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NOTE

First isolation of *Miamiensis avidus* (Ciliophora: Scuticociliatida) associated with skin ulcers from reared pharaoh cuttlefish *Sepia pharaonis*

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ABSTRACT: In the winter of 2015, a skin ulcer disease outbreak occurred in a pharaoh cuttlefish *Sepia pharaonis* population cultured on a land-based fish farm in China. Affected cuttlefish (about 60% of the population) were characterized as having developed ulcers on the dorsal skin, fin fringe, or distal mantle tip. Masses of a ciliated protozoan were isolated from skin ulcers. The ciliate was identified as *Miamiensis avidus* based on the morphological features of living and protargol-impregnated specimens. This identification was also supported by high sequence similarity of the small subunit ribosomal RNA gene (100%) and another ribosomal DNA region (including the 2 internal transcribed spacers and the 5.8S gene; 99%) with published sequences of fish parasitic *M. avidus* strains. *M. avidus* is known to be a histophagous marine fish parasite. This report describes the first case of *M. avidus* associated with skin ulcers in a cephalopod mollusk (Mollusca, Cephalopoda). This finding suggests that *M. avidus* may infect a phylogenetically broader range of hosts than what has previously been reported. Furthermore, *M. avidus* may pose a health risk to hatchery-reared cephalopods.

KEY WORDS: *Miamiensis avidus* · Pharaoh cuttlefish · *Sepia pharaonis* · Skin ulcers

INTRODUCTION

Protozoans in the phylum Ciliophora are also known as ciliates. In general, parasitic ciliates make up only a minor proportion of known ciliates and are fewer than free-living and mutualistic or communalistic species (Lynn 2012). Recently, infections in aquatic animals by ciliates have been increasingly reported (Jung & Woo 2012, Buchmann 2015). Especially in the order of Scuticociliatida, species such as *Philasterides dicentrarchi*, *Uronema nigricans*, and *Miamiensis avidus* have been demonstrated to be parasitic in some mariculture fishes (Ramos et al. 2007, Jung & Woo 2012). *M. avidus* was first isolated from seahorses in bay waters of Miami, Florida (USA), and was established as a facultative parasite on seahorses *Hippocampus erectus* (Thompson & Moewus 1964). Subsequent studies also revealed that *M. avidus* infects turbot *Scophthalmus maximus* and olive flounder *Paralichthys olivaceus* (Song et al. 2009, Jung & Woo 2012). Therefore, *M. avidus* has been regarded as an opportunistic parasite of fishes.

In this study, *M. avidus* was isolated for the first time from pharaoh cuttlefish *Sepia pharaonis* and was found to be associated with a skin ulcer disease in this species. The pharaoh cuttlefish is normally distributed from 35° N to 30° S and from 30° to 140° E in the Indo-Pacific (Nabhitabhata & Nilaphat 1999). The cuttlefish described in this study were cultured in an artificial environment for aquaculture trials. These animals were hatched from eggs originally obtained by keeping adult wild-caught cuttlefish in seawater tanks until spawning. Cuttlefish hatchlings were then cultured in tanks on a land-based fish

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farm. In winter of 2015, a skin ulcer disease erupted in this cultured cuttlefish population. In affected animals, masses of *M. avidus* that were active in ulcers present on dorsal mantle skin were identified as a probable cause of this disease. The affected cuttlefish were characterized by ulcers that developed on dorsal skin, fin fringe, or distal mantle tip. This finding suggests that *M. avidus*, previously only known as a facultative fish parasite, might also be a serious parasitic threat to cultured cephalopod species.

MATERIALS AND METHODS

Sampling cuttlefish and locality

Pharaoh cuttlefish *Sepia pharaonis* were cultured in an aquaculture farm on Xianshang Bay, China (29° 32′ N, 121° 45′ E). In total, 130 cuttlefish were reared in 2 rectangular tanks (4 m × 8 m) containing 48 m^3 of seawater with a daily water-exchange rate ranging from 60 to 80%. The culture water was obtained from local coastal water through sand filtration beds and a 200 µm mesh filter. The temperature and salinity of the tank water were maintained at 21 ± 1°C and from 24 to 30‰. The cuttlefish had reached a body length of 164.3 ± 8.2 SD mm (n = 10) and average weight of 470.9 ± 86.7 SD g when a skin ulcer epidemic occurred in this cultured population in November 2015. Ten cuttlefish with lesions were sampled for further analysis.

Sample collection and inspection

Skin and gill samples were collected after anesthetizing the cuttlefish in $MgCl_2$ solution (0.15 M) for 2 to 3 min (Messenger et al. 1985). Ulcerated and normal epidermis from the external mantle surface, as well as the gill (ctenidia) surface in the mantle cavity were scraped for parasitological analysis. Wet mount preparations were directly examined for the presence of any parasites under a light microscope (Nikon Eclipse Ni-U). In addition, smears were stained with Diff-Quik (Solarbio) to visualize their shape and nuclear apparatus following the manufacturer's instructions. If any putative parasites were discovered, further identification was conducted as described below.

Ciliate isolation and identification

During preliminary parasitic inspection, numerous ciliates of uniform shape were found inhabiting the lesioned tissues from all inspected cuttlefish. To isolate the ciliates from their hosts, ciliates were collected using a pipette and resuspended in 0.22 µm filtered seawater (FSW). Ciliate individuals were then spun down from seawater by centrifugation at 650 × *g* (5 min), followed by 2 washes in FSW. Ciliate cells were preliminarily identified based on the morphology of living forms obtained immediately from the hosts and starved cells that were maintained in FSW at 20°C for 48 to 72 h to reduce the granular particles contained in the ciliate cell. To facilitate further identification, buccal organization and infraciliatures of ciliates were revealed using the protargol impregnation method according to Wilbert (1975). Terminology and systematic classification follows the description of Lynn (2008). In addition, molecular identification of cultured ciliates was accomplished through sequence similarity searches involving the small subunit ribosomal RNA (SSU rRNA) gene and another ribosomal DNA (rDNA) region that includes the 2 internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene (ITS1-5.8S rDNA-ITS2). The procedure for DNA extraction, PCR, and sequencing is detailed in the Supplement, available at www.int-res.com/articles/suppl/d122p067_supp.pdf. The PCR primers used to amplify the SSU rRNA gene and ITS1-5.8S rDNA-ITS2 fragments are shown in Table 1.

Table 1. Oligonucleotide primers used in this study

Primer names	Primer sequences (5′–3′)	Reference
Euk-F	A(T/C)C TGG TTG AT(T/C) (T/C)TG CCA G	Medlin et al. (1988)
Euk-R	TGA TCC ATC TGC AGG TTC ACC T	Medlin et al. (1988)
ITS-F	GTA GGT GAA CCT GCG GAA GGA TCA TTA	Shang (2004)
ITS-R	TAC TGA TAT GCT TAA GTT CAG CGG	Shang (2004)
18S-900F	ACT AGG ACG GTA TCT GAT CG	Gao et al. (2012)
18S-900R	ACT AGG ACG GTA TCT GAT CG	Gao et al. (2012)

RESULTS

From a total of 130 cultured cuttlefish, about 60% developed visible lesions after 2 to 3 wk, when a small number of cuttlefish with skin lesions on the margin or rear of the mantle were noticed by the fish farm workers. Affected cuttlefish mostly died, partially due to the physical damage and irritation that was indicated by ink ejection in some morbid cuttlefish. A representative cuttlefish with skin lesions is shown in Fig. 1a. Lesions were located on the dorsal skin, lateral fin fringe, or distal tip of the mantle. The ulcers initially formed on the mantle epidermis, followed by downward penetration through the dermis to the underlying muscle tissue. The affected cuttlefish ejected ink more frequently than heathy cuttlefish. Inspection of the sampled cuttlefish revealed that in 100% of the examined specimens, a large number of ciliates were intimately associated with skin lesions. Moreover, ciliates appeared to be limited to external skin, especially in the ulcerated area, but were not observed on the gill surface and internal mantle surface.

Isolated ciliates presented a uniform ovoid shape with a rounded posterior and a pointed or tapering anterior end (Fig. 1b,c). In cross section, ciliates were slightly bilaterally flattened with a distinct depression at the buccal field (Fig. 1d) which normally extends to about 2/5 of cell length. Fixed ciliates are 43.3 ± 2.4 µm (mean ± SD; n = 10) in length and 18.2 ± 2.1 µm in width. The densely arranged cilia are about 10 µm long, and a single caudal cilium is about 15 to 20 µm in length. Cytoplasm is generally hyaline and colorless, filled with numerous food vacuoles (2.5 to 4.3 µm in diameter) in the freshly isolated individuals. In starved ciliates, which are usually more slender, no such granules are recognizable. One spherical macronucleus is centrally located in the body, and 1 micronucleus is often positioned near the macronucleus. In silver-impregnated specimens obtained by the protragol method, the somatic ciliature and buccal apparatus were revealed perfectly. There are about 13 to 14 somatic kineties (SK) longitudinally arranged. Membranelle 1 (M1) is small and consists of 2 longitudinal rows with about 5 basal bodies each. Membranelle 2 (M2) is distinctly larger than M1, consisting of 4 to 5 longitudinal rows of kinetosomes, and membranelle 3 (M3) is close to M2 and usually consists of 2 to 3 rows of basal bodies. The paroral membrane is close to M3 and is obvious in impregnated individuals. The present population corresponds closely to the original and subsequent descriptions of *Miamiensis avidus* in all morphological features (Thompson & Moewus 1964, Lynn 2008).

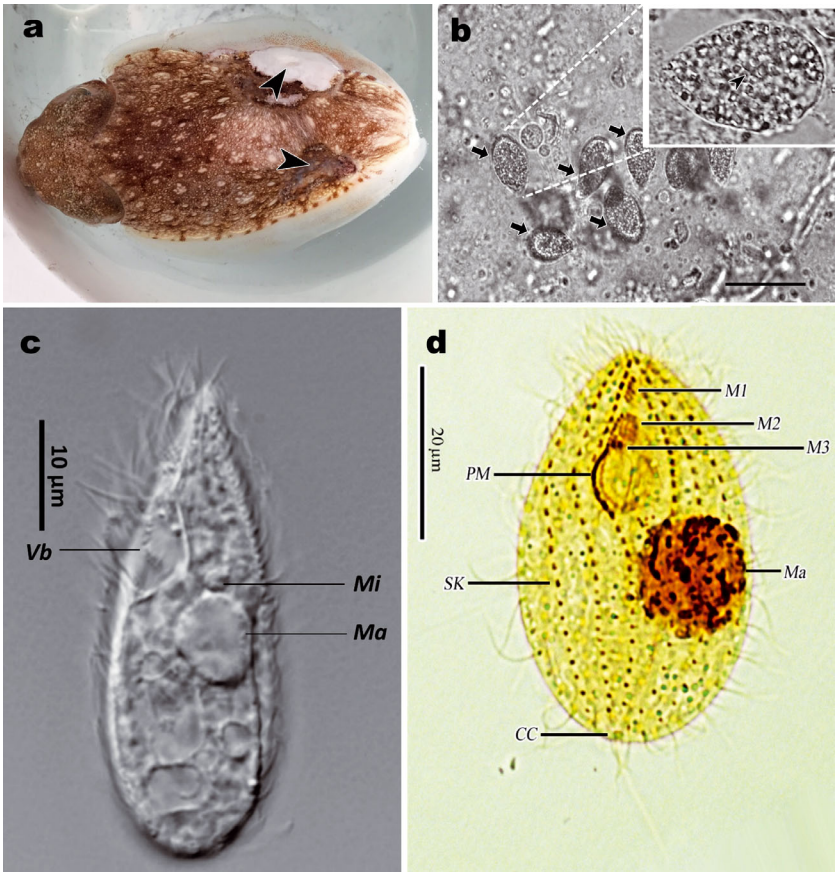

Fig. 1. (a) Diseased pharaoh cuttlefish *Sepia pharaonis*. This animal developed skin lesions, followed by downward penetration through the dermis and underlying muscle tissue. Ciliates were packed in the skin lesions in the mantle (arrowheads). (b) Microscopic morphology of ciliates from wet mount preparations of a skin sample scraped from an ulcer. Arrows indicate masses of ciliates. Inset shows possible food vacuoles (arrowhead) within the cytosol, indicating consumption of host tissue. Scale bar = 50 µm. (c) Differential interference contrast image of a fixed ciliate (in 0.5% glutaraldehyde in 0.45 µm filtered seawater [FSW]). Scale bar = 10 µm. (d) Protargol-impregnated ciliate (starved in FSW for 3 d) showing the cytostome and ventral infraciliature. Scale bar = 20 µm. CC: caudal cilium; M1–3, membranelles 1–3; Ma: macronucleus; Mi: micronucleus; PM: paroral membrane; SK: somatic kineties; Vb: vestibulum

Sequence similarity analysis of the SSU rRNA gene (1759 bp fragment) and the ITS1-5.8S-ITS2 region (557 bp) showed that the cuttlefish ciliate isolate shared a 100% sequence identity of the SSU rRNA gene with a fish pathogenic *M. avidus* strain retrieved from GenBank (accession no. AY550080). Similarly, its ITS1-5.8S-ITS2 sequence also shared high identity (99%) with that of an *M. avidus* strain (accession no. HM768743). The SSU rRNA gene and ITS1-5.8S-ITS2 region of the isolated ciliate have been deposited in GenBank and assigned accession numbers KU992658 and KU720303, respectively.

DISCUSSION

In November 2015, a skin ulcer disease occurred in cultured pharaoh cuttlefish, and *Miamiensis avidus* was found active in these ulcers. Moreover, the microscopic morphology of ciliates revealed numerous food vacuoles contained in the cytoplasm, indicating a probable consumption of host epithelium (Fig. 1b inset). These ciliates matched closely in morphology to earlier descriptions of *M. avidus* (Thompson & Moewus 1964). In addition, a bacterial screen showed that the majority of bacterial isolates from both healthy skin and ulcers were *Pseudoalteromonas* sp. (see Fig. S2 in the Supplement), which are common commensal microbes in many marine organisms (Holmström & Kjelleberg 1999). These observations suggest that the skin ulcers in *Sepia pharaonis* in this study were more likely to be related to activities of *M. avidus* than to bacterial infections.

The ciliate isolated from cuttlefish was initially identified based on morphological features. To some extent, this approach for identification is subjective, and relies heavily on qualitative staining techniques and researchers' experience and knowledge. Thus, 2 gene markers (SSU rRNA and ITS1-5.8S rDNA-ITS2) were also employed for the molecular identification. In general, the SSU rRNA gene is rich in taxonomic characters, and its sequence analysis approach is a universally applicable tool (Gao et al. 2012). Since a previous study suggested that the approach of SSU rRNA sequence analysis is limited in its capacity to differentiate between closely related species (Jung et al. 2011b), a further molecular identification was carried out based on the sequence of a DNA fragment containing 5.8S rDNA and upstream and downstream flanking ITS regions. The sequence analysis showed that our ciliate isolates shared high sequence similarities of SSU rRNA (100%) and ITS1-5.8S-ITS2 (99%) with those of *M. avidus* isolates that are parasitic to olive flounder (Jung et al. 2005, 2011a). It is noteworthy that the sequence analyses of ITS1-5.8S rDNA-ITS2 revealed a 99% identity of our specimen to that of an *M. avidus* strain pathogenic to flounder (accession no. HM768743) but only 82% to an *M. avidus* strain isolated from elsewhere (JN885095). This finding indicates that the ITS1-5.8S rDNA-ITS2 sequence may be a suitable maker to resolve *M. avidus* strains.

During a microscopic examination, affected cuttlefish were all loaded with *M. avidus* in the lesion sites of the mantle skin. Unlike in the cuttlefish, the main signs registered in olive flounder infected by *M. avidus* include abdominal distension, dark body color, hemorrhages, enteritis, loss of scales, and dermal necrotic lesions (Song et al. 2009, Takagishi et al. 2009). The invasion of *M. avidus* in these fish hosts may cause both external ulceration and systemic infections of the brain, liver, gills, and digestive tract leading to high host mortalities through systemic invasions. Moreover, another study revealed in an immersion infection experiment that *M. avidus* can aggressively invade seemingly healthy fish from seawater (Jung et al. 2007).

Ciliate species that are not exclusively parasitic can be associated with diseases of significant economic consequence in aquaculture (Harikrishnan et al. 2010). For instance, *M. avidus*, as a facultative parasite, heavily infects cultured turbot and olive flounder (Song et al. 2009, Jung & Woo 2012). Conditions in aquaculture enhance the establishment of parasites. In our case, it was possible that *M. avidus* may have entered the aquaculture tanks via insufficiently disinfected water or live feed, and this opportunistic parasite was likely to establish and proliferate in the culture system because it is able to reproduce in the water and can directly infect farmed animal hosts confined in indoor tanks or ponds. Moreover, squids or octopus in captivity readily sustain mantle tip damage and or fin abrasions during capture or confinement in a culture system (Leibovitz et al. 1977, Hulet et al. 1979, Hanlon et al. 1984). It is conceivable that the presence of a lesion in the cuttlefish skin (see Fig. 1a) may serve as an entry point for parasites. In addition, cuttlefish spend more time resting at the bottom of the tank than swimming during the day. The low mobility may facilitate attachment of ciliates onto the skin surface following a skin abrasion. As a result, animals develop a cutaneous ulcer following the invasion of opportunistic parasites into broken skin lacking a protective mucus covering.

To our knowledge, this is the first case of a cephalopod host for *M. avidus*. This finding indicates that *M.*

avidus can also infect invertebrate hosts, especially cultured or confined animals.

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