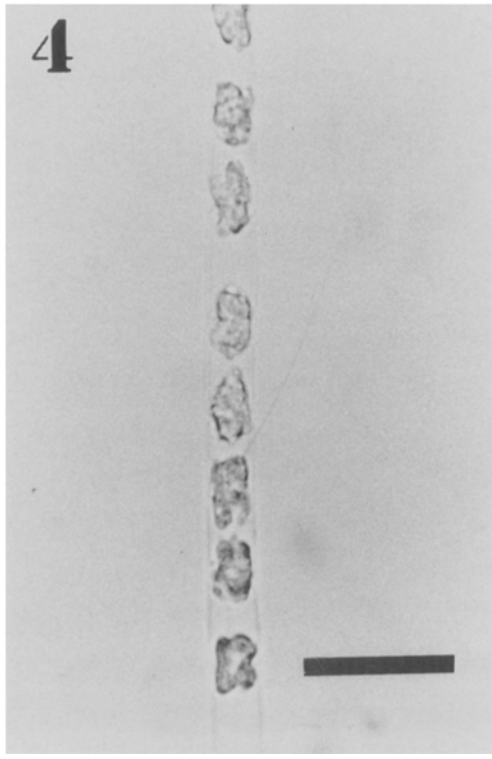
Histopathology Two diseased soft-shelled turtles were fixed in 10% phosphate-buffered formalin solution. The fixed tissues were processed into paraffin and sections of 3-5 μm were stained with haematoxylin and eosin (H & E), Grocott, and periodic acid Schiff's (PAS) reagent, using routine histological methods (Hendickson, 1985).

Identification For morphological observation, a GY agar block cut from an actively growing edge of a colony was put into 40 ml of the GY broth and incubated at 25°C for 4 d to grow mycelia. The mycelia were then washed twice with sterile tap water and resuspended in sterile tap water to induce zoospore formation. The isolated fungi were identified according to Sparrow (1960) and Scott (1961). One of the isolated strains, NJM 9525, was used for all experiments. Isolate NJM 8997 of *Aphanomyces piscicida* Hatai isolated from ayu, *Plecoglossus altivelis* Temminck & Schlegel, with mycot-









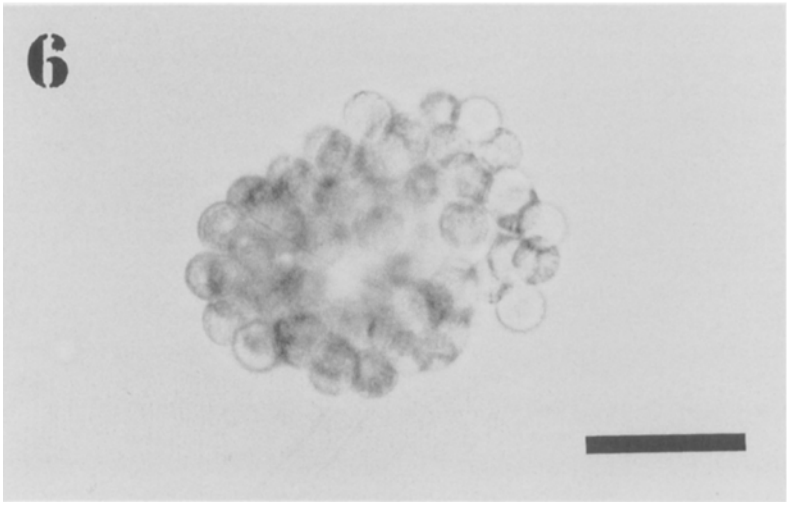


Fig. 1. Characteristic external signs of infected soft-shelled turtle. Scale: 1 cm.
Fig. 2. Microscopical examination of fresh mounts prepared from the infected carapace. Note primary zoospores which encysted in a cluster at the top of the zoosporangium. Scale: 100 μm.
Fig. 3. Histologic section from the affected turtle. No mycotic granuloma were present in the lesions. Grocott stain. Scale: 100 μm.
Fig. 4. Primary zoospores produced in a single row within a zoosporangium. Scale: 50 μm.
Fig. 6. Zoospores produced at the terminal of a hypha. Scale: 50 μm.

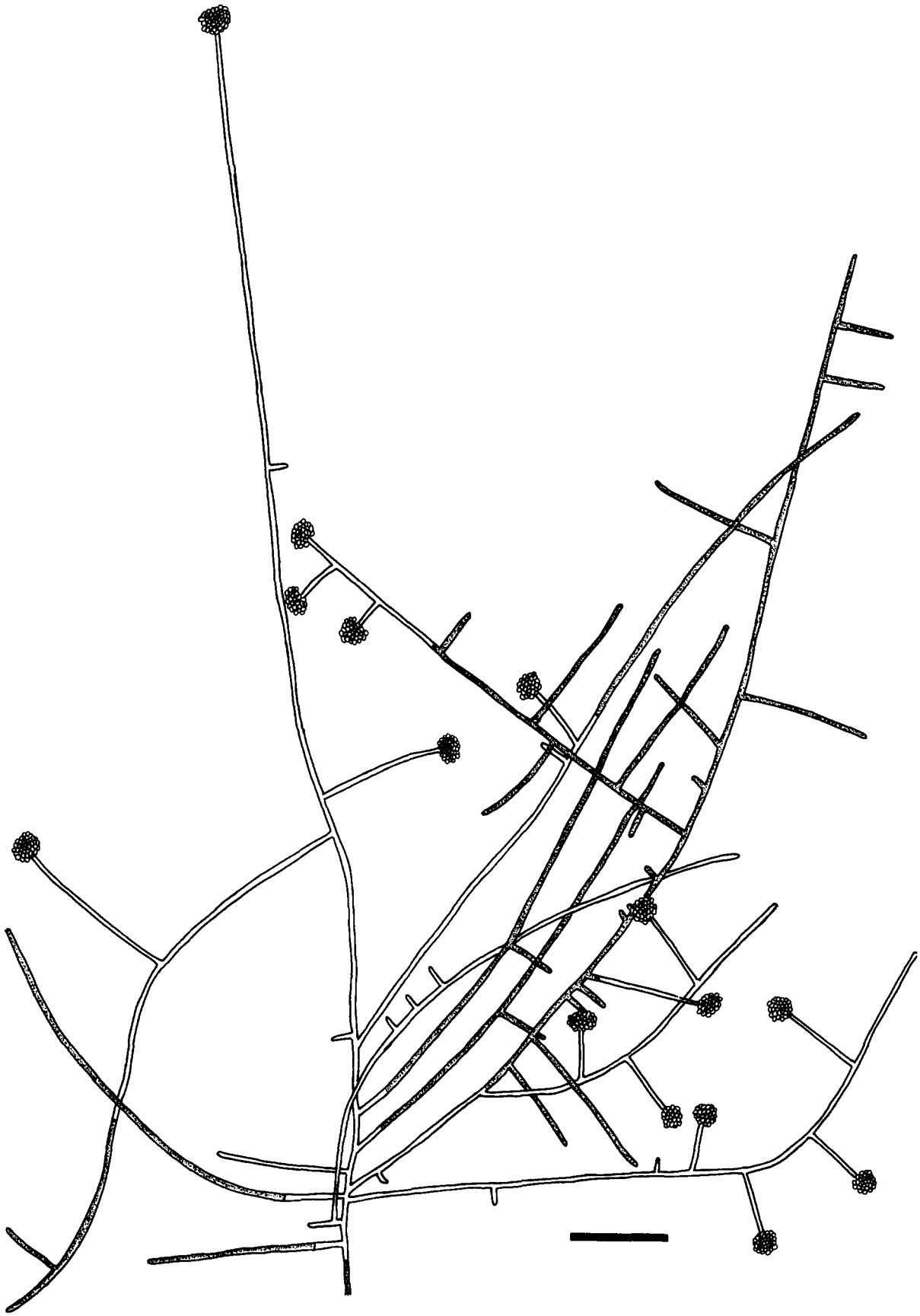


Fig. 5. Zoospores production of *Aphanomyces* sp., NJM 9525. Type of zoosporangia is complex. Scale: 300 μm.

ic granulomatosis in 1989 was used for comparison of growth at various temperatures.

Temperature range for growth A sample of an actively growing colony on GY agar was cut with a No. 2 cork borer and placed on the center of 20-ml GY agar plates (8.25 cm in diam), then incubated at eight different temperatures: 5, 10, 15, 20, 25, 30, 35 and 40°C. Mycelial growth was determined by measuring the colony diameter with vernier callipers daily for 14 d. The growth of the isolate was compared with that of *Aphanomyces piscicida* NJM 8997.

Effects of NaCl and six potential fungicides NaCl and six fungicides were tested against both hyphae and zoospores in GY broth using the two-fold dilution method, with the following concentrations: tachigaren 31-1,000 μg/ml, polyphenone 62.5-1,000 μg/ml, malachite green 0.13-2 μg/ml, cycloheximide 0.78-12.5 μg/ml, formalin 16-125 μg/ml, metalaxyl 125-1,000 μg/ml and NaCl 0.13-2.50% (w/v). Two sets of each chemical were prepared. Small 3-d-old colonies were inoculated into one set, and 3×10^2 zoospores/ml into the other. The germination of zoospores and viability of hyphae were observed under light microscopy daily during incubation at 20°C for 7 d.

Artificial infection A zoospore suspension of isolate NJM 9525 was prepared as described above, and the number of zoospores adjusted to approximately 5,000 per ml of sterile tap water. Twelve healthy soft-shelled turtles ranging from 3.1 to 4.2 cm in size (carapace length) were used for artificial infection. They were divided into four groups. The turtles in the first and third groups were injured by scraping the center of the carapace with a scalpel. The turtles in the second and the fourth groups were left intact. All groups were immersed in the zoospore suspension for 1 h, then kept in water in separate 1,000-ml beakers for 28 d. The water was changed daily. Oxytetracycline at 10 μg/ml was added to the water to control bacteria in the third and fourth groups. Water temperature was maintained at 25°C.

Results

Gross signs Integumental necrosis and ulceration with the appearance of white cotton were characteristic external signs of infection in soft-shelled turtle (Fig. 1). Microscopical examination of fresh mounts prepared from the infected carapaces showed primary zoospores which encysted in a cluster at the top of the zoosporangium (Fig. 2). *Aphanomyces* infection was suspected from these typical features.

Histopathology Histologic examinations of the carapace and flipper revealed lesions containing irregulary branching, aseptate fungal hyphae, measuring between 10-15 μm wide, consistent with organisms of *Aphanomyces* sp. Keratin and prickle cell layers were disrupted and replaced by masses of nonbranching hyphae. Histologic examination of sections from the turtle samples indicated that mycotic granuloma were not present in the lesions (Fig. 3).

Identification Colonies of isolate NJM 9525 showed white flat mycelium on GY agar. Hyphae were 7.5-15 μm in diam, coarse, and abundantly branched. Primary zoospores were produced in a single row, and linked by thin strands of cytoplasm, within a zoosporangium (Fig. 4). Zoosporangia observed in the isolate were complex, its entire thallus being converted into zoosporangial units, with short or long lateral evacuation tubes, and isodiametric, 100-500 μm in length (Fig. 5). Clusters of zoospores were also produced at the terminals of hyphae (Fig. 6). The production of the primary zoospores was achlyoid. The primary encysted zoospores were spherical, 10-15 μm in diam. No sexual stages were observed on a hemp seed incubated in sterile tap water.

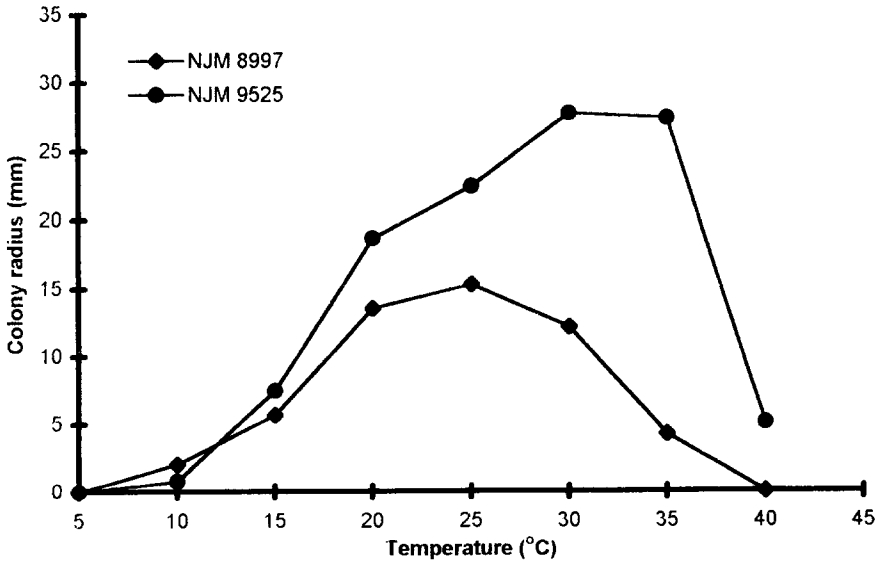


Fig. 7. Effects of temperature on the growth of *Aphanomyces* sp., NJM 9525 isolated from soft-shelled turtle and *Aphanomyces piscicida* NJM 8997 isolated from ayu.

Table 1. Minimum inhibitory concentration (MIC) of seven chemicals against hyphal growth and zoospore germination of *Aphanomyces* sp., NJM9525.

Chemical	MIC (μg/ml)[a]	
	Hypha	Zoospore
Tachigaren	250	125
Polyphenone	1000	125
Malachite green	0.25	<0.13
Cycloheximide	6.25	<0.78
Formalin	63	<16
Metaraxyl	>1000	>1000
NaCl	2%	1.5%

a) Measured at 7 d after inoculation.

The isolate NJM 9525 was identified as *Aphanomyces* sp. from the morphological observation, especially the mode of zoospore formation.

Temperature range for growth The growth curves after 7 d of *Aphanomyces* sp. NJM 9525 differed from those of *A. piscicida* NJM 8977 (Fig. 7). The optimal temperature for growth of the isolate NJM 9525 was 30°C, but that of the isolate NJM 8997 was 25°C. Neither isolate grew at 5°C.

Effects of NaCl and six potential fungicides Table 1 shows the minimum inhibitory concentration of NaCl and the six potential fungicides agianst hyphal growth and zoospore germination of *Aphanomyces* sp. NJM 9525. Malachite green was the most effective drug, completely inhibiting hyphal growth at 0.25 μg/ml, and inhibiting zoospore germination at 0.13 μg/ml.

Histopathology Microscopical examination of the lesions of the first and the third groups revealed numerous fungal hyphae after 48 h, which were irregulary branching, aseptate and approximately 10-15 μm in diam. The soft-shelled turtles in the second and the fourth groups showed small white cotton-like hyphae within 1 wk. In the first group, one turtle died on the 6th day and one on the 14th day, while one survived. The turtles in the second group died on the 6th, 7th and the 12th days (Table 2). There were no mortalities in the third and the fourth groups, which had been treated with 10 μg/ml oxytetracycline. After 28 d, all the turtles were fixed in 10% phosphate-buffered formalin solution and examined histopathologically.

Table 2. Mortality of soft-shelled turtles experimentally exposed to zoospores of *Aphanomyces* sp., NJM 9525.

Group[a]	Carapace	OTC bath[b]	Days after exposure[c]			
			5	10	15	20
1	Injured	−	0[d]	1	1	0
2	Intact	−	0	2	1	0
3	Injured	+	0	0	0	0
4	Intact	+	0	0	0	0

a) Three soft-shelled turtles were used in each group.
b) Oxytetracycline at 10 μg/ml was added (+) or not (−) to the rearing water.
c) Each soft-shelled turtle was exposed to sterile tap water with 5×10^3 zoospores/ml.
d) No. of dead soft-shelled turtles.

The result of gross findings and histopathological examination of soft-shelled turtles artificially infected with *Aphanomyces* sp. NJM 9525 were the same as in the naturally infected turtles, and the artificially introduced fungus could be reisolated from the lesions.

Discussion

Skin diseases are prevalent in reptiles (Elkan and Cooper, 1980; Marcus, 1981; Frye, 1991). In December 1993, *Aphanomyces* sp. and *Pythium* were seen growing externally on the neck of a turtle being kept in the AAHRI aquarium facility (Valairatana and Willoughby, 1994). Reptiles have a thick skin which is sometimes heavily keratinized, usually protected by scales (of ectodermal origin) but with scale pockets in between. In addition, chelonians have a "shell" consisting of both osseous (dermal bone) and epithelial elements. The latter form shields or "scutes" (Zangerl, 1969). The "shell" (carapace and plastron) of chelonians may be damaged by trauma or burning. Changes may also arise due to nutritional, metabolic and genetic factors or infection. Lesions can be repaired by cleaning, disinfection, application of plastic skin dressing (mild lesions) or epoxyresin (severe lesions). The healing process may take a long time (Jackson, 1978; Holt, 1981).

In aquatic chelonians, failure to shed shell plates may be due to poor basking facilities or osteodystrophy, and inadequate lighting may contribute.

Fungal lesions of the skin have been reported in many species. Aquatic reptiles appear to be particularly susceptible. The fungi involved are often not isolated, but *Aspergillus* has been reported. Egusa (1970) has reported a fungal infection of cultured soft-shelled turtle, *Pelodiscus sinensis* (Wiegmann), caused by *Mucor* sp. The disease first occurred in Ooita Pref., Japan, 1969, its gross feature being clouding of the carapace. Ulcerative epidermitis associated with a *Mucor* sp. infection has been described in a large group of hatchling Florida softshell turtles, *Trionyx ferox* (Schneider) (Jacobson et al., 1980). In the present study, although there was no histologic evidence of mycotic granuloma involvement, death of these turtles was believed to be associated with widespread cutaneous disruption. Epidermal necrosis could have resulted in loss of the keratinized barrier, resulting in dehydration, loss of essential ions, osmotic imbalances or the potential entry of bacteria into the body.

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