Research article

Outbreak of Nontuberculous Mycobacteriosis with Dematiaceous Fungus Co-infection in Aquarium-reared Sharphead Flyingfish (*Hirundichthys oxycephalus*)

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ABSTRACT—An episode of serial mortality occurred in a population of wild-caught, aquarium-reared sharphead flyingfish *Hirundichthys oxycephalus*. Most diseased fish had ulcerative hemorrhagic lesions on the skin, especially on the head, and displayed multiple, fine, whitish nodules scattered on the internal organs, including the kidney, spleen and liver. Histopathological examination revealed the nodules to be granulomatous lesions consisting of epithelioid cells with a thin outermost rim of fibroblasts and connective tissue, and the central caseous core of the granulomas was associated with acid-fast bacilli. Though the granulomatous lesions were systemically present in the major internal organs, the most severe lesions were in the kidney and spleen. In addition, melanized fungal hyphae were also found in the kidney and spleen. Acid-fast bacterial strains were isolated from affected fish and most were identified as *Mycobacterium marinum* by molecular examination. A few dematiaceous fungal strains were also isolated from some fish and identified as *Exophiala aquamarina* by morphological and molecular examination. This is a novel description of a case of co-infection of a nontuberculous mycobacterium (*M. marinum*) and a dematiaceous fungus (*E. aquamarina*) in a flyingfish species.

Key words: nontuberculous mycobacteriosis, dematiaceous fungus, sharphead flyingfish, co-infection

Nontuberculous mycobacterial infections are chronic bacterial diseases caused by *Mycobacterium* spp. other than *M. tuberculosis* complex and *M. leprae* (Jacobs *et al.*, 2009). Nontuberculous mycobacteria (NTM) are gram-positive, acid-fast, non-motile water-borne bacteria, ubiquitously found in freshwater and saltwater environments. In aquatic animals, disease associated with NTM infections is commonly reported in aquaria and aquaculture systems as fish (piscine) mycobacteriosis, and has been especially associated with *M. marinum*, *M. fortuitum* or *M. chelonae* infection (Jacobs *et al.*, 2009; Hashish *et al.*, 2018). Piscine mycobacteriosis can present as chronic multifocal granulomatous formations in the visceral organs, as seen in various fish species, including aquarium-reared tropical fishes (Gauthier *et al.*, 2009; Jacobs *et al.*, 2009; Hashish *et*

al., 2018). Recently, Gupta *et al.* (2018) provided evidence of five distinct monophyletic groups and proposed division of the genus *Mycobacterium* as five separate genera: *Mycobacteroides* ("Abscessus–Chelonae" clade), *Mycolicibacterium* ("Fortuitum–Vaccae" clade), *Mycolicibacter* ("Terrae" clade), *Mycolicibacillus* ("Triviale" clade) and *Mycobacterium* ("Tuberculosis–Simiae" clade).

It has been postulated that *M. marinum* is the most common causative agent of mycobacteriosis in marine fish species (Smith, 1997; Jacobs *et al.*, 2009; Hashish *et al.*, 2018), resulting in considerable economic losses for farm-fish and aquarium-fish production (Hashish *et al.*, 2018). In addition, *M. marinum* has been described as a candidate zoonotic aquatic microorganism capable of causing dermal granulomatous lesions in immunocompetent human patients, a condition diagnosed as 'fish tank granuloma' (Wu *et al.*, 2012; Johnson *et al.*, 2015).

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Exophiala spp. are amorphic melanized fungi with brown to black hyphae. Based on this physical feature they are classified in the Chaetothyriales order of dematiaceous fungi (or black yeasts) and are known to be agents of infection in both fish and amphibians (Kurata *et al.*, 2008; de Hoog *et al.*, 2011; Hatai *et al.*, 2012).

Exophiala aquamarina is a waterborne pathogen of fish, characterized by olivaceous to black-colored velvety colonies, ellipsoidal to cylindrically shaped conidia with annelated zones, occasionally with spirally twisted hyphae, and with an optimum temperature range of 24–30°C (de Hoog *et al.*, 2011). This fungus has been isolated from various disseminated lesions in a wide range of marine fish species, including leafy seadragon *Phycodurus eques*, weedy seadragon *Phyllopteryx taeniolatus*, winter flounder *Pseudopleuronectes americanus*, and little tunny *Euthynnus alletteratus* (de Hoog *et al.*, 2011).

The sharphead flyingfish *Hirundichthys oxycephalus* is a marine teleost found in temperate to tropical waters of the Indo-West Pacific, from southern Taiwan to northwestern Kyushu (Ichimaru, 2008; Chou *et al.*, 2015; Murwani *et al.*, 2020). Wild populations of this species are declining partly because of strong pressure from the harvesting of flyingfish for fish roe (Chou *et al.*, 2015). Flyingfishes, including the sharphead flyingfish, have been reared in Japanese aquaria for the sake of exhibition and study.

Here, we describe infection of a NTM, as well as co-infection of a NTM and a dematiaceous fungus in aquarium-reared sharphead flyingfish as the first description in a flyingfish species.

Materials and Methods

Animals and sample collection

Wild-caught sharphead flyingfish, captured at 35°08′18.2″ N, 132°02′20.8″ E or 35°0′54.7092″ N, 132°11′39.516″ E, were reared in circular (4-m diameter, with approximately 13.8-t capacity) or rectangular (2.3 × 3 × 2 m, approximately 15-t capacity) aquaria containing natural seawater maintained at 23°C. The fish first began to die in December 2017; cumulative mortality reached as high as approximately 41.3% during the period April to September 2018. Most fish had ulcerative hemorrhagic skin lesions, and a few exhibited bradykinesia and anorexia. Fish died as early as within 2 mo since they had been introduced, and numerous whitish micronodules were apparent on their internal organs.

Fifty-three freshly dead or moribund fish were removed from the aquaria, between February and September 2018. Moribund individuals were euthanized by cranial maceration immediately after immobilization by submersion in ice water. The collected fish were routinely dissected for observations of their external and internal gross features. From 49 of these

fish, tissue blocks were collected from the major organs (including the liver, spleen, kidney, heart, gills, and intestine) as well from ulcerative skin lesions. Each tissue sample was divided into three pieces, for use in histopathology (fixed in 10% phosphate-buffered formalin solution) and microbiology and molecular biology (frozen), to determine the pathogenesis of the present case. Except, of the total 53 fish, the tissues of two fish were fixed with 99% ethanol only and processed for molecular biological examination; and the other two remaining fish were individually stored frozen or formalin-fixed and used for microbiology and histopathology, respectively.

Tissues for histopathology

Formalin-fixed tissues of the liver, kidney, spleen, heart, gills, intestine, brain, and ulcerative skin lesions were processed routinely to prepare paraffin sections for histology. The sections were stained with hematoxylin and eosin (H&E). Some selected sections were stained with Ziehl–Neelsen (ZN), periodic acid–Schiff (PAS) reaction, Grocott's modified methenamine–silver nitrate method, or counterstained with H&E (Grocott–H&E) and Schmorl's method.

Evaluation of granulomatous lesions associated with acid-fast bacilli in the liver, kidney, and spleen was performed by observing 10 fields in each section stained with ZN, under ×400 magnification using light microscopy. The number of granulomas with acid-fast organisms was counted in each field, and the average number was calculated from the sum of them in the 10 fields. This procedure was done in triplicate (by three individual observers) and the outcomes averaged. Next, the average numbers of granulomatous lesions were classified as: no granuloma (–); 1–3 granulomas (+, rare); 4–10 granulomas (++, moderate); or ≥11 granulomas (+++, abundant). Finally, the percentage of each category was calculated for the three organs examined (*i.e.* liver, kidney, and spleen) (Table 1).

Microbiological analysis: NTM isolation and photochromogenicity test

Selected frozen tissue samples (*i.e.* liver, kidney and/or spleen) from 50 fish were processed for NTM isolation. These tissues were homogenized with 0.5 mL of sterilized ultrapure water and decontaminated by adding 1 mL of 4% NaOH for (at most) 15 min, and then neutralized by adding at least 6 mL of pH 6.8 phosphate buffer. After neutralization, the samples were centrifuged at 6,500 × *g* for 20 min before discarding the supernatant and then resuspending the pellet in 1 mL of Middlebrook 7H9 broth base (Becton™, Dickinson and Company), which was supplemented with 10% OADC enrichment (Becton™, Dickinson and Company) and 0.5% Tween 80 (Sigma-Aldrich). Aliquots (100 μL each of the sample) were inoculated on Middlebrook 7H11 agar supplemented with 10% OADC enrichment and

Table 1. Evaluation of granulomatous formations associated with *Mycobacterium marinum* in the liver, kidney and spleen samples from the total 50 sharphead flyingfish examined.

Organ	No granulomas (−)	Rare (+)	Moderate (++)	Abundant (+++)
Liver	32.0% (16/50)*	24.0% (12/50)	20.0% (10/50)	24.0% (12/50)
Kidney	6.12% (3/49)	8.16% (4/49)	10.2% (5/49)	75.5% (37/49)
Spleen	6.00% (3/50)	10.0% (5/50)	12.0% (6/50)	72.0% (36/50)

Average numbers of the granulomas in 10 fields under ×400 magnification were classified into four categories as follows: No granulomas (−); Rare (+): 1–3 granulomas; Moderate (++): 4–10 granulomas; Abundant (+++): 11 or more granulomas.

* Percentage of each category of granulomatous lesions in each organ examined.

0.5% Tween 80 and spread on 2% Ogawa egg slant (Kyokuto Pharmaceutical Industrial Co., Ltd.). These media were incubated at 25°C for 2 mo; they were checked daily for the first week, and thereafter once a week. Selected colonies were stained with ZN on glass slides to check their acid-fast stainability. After that, the photochromogenicity of slow-growing colonies of 17 isolates were examined on 2% Ogawa egg slants, following the method of Fukano *et al.* (2015).

Fungal isolation

Fungal isolations were conducted with approximately 2 mm³ of each the frozen liver, kidney and/or spleen tissue samples from each fish. These tissue samples were inoculated on glucose yeast extract (GY) agar (Hatai and Egusa, 1979) and incubated at 25°C for 2 mo. The addition of 500 μg/mL each of streptomycin sulfate (Meiji Seika Pharma Co., Ltd.) and ampicillin sodium (Kyoritsu Seiyaku Corp.) was required for inhibition of bacterial growth. Fungal colonies were subcultured on GY agar to obtain pure cultures. From the pure cultures, representative isolates were used for more-detailed morphological identification using the slide culture technique with potato dextrose agar (PDA) (Nissui Pharmaceutical Co., Ltd), at 25°C, for 3 wk (Riddell, 1950). The slide cultures were examined with light microscopy, once a week, for evidence of conidiogenesis, and the samples were eventually fixed and stained with lactophenol cotton blue (LPCB) for permanent preparations.

Molecular diagnosis of nontuberculous mycobacteria (NTM)

The spleen tissue was selected for sequencing because gross lesions were more clearly observed in the spleen of most of the fish. For extraction of DNA from spleen tissue, a QIAamp DNA Mini Kit (QIAGEN) was used, following the manufacturer's instructions. However, genomic DNA of the mycobacterial isolate was also extracted through boiling, as described below. Each single colony collected from the acid-fast isolates on Middlebrook 7H11 agar was transferred into Middlebrook 7H9 broth. A total volume of 800 μL of

the 7H9 broth was centrifuged at 3,000 × *g* for 20 min after being allowed to grow for 2 wk. After removing the supernatant, the pellet was then resuspended in 120 μL of sterilized ultrapure water, frozen at −20°C, and then thawed to perform cell lysis. The suspension was boiled at 95°C for 15 min and centrifuged at 3,000 × *g* for 10 min. The supernatant was then collected and used as a template for the PCR analyses. PCR analysis was carried out to amplify three housekeeping genes: 16S rRNA, RNA polymerase b-subunit (*rpoB*), and 65 kDa heat-shock protein (*hsp65*), using primer sets 8F–1047R, MycoF–MycoR, and Tb11–Tb12, respectively (Fukano *et al.*, 2017). PCR was performed with GoTaq® DNA Polymerase (Promega), under the following amplification conditions (modified from Fukano *et al.*, 2017): 95°C for 10 min, 35 cycles of 95°C for 40 s, 55°C for 40 s and 72°C for 90 s, and a final cycle of 72°C for 5 min. The amplicons were purified with NucleoSpin® Gel and PCR Clean-up (MACHEREY-NAGEL GmbH & Co. KG). The purified amplicons were sequenced by FASMAC (Kanagawa, Japan). Sequences were aligned using ClustalW in MEGA X (Kumar *et al.*, 2018) and the sequence similarities were analyzed through BLAST (GenBank, http://www.ncbi.nlm.nih.gov/blast.cgi).

Phylogenetic analysis was conducted using a multilocus sequence concatenated with the 16S rRNA, *rpoB* and *hsp65* genes, and then reconstructing the sequences using the neighbor-joining method with Kimura's two-parameter distance correction model and 1,000 bootstrap replicates, based on the latest taxonomic categories described by Gupta *et al.* (2018).

The representative four isolates suspected to be *M. marinum* were selected to confirm the presence of IS*2404* and IS*2606* of the insertion sequences, relating to mycolactone-producing mycobacteria (MPM), using the primer sets MU5–MU6 and MU7–MU8, respectively, through the cycles as described by Stinear *et al.* (1999). The examination was performed with reference strains that included *M. marinum* ATCC BAA535, *M. marinum* JCM17638, and *M. pseudoshottsii* JCM15466.

Molecular diagnosis of fungi

From the representative 15 pure-culture fungal isolates taken from 9 individuals, small pieces of agar blocks with colonies were transferred into GY broth and cultured at 25°C for 2 wk. For molecular diagnosis, genomic DNA of the fungal isolates was extracted from hyphal masses floating in the broth. A few hyphal masses (approximately 5-mm diameter) in the broth were collected and transferred into 400 μL of Lysis Buffer PL1 for a NucleoSpin Plant II kit (MACHEREY-NAGEL GmbH & Co. KG) with 0.5-mm diameter zirconia beads (Tomy Seiko Co., Ltd), and then disrupted using Micro Smash™ MS-100 (Tomy Seiko Co., Ltd) at 4,300 rpm for 100 s. The extraction protocol was carried out in accordance with the manufacturer's instructions. The extracted DNA samples from the spleen tissue of fish examined for NTM analysis, described above, were also used for molecular analysis of the fungal pathogen.

PCR analysis of the fungal isolates was performed according to Ohkura *et al.* (2009), with the internal transcribed spacer (ITS) region as the target gene. The ITS regions were amplified with the fungi-specific primers ITS5 and ITS4. However, molecular analysis of the tissue-extracted DNA samples was done with nested PCR. The first-round PCR products with the ITS5 and ITS4 primers were diluted 1,000 times with sterile distilled water, and then used as templates for second-round PCR, with ITS4 and ITS3 as the primer set. The second PCR was performed with the amplification cycles followed by Muñoz-Cadavid *et al.* (2010). Phylogenetic analysis of the ITS regions was performed with the same protocol.

Results

Gross features of infection

Most fish examined showed ulcerative hemorrhagic skin lesions on the head, snout, mandible and/ or dorso-anterior region of the trunk (Fig. 1A). Most of these fish also showed splenomegaly and/or renomegaly with associated renal discoloration. In addition, numerous whitish micronodules were apparent on the kidney, spleen, liver and heart (Fig. 1B). Epicardial tissue thickening and cloudy myocardial fibers were observed in many of the heart samples.

Histopathology

Histopathological examination of the formalin-fixed tissues revealed granulomatous lesions in the parenchyma of liver and/or spleen in 48 of the 50 fish examined. Similar lesions were observed in interstitial tissue of the trunk kidney, in the connective tissue, and in subcutaneous to muscular layers of the skin with and without ulcerative lesions on the snout, mandible, and/ or dorso-anterior region of the trunk (Fig. 2A & B). In some fish, no granulomas or acid-fast bacilli were observed in the ulcerative skin lesions.

The granulomas were characterized by a central necrotic core, surrounded by oval or spindle-shaped epithelioid cells, and a thin outermost rim of fibroblasts and connective tissue. ZN staining revealed variable numbers of acid-fast bacilli in the epithelioid cells and the central necrotic cores (Figs. 2C & 3). No Langhans-type or foreign-body-type giant cells were observed. Histopathological evaluation of the granulomatous lesions associated with the acid-fast bacilli revealed that the kidney and spleen had more severe lesions than the liver of fish examined (Fig. 2D–F; Table 1).

In addition to the granulomatous lesions associated with acid-fast bacilli, melanized fungal hyphae with visible septa, which stained positively with Schmorl's method, were found in the kidney, liver, brain, and ulcerative skin lesions on the head and mandible, in some individuals. Most of these hyphae were surrounded by granulomas with histological features similar to the hyphae found in the mycobacterial granulomas;
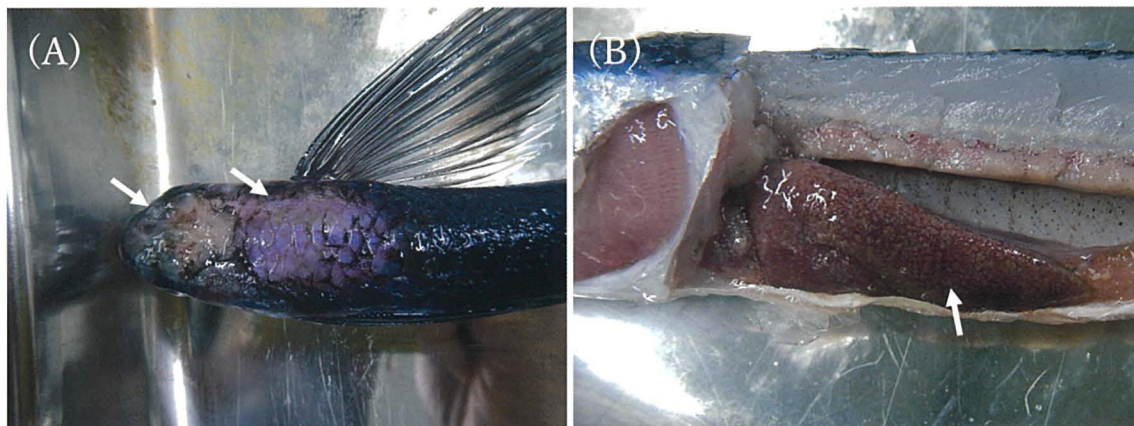

Fig. 1. Gross features observed in diseased sharphead flyingfish *Hirundichthys oxycephalus*: (A) an ulcerative skin lesion on the head and dorso-anterior region of the trunk (arrows); (B) signs of splenomegaly associated with scattering micronodules (arrow).
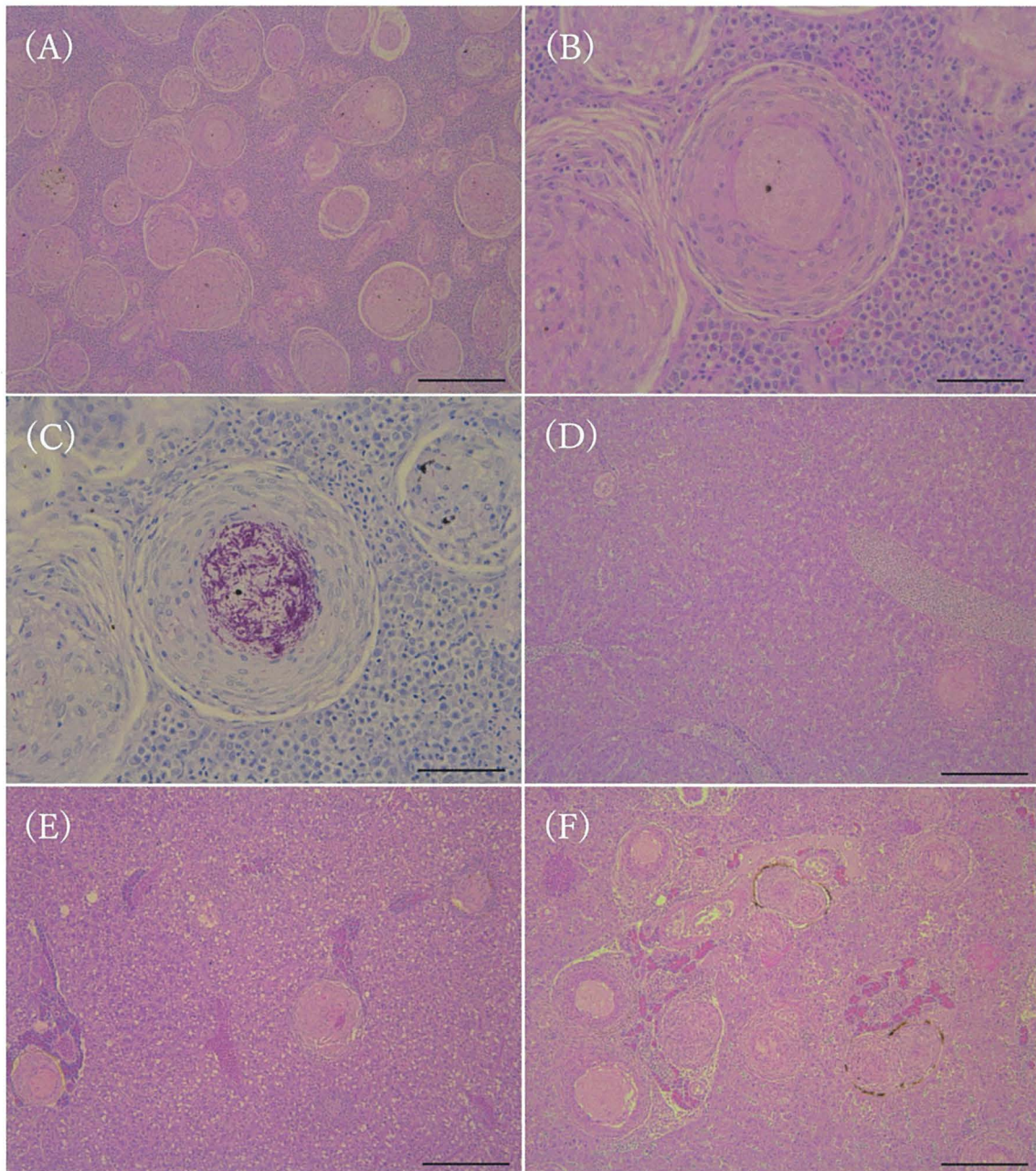
Fig. 2. (A) Granulomas in the kidney of a sharphead flyingfish displaying mycobacteriosis (H&E stain, scale bar = 200 μm). (B) The granulomas were composed of a central necrotic core surrounded by round or spindle-shaped epithelioid cells and a thin outermost rim of fibroblasts and connective tissue (H&E stain, scale bar = 50 μm). (C) Numerous acid-fast bacilli were observed within the epithelioid cells and the central necrotic area (ZN stain, scale bar = 50 μm). (d–f) Liver tissue showing three of four categories of granulomatous lesions: (D) +, Rare; (E) ++, Moderate; (F) +++, Abundant (H&E stain, scale bar = 200 μm).

however, the outermost rims (with fibroblasts and connective tissue) of the mycotic granulomas were not well-developed compared with those seen in mycobacterial granulomas. A few hyphae were also found to cohabit in the mycobacterial granulomas (Fig. 4A). In some fish with severe fungal invasion, the hyphae also penetrated the blood vessels in the kidneys (Fig. 4B) and into bony tissue.

Culture of the nontuberculous mycobacteria

In total, 159 bacterial strains were isolated from 94% (47/50) of the fish examined, through culturing on Middlebrook 7H11 agar. Of these strains, 154 isolates were slow-growing and showed ivory-white and smooth-type colonies, of approximately 1-mm diameter, 14 days after cultivation (Fig. 5A). Five representative isolates of the slow-growers were labeled as NJB1728-216S, NJB1728-510S, NJB1728-615S, NJB1728-715S

and NJB1728-910S. NJB1728-216S, NJB1728-510S, NJB1728-615S, and NJB1728-910S were used to confirm the presence of the insertion sequences IS2404 and IS2606. Additionally, NJB1728-216S, NJB1728-510S, NJB1728-615S, NJB1728-715S were also used for the phylogenetic analysis. From two of the 47 fish, five rapidly growing isolates, characterized by smooth, non-pigmented cream coloration and umbonate colonies with approximately 0.5-mm diameter, were also isolated at 5 days after cultivation. Two representative strains of these five rapid-growers were labeled as NJB1728-411SL and NJB1728-521SL. Smear preparations using all of the representative isolates, stained with ZN, revealed acid-fast bacilli ranging from 2 to 3 μm in length (Fig. 5B). Colonies of the selection of 17 isolates of slow-growers showed ivory-white coloration under dark conditions, which changed to yellow coloration after irradiation with a 60-watt fluorescent lump, indicating photochromogenic strains.
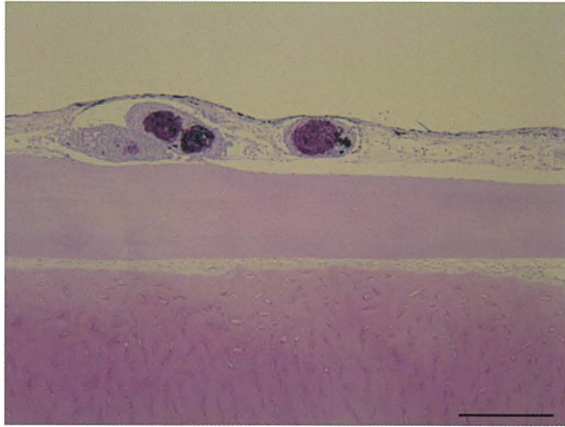

Fig. 3. Granulomas in the ulcerative skin lesions on the head in a sharphead flyingfish displaying mycobacteriosis (ZN stain, scale bar = 200 μm).

From the results of the photochromogenicity tests, the slow-growing isolates (which were the major acid-fast bacterial isolates in the present case) could be classified as Runyon Group I, whereas the rapid-growing isolates could be classified as Runyon Group IV (Runyon, 1959).

Fungal culture

Dematiaceous fungal colonies were isolated from tissues of 10 of 50 fish examined. The selected isolates were labeled as NJM1728-216K, NJM1728-216S, NJM1728-5131L, and NJM1728-615K. Of these, NJM1728-216K and NJM1728-216S were used for the slide culture. The fungal colonies on GY agar were slow growing, slightly domed, velvety to floccose, and gray to olive in color (Fig. 5C). On the slide culture for the olive-colored colony, the conidia showed blastic conidiogenesis, with an ellipsoidal to cylindrical form of 3–4 × 2–3 μm, and with short conidiophores (Fig. 5D). Parts of the hyphae had a spirally twisted structure.

PCR and phylogenetic analysis

From 44 individuals, each DNA sequence for the *hsp65* gene extracted from the frozen spleen sample presented a 100% match to sequences extracted from the slow-growing bacterial strains isolated from the same spleen sample. Moreover, these partial nucleotide sequences for *hsp65* entirely matched (100%) that of *Mycobacterium marinum* (Table 2).

The 16S rRNA, *rpoB*, and *hsp65* gene sequences of the slow grower (NJB1728-510S) and the rapid growers (NJB1728-411SL, NJB1728-521SL) were submitted to the DDBJ database under the following accession numbers: LC549647.1, LC549669.1, and LC549670.1 (for the 16S rRNA, *rpoB*, and *hsp65* genes of NJB1728-510S, respectively); LC549648.1, LC570840.1, and LC570842.1 (for the 16S rRNA, *rpoB*, and *hsp65* genes
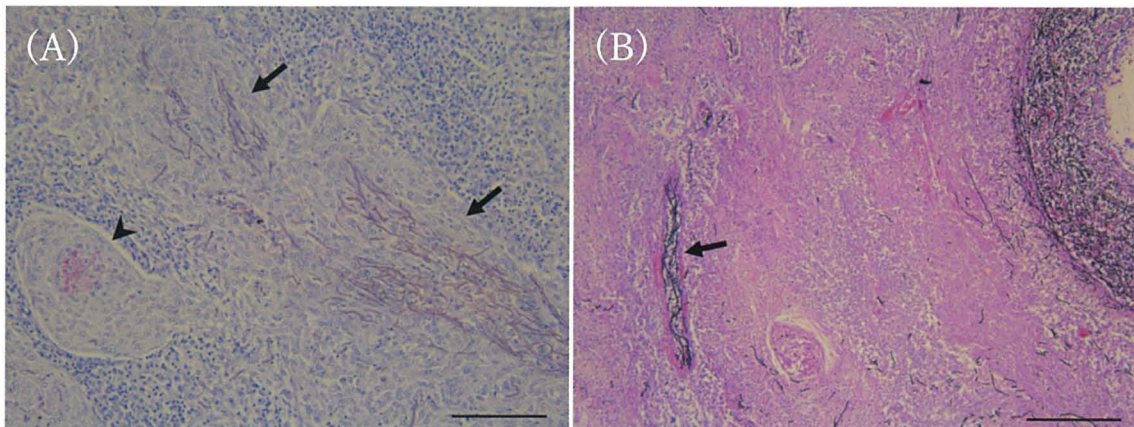

Fig. 4. (A) Example of a mycotic granuloma in tissue of a sharphead flyingfish. Note the abundant, brown-colored hyphae of dematiaceous fungi in the granuloma (arrows); the outermost rim was not as well-developed as in the mycobacterial granuloma (arrowhead) (ZN stain, scale bar = 100 μm). (B) The hyphae penetrated the blood vessels of the kidney in some fish with severe fungal invasion (arrow) (Grocott's–H&E stain, scale bars = 200 μm).
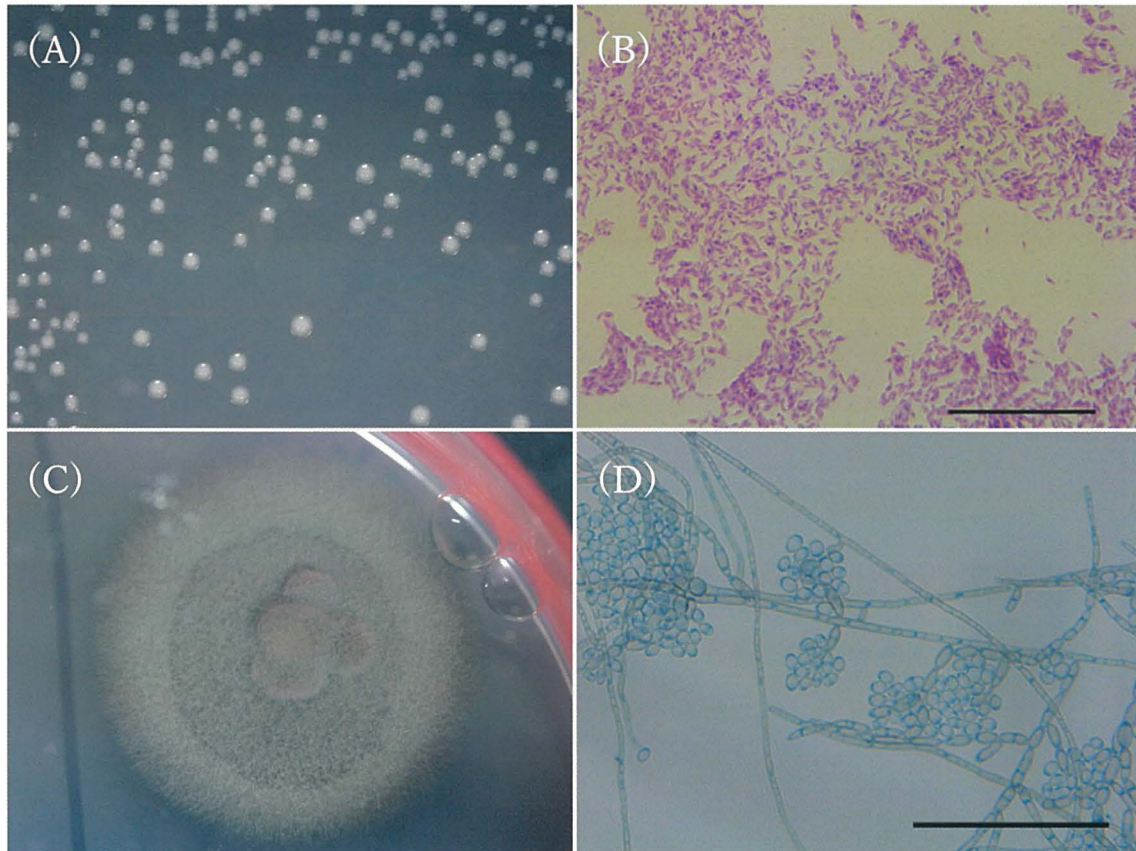
Fig. 5. (A) Colonies of the slow-growing isolate *Mycobacterium marinum* NJB1728-510S.   (B) Smear preparation of NJB1728-216S, revealing acid-fast bacilli, 2–3 μm in length (ZN stain, scale bar = 20 μm).   (C) Colony of *Exophiala aquamarina* NJM1728-615K on GY agar.   (D) Conidia of *E. aquamarina* NJM1728-216K on slide culture (scale bar = 20 μm).

of NJB1728-411SL, respectively); and LC570839.1, LC570841.1, and LC570843.1 (for the 16S rRNA, *rpoB*, and *hsp65* genes of NJB1728-521SL, respectively).   A phylogenetic analysis was carried out using multilocus sequences concatenated with 899 bp of the 16S rRNA, 730 bp of *rpoB*, and 401 bp of *hsp65* of representative spleen tissue samples (labeled as 1728-206S, 1728-510, and 1728-910) and the six isolates (NJB1728-216S, NJB1728-510S, NJB1728-615S, NJB1728-715S, NJB1728-411SL, and NJB1728-521SL) (Fig. 6).

The representative slow-growing isolates, NJB1728-216S, NJB1728-510S, NJB1728-615S and NJB1728-910S, suspected to be *M. marinum*, showed positive features for IS*2404*, while no apparent bands were detected for the IS*2606* sequence with the present isolates (Fig. 7).

DNA sequences for the ITS regions from the fungal isolates and those from the tissue samples were 100% identical.   The sequence analysis of this region confirmed 100% identity as *Exophiala aquamarina*.   The results of phylogenetic analysis with the representative isolate NJM1728-5131L and the tissue sample 1728-425S under 555 bp of the ITS region, are shown in Fig. 8.   The sequence for NJM1728-5131L was

deposited in the DDBJ database under accession number LC547432.1.   The results for all fish examined are summarized in Table 2.

Discussion

Mycobacteriosis caused by nontuberculous mycobacteria (NTM) can result in chronic disease in wild, cultured and aquarium fish (Hashish *et al*., 2018).   *M. marinum* is a slowly growing member, in Runyon Group I of the classification of NTM; its colonies are white, turbid, and smooth, like other mycobacteria, and can be grown with egg-base, agar-base, or broth medium (such as Middlebrook 7H11, 7H10, 7H9, and Lowenstein–Jensen media), with an optimal growth temperature of 30°C (Kent *et al*., 2006; Ostland *et al*., 2008; Aubry *et al*., 2017).

In this study, the characteristics of the major bacterial isolates, such as growth rate, coloration and form of the colony, and photochromogenicity, corresponded with *M. marinum*, as previously described by Aubry *et al*. (2017).

The sequencing and phylogenetic analyses likewise proved high similarities among the isolates, the

Table 2. Summary of the results for all 53 sharphead flyingfish examined in this study.

Fish ID	NTM			Dematiaceous fungi		
	ZN	Species (% of best match in 401-bp *hsp65*)		Schmorl's	Species (% of best match in 379-bp ITS)	
		Tissues	Isolates		Tissues	Isolates
1728-206	ND	*M. marinum* (100)	ND	ND	ND	–
1728-216	ND	*M. marinum* (100)	NJB1728-216S, *M. marinum* (100)	ND	ND	NJM1728-216K, *E. aquamarina* (100)
1728-219	+	ND	ND	+	ND	–
1728-223	ND	*M. marinum* (100)	ND	ND	*E. aquamarina* (100)	–
1728-411	+	*M. marinum* (100)	NJB1728-411S, *M. marinum* (100)*	–	ND	–
1728-4201	+	*M. marinum* (100)	NJB1728-4201S, *M. marinum* (100)	+	ND	NJM1728-4201K, *E. aquamarina* (99.7)
1728-4202	+	*M. marinum* (100)	NJB1728-4202S, *M. marinum* (100)	–	ND	–
1728-425	+	*M. marinum* (100)	NJB1728-425S, *M. marinum* (100)	–	*E. aquamarina* (100)	NJM1728-425S, *E. aquamarina* (100)
1728-426	+	*M. marinum* (100)	NJB1728-426S, *M. marinum* (100)	+	ND	NJM1728-426S, *E. aquamarina* (100)
1728-428	+	*M. marinum* (100)	NJB1728-428S, *M. marinum* (100)	–	ND	–
1728-501	+	*M. marinum* (100)	NJB1728-501S, *M. marinum* (100)	–	–	–
1728-508	–	–	–	–	ND	–
1728-510	+	*M. marinum* (100)	NJB1728-510S, *M. marinum* (100)	+	–	NJM1728-510S, *E. aquamarina* (100)
1728-512	+	*M. marinum* (100)	NJB1728-512S, *M. marinum* (100)	–	–	–
1728-5131	+	*M. marinum* (100)	NJB1728-5131S, *M. marinum* (100)	+	–	NJM1728-5131L, *E. aquamarina* (100)
1728-5132	+	–	NJB1728-5132S, *M. marinum* (100)	–	–	–
1728-518	+	*M. marinum* (100)	NJB1728-518S, *M. marinum* (100)	–	ND	–
1728-519	+	*M. marinum* (100)	NJB1728-519S, *M. marinum* (100)	+	ND	ND
1728-521	+	*M. marinum* (100)	NJB1728-521S, *M. marinum* (100)*	+	ND	–
1728-5221	+	*M. marinum* (100)	NJB1728-5221S, *M. marinum* (100)	–	ND	–
1728-5222	+	*M. marinum* (100)	NJB1728-5222S, *M. marinum* (100)	–	ND	–
1728-528	+	*M. marinum* (100)	NJB1728-528S, *M. marinum* (100)	–	ND	–
1728-531	–	–	–	–	ND	–
1728-601	+	*M. marinum* (100)	NJB1728-601S, *M. marinum* (100)	–	ND	–
1728-605	–	–	–	–	ND	–
1728-607	+	–	NJB1728-607S, *M. marinum* (100)	–	ND	–
1728-615	+	*M. marinum* (100)	NJB1728-615S, *M. marinum* (100)	+	ND	NJM1728-615K, *E. aquamarina* (100)
1728-617	+	*M. marinum* (100)	NJB1728-617S, *M. marinum* (100)	–	ND	–
1728-624	+	*M. marinum* (100)	NJB1728-624S, *M. marinum* (100)	–	ND	–
1728-6251	+	ND	NJB1728-6251S, *M. marinum* (100)	–	ND	–
1728-6252	+	*M. marinum* (100)	NJB1728-6252S, *M. marinum* (100)	–	ND	–
1728-7031	+	*M. marinum* (100)	NJB1728-7031S, *M. marinum* (100)	–	ND	–
1728-7032	+	*M. marinum* (100)	NJB1728-7032S, *M. marinum* (100)	–	ND	–
1728-705	+	*M. marinum* (100)	NJB1728-705S, *M. marinum* (100)	–	ND	–
1728-715	+	*M. marinum* (100)	NJB1728-715S, *M. marinum* (100)	–	ND	–
1728-717	+	*M. marinum* (100)	NJB1728-717S, *M. marinum* (100)	–	ND	–
1728-718	+	*M. marinum* (100)	NJB1728-718S, *M. marinum* (100)	–	ND	–
1728-719	+	*M. marinum* (100)	NJB1728-719S, *M. marinum* (100)	–	ND	–
1728-721	+	*M. marinum* (100)	NJB1728-721S, *M. marinum* (100)	–	ND	–
1728-724	+	*M. marinum* (100)	NJB1728-724S, *M. marinum* (100)	–	ND	–
1728-728	+	*M. marinum* (100)	NJB1728-728S, *M. marinum* (100)	–	ND	–
1728-730	+	*M. marinum* (100)	NJB1728-730S, *M. marinum* (100)	–	ND	–
1728-731	+	*M. marinum* (100)	NJB1728-731S, *M. marinum* (100)	–	ND	–
1728-804	+	*M. marinum* (100)	NJB1728-804S, *M. marinum* (100)	–	ND	–
1728-809	+	*M. marinum* (100)	NJB1728-809S, *M. marinum* (100)	–	ND	–
1728-8101	+	*M. marinum* (100)	NJB1728-8101S, *M. marinum* (100)	–	ND	NJM1728-8101K, *E. aquamarina* (100)
1728-8102	+	*M. marinum* (100)	NJB1728-8102S, *M. marinum* (100)	–	ND	–
1728-811	+	*M. marinum* (100)	NJB1728-811S, *M. marinum* (100)	–	ND	–
1728-818	+	*M. marinum* (100)	NJB1728-818S, *M. marinum* (100)	–	ND	–
1728-824	+	*M. marinum* (100)	NJB1728-824S, *M. marinum* (100)	–	ND	–
1728-828	+	*M. marinum* (100)	NJB1728-828S, *M. marinum* (100)	+	ND	NJM1728-828K, *E. aquamarina* (100)
1728-907	+	*M. marinum* (100)	NJB1728-907S, *M. marinum* (100)	–	ND	–
1728-910	+	*M. marinum* (100)	NJB1728-910S, *M. marinum* (100)	–	ND	–

* Rapid-growing *Mycobacterium* was also isolated, besides the slow-growing.
+: Ziehl–Neelsen (ZN) stain revealed the presence of acid-fast bacilli, or Schmorl's method revealed the presence of melanized fungal hyphae.
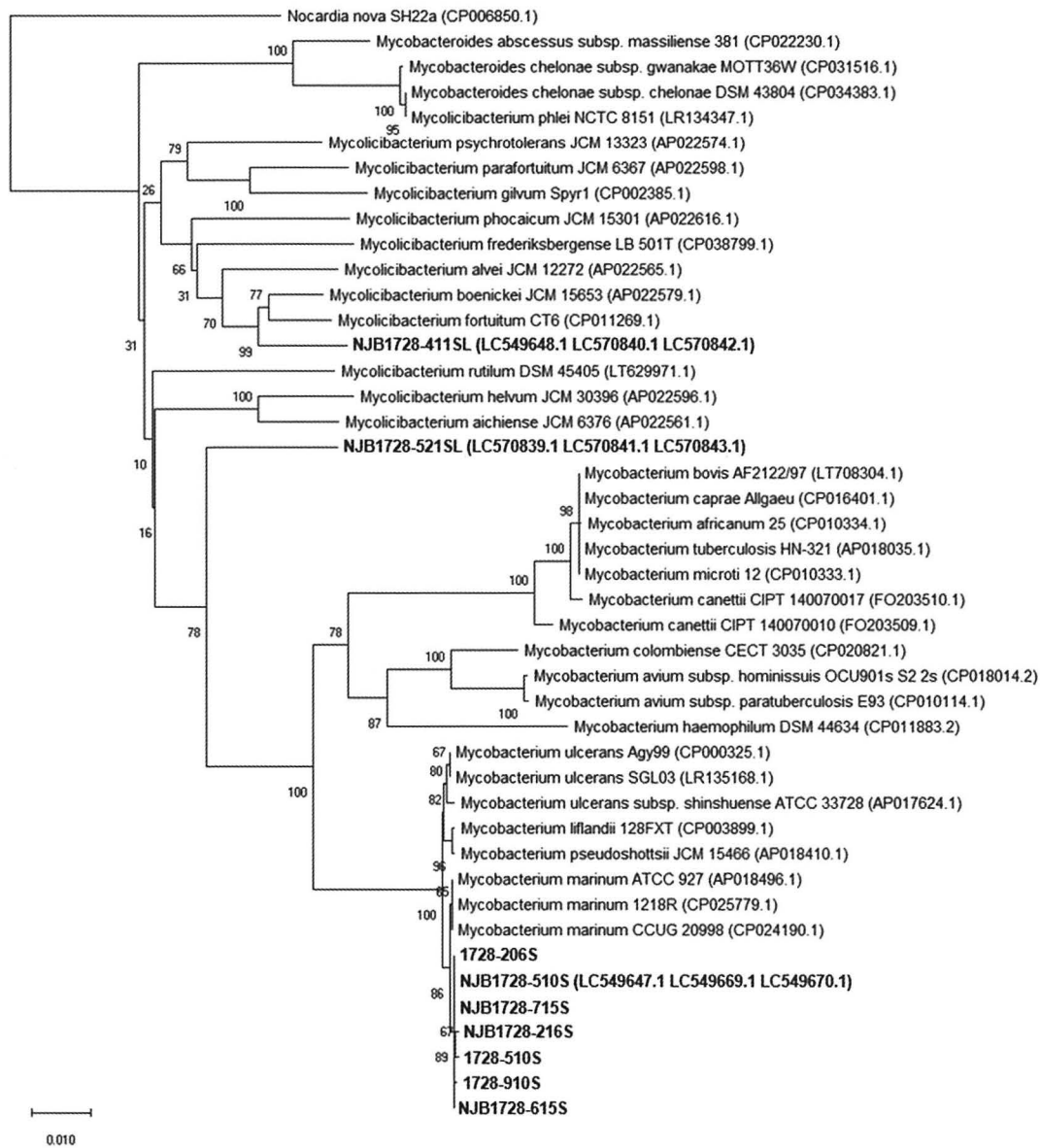–: acid-fast bacilli or dematiaceous fungi were not detected.
ND: not done.

Fig. 6. Neighbor-joining tree generated from a concatenated 2,030-bp sequence of the 16S rRNA, *rpoB*, and *hsp65* genes from spleens of sharphead flyingfish (1728-206S, 1728-510S, 1728-910S) and the six isolates of *Mycobacterium marinum* (NJB1728-216S, NJB1728-510S, NJB1728-615S, NJB1728-715S, NJB1728-411SL, NJB1728-521SL) with Kimura's two-parameter distance correction model.   Bootstrap values are indicated at nodes as a percentage of 1,000 replicates.
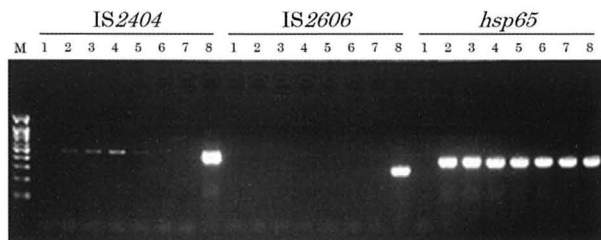

Fig. 7. Results of gel electrophoresis of PCR products to amplify IS*2404*, IS*2606* or the *hsp65* gene in 2% agarose gel: lane 1, water; lane 2, NJB1728-216S; lane 3, NJB1728-510S; lane 4, NJB1728-615S; lane 5, NJB1728-910S; lane 6, *Mycobacterium marinum* ATCC BAA535; lane 7, *M. marinum* JCM17638; lane 8, *M. pseudoshottsii* JCM15466; lane M, 100-bp DNA Ladder RTU (GeneDireX Inc.).

fish tissue samples, and *M. marinum*. PCR analysis targeting the insertion sequences of IS*2404* and IS*2606* found that most type strains of *M. marinum* could be distinguished from other mycolactone-producing myco-bacteria based on the absence of both these sequences (Stinear *et al.*, 1999). However, it has been revealed that some strains of *M. marinum* possess varying copy numbers of IS*2404* and IS*2606*, which consequently affects PCR identification of the genes (Stinear *et al.*, 1999; Chemlal *et al.*, 2002; Stragier *et al.*, 2007). In the present study, several isolates showed positive results for IS*2404* but not for IS*2606*. These fea-tures corresponded to those previously reported for *M. marinum* CC240299 isolated from koi carp *Cyprinus*
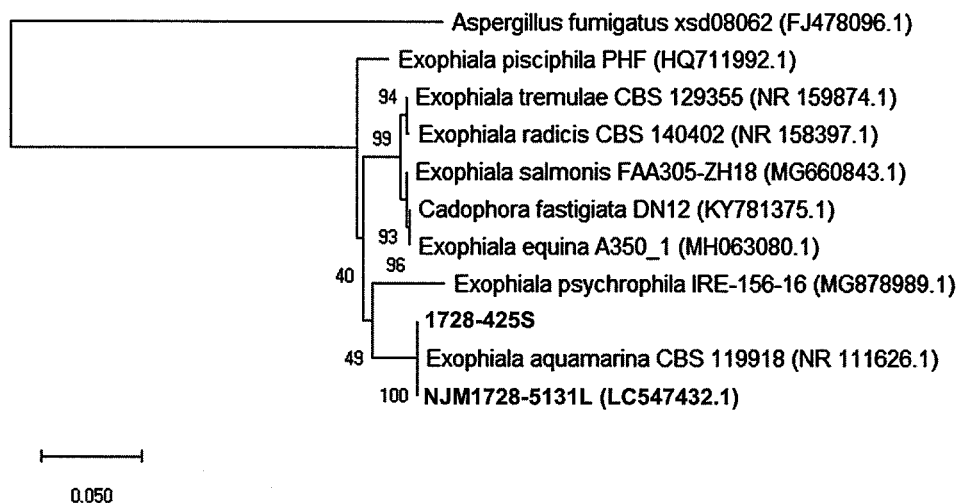
Fig. 8. Neighbor-joining tree generated from a 555-bp sequence of ITS1, 5.8S ribosomal RNA, and ITS2 from a representative dematiaceous fungal isolate (NJM1728-5131L) and from a tissue sample (1728-425S) in the present case, using Kimura's two-parameter distance correction model.   Bootstrap values are indicated at nodes as a percentage of 1,000 replicates.

carpio in Israel (Stragier et al., 2007) and for M. marinum ITM06-3844 isolated from an Acipenser baerii–A. guldenstaedtii hybrid sturgeon (Stragier et al., 2008). From these overall results, the present disease episode in sharphead flyingfish could be diagnosed as mycobacteriosis, with M. marinum as the likely pathogen. To our knowledge, bacterial infection has never been reported in any flyingfish species. This is the first case of mycobacteriosis caused by M. marinum in a flyingfish species.

We isolated both slow- and rapid-growing mycobacteria from two of the diseased flying fish examined; however, the representative rapid-growing strains, NJB1728-411SL and NJB1728-521SL, could not be identified to species. NTM are ubiquitous microorganisms that have been isolated from various substances, including fresh and marine waters, drinking water, dust from agricultural fields, garden and potting soils, and the aerosols of natural and drinking waters (Marumo et al., 2010; Falkinham, 2015). The rapid growers were isolated in only two of 50 fish examined. Therefore, we speculated that the rapid growers might be contaminated environmental microorganisms. However, the possibility remained of a mixed mycobacterial infection, as has been reported in Australian lungfish Neoceratodus fosteri, Chinese sturgeon Acipenser sinensis, and Amur sturgeon Acipenser schrenckii (Zhang et al., 2015; Strike et al., 2017).

Histopathological examination of piscine mycobacteriosis has shown granulomas primarily in the various internal organs, but especially the spleen, kidney and liver, during the earlier stage of disease (Weerakhun et al., 2008; Jacobs et al., 2009), but with some rare exceptions, such as no granulomas in the kidney of reared mullet with M. marinum (Antuofermo et al., 2017) or rare granulomas in the spleen of zebrafish Zebra danio artificially infected with M. marinum (Swaim et al., 2006). In

the present study, the granulomatous lesions were the most evident and more severe in the kidney and spleen compared with in the liver, and the gross features and histopathology were quite similar to that described in infected goldfish Carassius auratus (Pourahmad et al., 2014). Thus, it was likely that variations in the gross and histopathological features of piscine mycobacteriosis might be related to the difference of host fish species.

The results of the morphological, sequencing and phylogenetic analyses revealed that the dematiaceous fungal isolates of the present case should be identified as E. aquamarina, with pathological characteristics that corresponded with those described previously by de Hoog et al. (2011), with an exception for brain involvement in a few fish examined in the present case. In addition, the present case was characterized by co-infection with M. marinum and E. aquamarina, as observed in 13 of 53 fish examined (24.5%). To our knowledge, a case of co-infection with acid-fast bacilli and an imperfect fungus was first described in humphead wrasse Cheilinus undulatus (Wada et al., 1993); however, co-infection with a NTM and a dematiaceous fungus in fish constitutes a novel description.

Exophiala aquamarina infection on its own can affect some fish species (de Hoog et al., 2011); however, no case of single-infection mycosis was detected in the present study. This finding leads to a hypothesis that, in the pathogenesis of the present case, M. marinum might function as the primary pathogen, and E. aquamarina might secondarily infect the fish.

Because some strains of M. marinum have been isolated from cutaneous lesions in humans, this NTM species is considered an important, candidate aquatic zoonotic pathogen (Broutin et al., 2012; Slany et al., 2013). Further investigations of aquatic environments that include the epidemiology of this NTM species

should be required to recognize the potential threat of this mycobacterium to public health.

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飼育下ホソアオトビに発生した黒色真菌感染を伴う非結核性抗酸菌症の発生事例

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国内飼育ホソアオトビ群において，連続死亡事例が発生した。それらトビウオ群の頭部皮膚には潰瘍性出血病変を認め，腎臓，脾臓，肝臓などの臓器に白色の微小結節が散在していた。病理組織学的検査により，中心に抗酸菌を含む肉芽腫が全身臓器に確認され，特に腎臓と脾臓で病変が顕著であった。いくつかの個体の腎臓あるいは脾臓において，抗酸菌だけでなく黒色真菌の菌糸も認められた。病変部からは抗酸菌が分離され，分子生物学的解析の結果，大部分の分離菌株は *Mycobacterium marinum* と同定された。さらに真菌培養において黒色真菌が得られ，形態学的および分子生物学的解析の結果，分離真菌は *Exophiala aquamarina* と同定された。本研究が魚類における非結核性抗酸菌（*M. marinum*）と黒色真菌（*E. aquamarina*）の重複感染症例の初報告である。