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NOTE

Aphanomyces species associated with epizootic ulcerative syndrome (EUS) in the Philippines and red spot disease (RSD) in Australia: preliminary comparative studies

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ABSTRACT: Fungi morphologically consistent with class Oomycetes were recovered on primary culture from 20 of 22 ulcers on 21 fish with epizootic ulcerative syndrome (EUS) collected from 5 sites in the Philippines. Eleven primary isolates, and the unifungal cultures derived from them, were identified as *Aphanomyces* spp.; the remaining 9 primary isolates were lost through contaminant overgrowth. The *Aphanomyces* isolates were morphologically and culturally indistinguishable from those reported from red spot disease (RSD) in Australia. Comparison of 4 representative *Aphanomyces* isolates from Australian fish with RSD and 3 representative *Aphanomyces* isolates from Philippine fish with EUS, using SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis), revealed similar peptide banding profiles, indicative of a single *Aphanomyces* species. These findings, combined with epizootiological and pathological similarities between EUS and RSD, suggest the 2 syndromes are identical, and that a single *Aphanomyces* sp. may be the primary infectious cause.

KEY WORDS: *Aphanomyces* · Ulcer · EUS · Philippines · Australia

Since 1980, severe periodic outbreaks of epizootic ulcerative syndrome (EUS) have affected wild and cultured freshwater fishes, as well as wild estuarine fishes, in many countries of South and Southeast Asia (Lilley et al. 1992). Typically affected fish have one or more large dermal ulcers with varying degrees of destruction of underlying tissues, and mortality rates are often high. In the Philippines, periodic outbreaks of EUS have occurred since 1985; many fish genera, including *Mugil*, *Arius* and *Scatophagus*, are affected (Llobrera & Gacutan 1987, Reantaso 1991).

In eastern and northern Australia, periodic outbreaks of a similar syndrome, known colloquially as 'red spot disease' (RSD), have occurred since 1972 in wild freshwater and estuarine fish (McKenzie & Hall 1976, Pearce 1990). Affected fish genera include those listed above for the Philippines.

The patterns of spread of both EUS and RSD suggest involvement of one or more primary infectious agents (Rodgers & Burke 1981, Roberts et al. 1992). Although a number of viruses or bacteria have been recovered inconsistently from fish with EUS (Frerichs et al. 1986, Hedrick et al. 1986, Llobrera & Gacutan 1987, Roberts et al. 1992, Torres et al. 1992) and RSD (Burke & Rodgers 1981, Callinan & Keep 1989, Pearce 1990), no aetiological agent has been conclusively identified for either condition.

Numerous invasive fungal hyphae, morphologically consistent with fungi of the classes Oomycetes or Zygomycetes, are present in histological sections of advanced ulcers from EUS-affected fish (Tonguthai 1985, Bondad-Reantaso et al. 1992, Roberts et al. 1993) and in early and advanced ulcers on RSD-affected fish (McKenzie & Hall 1976, Callinan et al. 1989, Pearce 1990). Severe necrotising granulomatous dermatitis and myositis are associated with the invasive hyphae. While it is possible that these fungi are opportunists which have invaded ulcers initiated by other agents, the similar fungal morphology and host tissue responses in numerous species of fish with EUS and RSD suggest the fungi may be primary infectious agents in both conditions. Several studies have attempted to identify them. Fraser et al. (1992), using methods which minimise contamination of inocula, isolated an apparently single *Aphanomyces* sp., an oomycete fungus, from 27 of 28 RSD lesions on 3 species of estuarine fish from widely separated river systems in eastern Australia. These isolates were morphologically and

culturally very similar, and the authors suggested the putative species was the cause of the typical RSD granulomas. By contrast, *Achlya* spp. have been recovered from EUS-affected fish in Thailand (Pichyankura & Bodhalimik 1983) and Sri Lanka (Subasinghe et al. 1990). However, these isolates may have been contaminants; *Achlya* spp. occur commonly in fresh water in Southeast Asia and, in our experience, the isolation methods used in these studies are likely to result in a high rate of recovery of contaminant fungi. More recently, Roberts et al. (1993) recovered 3 putative *Aphanomyces* pathogens from EUS-affected striped snakehead *Channa striatus* in Thailand. These isolates were morphologocially and culturally consistent with those recovered by Fraser et al. (1992), except that the RSD isolates typically had 1 or 2 lateral evacuation tubes on each sporangium, rather than the 4 reported as typical for the Thai EUS isolates.

Materials and methods. In November and December 1991, EUS-affected fish were collected from 4 widely separated freshwater sites and 1 coastal, fresh-to-brackish water site on Luzon Island, the Philippines; they were then transported live, in local water, to a laboratory. Twenty-one fish (14 striped snakehead, 3 mullet *Mugil* sp., 2 bar-eyed goby *Glossogobius giurus*, 1 walking catfish *Clarias batrachus*, and 1 three-spot gourami *Trichogaster trichopterus*) with typical early to advanced dermal ulcers were killed, and muscle tissue from 22 lesions on these fish was cultured for fungi. The methods of Fraser et al. (1992) were used, with the following modifications: media were inoculated in a Class I microbiological safety cabinet with the airflow system disconnected, and inoculated plates were incubated at 28 to 30°C. Cultures were examined daily and the methods of Fraser et al. (1992) used to obtain contaminant-free primary fungal cultures and to derive unifungal cultures from them.

Results and discussion. Broad, non-septate, sparsely branching fungal hyphae, indistinguishable from *Aphanomyces* spp., grew from muscle tissue inocula from 20 of the 22 lesions cultured (Table 1). Of these primary cultures 9 were lost due to overgrowth of bacteria or of fungi morphologically distinct from those present in lesions. The remaining 11 primary cultures, and the unifungal cultures derived from them, were similar in morphology and growth rate, producing flat, slightly

Table 1. Recovery of *Aphanomyces* isolates from EUS lesions on fish from the Philippines. nfg: no fungal growth. •: no data

Collection site	Fish species	*Aphanomyces*-like growth on primary culture	*Aphanomyces* on primary and unifungal cultures
Coastal lagoon, Buguey (Cagayan Province, Northern Luzon)	*Mugil* sp.	+	+
	Mugil sp.	+	+
	Mugil sp.	nfg	•
	Glossogobius giuris	+	+
	G. giuris	nfg	•
Ricefield, Buguey (Cagayan Province, Northern Luzon)	*Channa striatus*		
	Lesion 1	+	+
	Lesion 2	+	+
	C. striatus	+	+
	C. striatus	+; lost	•
Pond, Bautista (Pangasinan Provice, Central Luzon)	*C. striatus*	+	+
	C. striatus	+: lost	•
	C. striatus	+; lost	•
	C. striatus	+; lost	•
	C. striatus		
Swamp, Pulinan (Bulacan Province, Central Luzon)	*C. striatus*	+; lost	•
Laguna Lake (Laguna Province, Southern Luzon)	*C. striatus*	+	+
	C. striatus	+	+
	C. striatus	+	+
	C. striatus	+; lost	•
	C. striatus	+; lost	•
	C. striatus	+; lost	•
	Clarias batrachus	+	+
	Trichogaster trichopterus	+; lost	•

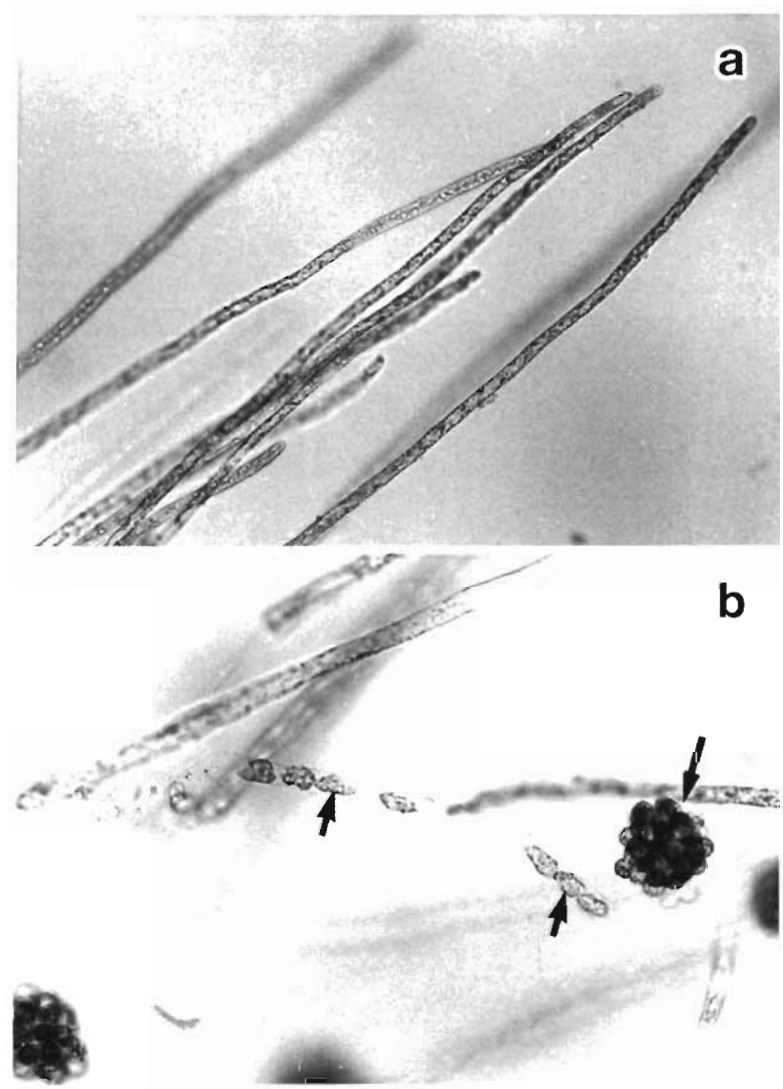

Fig. 1. *Aphanomyces* sp. recovered from skeletal muscle underlying a dermal ulcer on a striped snakehead *Channa striatus* from a freshwater pond, Bautista, Pangasinan Province, Central Luzon. (a) Vegetative hyphae (Lugol's iodine; ×110); (b) spore cluster (long arrow) and primary spores (short arrows) within sporangium (Lugol's iodine; ×225)

opaque colonies with an uneven white velvety surface and without aerial hyphae. Both primary and unifungal cultures were identified as *Aphanomyces* spp. (Scott 1961); all had filamentous sporangia indistinguishable from hyphae, and an 'achlyoid' manner of primary spore discharge and encystment (Fig. 1). Furthermore, colonial morphology, temperature-growth relationships, and microscopic morphology of the Philippine isolates were consistent with those of *Aphanomyces* isolates from RSD-affected fish (Fraser et al. 1992) and EUS-affected fish from Thailand (Roberts et al. 1993) except that, like the Australian isolates, the Philippine isolates typically had 1 or 2 lateral evacuation tubes per sporangium, not the 4 described as typical for the Thai isolates. No other fungi, morphologically consistent with those present in lesions, were recovered in cultures.

Histopathological examination of ulcer sections from all 21 fish cultured showed lesions typical of EUS and indistinguishable from those of RSD (Fig. 2).

Identification of *Aphanomyces* spp. is based on differential morphology of oogonia and antheridia (Scott 1961). None of these structures were observed in the present study, none were observed in RSD isolates by Fraser et al. (1992), and none were described for Thai EUS isolates by Roberts et al. (1993). Total protein electrophoresis provides useful, but not definitive, information concerning relationships within and between species of oomycete fungi (Bielenin et al. 1988, Chen et al. 1991). In order to examine possible relationships between isolates, peptide extracts from 4 representative RSD *Aphanomyces* isolates, 3 representative EUS *Aphanomyces* isolates, as well as an *Aphanomyces laevis* type isolate, an *Aphanomyces cochloides* type isolate, and an *Aphanomyces euteiches* type isolate were compared electrophoretically (Fig. 3). Fungal mats were derived from GY broth (Griffin 1978, in Dykstra et al. 1986) cultures incubated at 30°C in the dark for 15 d. Mats were then rinsed in distilled water, dried, and stored at

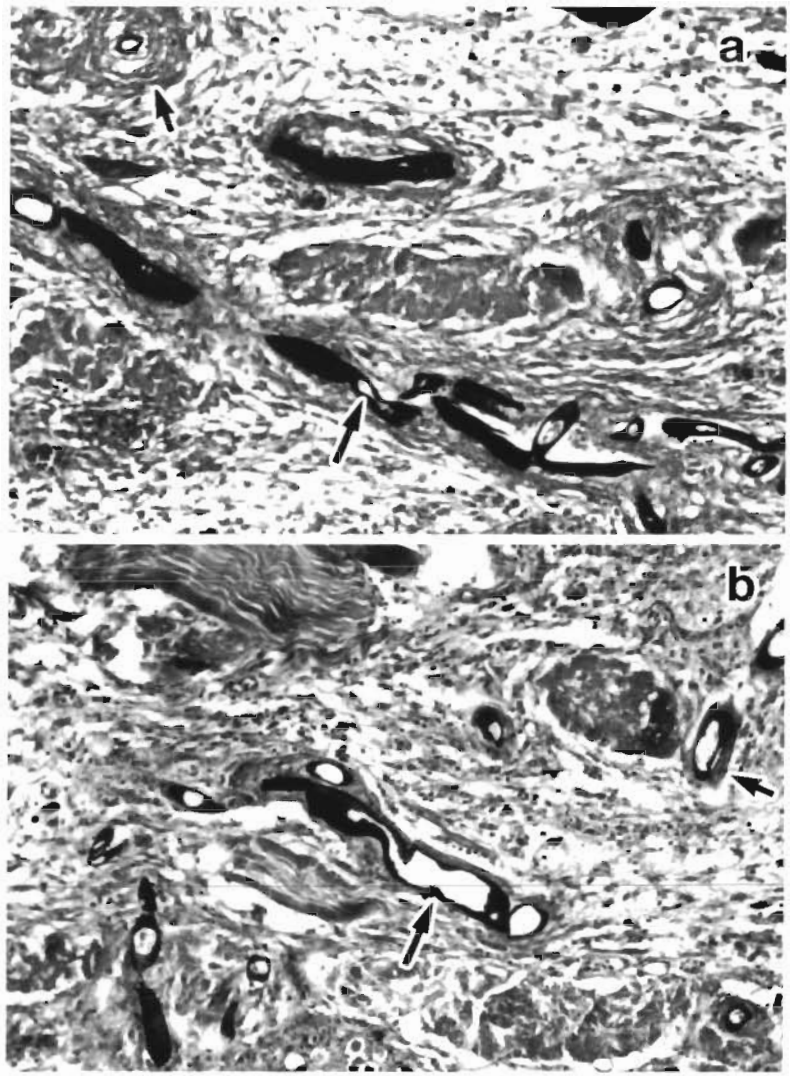

Fig. 2. Skeletal muscle underlying dermal ulcers in EUS-affected fishes from Buguey, Cagayan Province, Northern Luzon. There is severe necrotising myositis and early granuloma formation (short arrows) associated with invading fungal hyphae (long arrows) (Gomori methenamine silver and haematoxylin and eosin; ×300). (a) Striped snakehead *Channa striatus* from a ricefield; (b) mullet *Mugil* sp. from a coastal lagoon

–80°C. Prior to extraction, frozen fungal mats were placed in liquid nitrogen and ground to a powder with mortar and pestle. 100 mg aliquots of fungi were then extracted by boiling for 5 min in 500 µl of reducing mixture (2% sodium dodecyl sulphate, 5% 2-mercaptoethanol, 10% glycerol in 62.5 mM Tris-HCl at pH 6.8). The mixture was clarified at 5000 × *g* and 20 µl of the supernatant was loaded into 12% acrylamide and electrophoresced under reducing conditions according to Laemmli (1970). Peptides resolved by SDS-PAGE were visualised by silver staining (Tsai & Frasch 1982).

Band similarities or differences between isolates were assessed on the basis of band clusters over specific molecular weight class ranges. On this basis, the banding profiles of isolates from striped snakehead (Bautista), striped snakehead (Laguna), sea mullet (Queensland) and yellowfin bream (Clarence) were very similar over the 14 to 94 kDa range, indicating a close degree of relatedness between these Philippine and Australian strains. The remaining EUS and RSD isolates had similar banding profiles to the above group and to each other, but showed various band cluster differences across this molecular weight range, suggesting they may be less closely related to the above isolates. By contrast, *Aphanomyces cochloides*, *A. laevis*, and *A. euteiches* differed greatly from all EUS and RSD isolates and from each other in several band clusters, notably in the 35 to 94 kDa range. The overall similarity in banding profiles between *Aphanomyces* isolates from RSD and EUS supports the concept that they represent strains within a single species, and this putative species can be clearly differentiated from *A. cochloides*, *A. laevis* and *A. euteiches* by the method used. However, further comparative studies are necessary to validate the hypothesis that a single species is involved.

The high rate of recovery, in the current study, of morphologically and culturally similar *Aphanomyces*

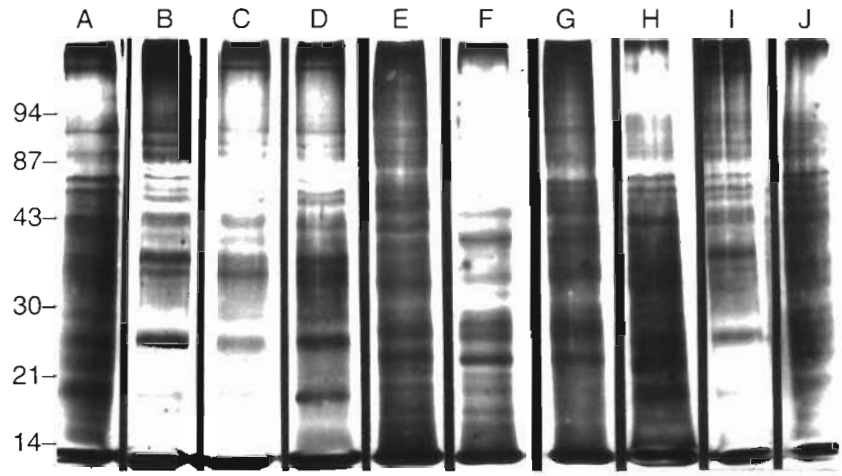

Fig. 3. *Aphanomyces* spp. Silver-stained SDS-PAGE polypeptide profiles of representative isolates from EUS-affected fish from the Philippines and RSD-affected fish from Australia, and type isolates of *A. cochloides*, *A. laevis*, and *A. euteiches*. Lane A: isolate from mullet *Mugil* sp., Buguey lagoon, Cagayan Province, Northern Luzon; B: isolate from striped snakehead *Channa striatus*, Bautista, Pangasinan Province, Central Luzon; C: isolate from striped snakehead *Channa striatus*, Laguna Lake, Laguna Province, Southern Luzon; D: isolate from sea mullet *Mugil cephalus*, Saltwater Creek, north Queensland; E: *Aphanomyces cochloides* (IMI 300493, International Mycological Institute, Kew, UK); F: *Aphanomyces laevis* (CBS 107.52; Centraalbureau voor Schimmelcultures, Baarn, The Netherlands); G: *Aphanomyces euteiches* (IMI 300494, International Mycological Institute, Kew, UK); H: isolate from sand whiting *Sillago ciliata*, Richmond River, New South Wales; I: isolate from yellowfin bream *Acanthopagrus australis*, Clarence River, NSW; J: isolate from sea mullet *Mugil cephalus*, Richmond River, NSW. Molecular weight standards are indicated on the left

isolates from EUS-affected fish in the Philippines, combined with the epizootiological and pathological similarities between EUS and RSD, indicates these conditions are identical; we therefore propose the term EUS be used to designate the condition in Australia. The study results also suggest that a single *Aphanomyces* sp. is associated with EUS in Australia, the Philippines, and elsewhere in Asia, and that it may be the primary infectious cause. In recent decades, this agent could have spread throughout the region, possibly through movements of fish or water, in a manner similar to the spread throughout Europe of *Aphanomyces astaci*, the cause of crayfish plague (Alderman et al. 1987).

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