academicJournals

Vol. 8(38), pp. 3441-3452, 17 September, 2014
DOI: 10.5897/AJMR2014.6874
Article Number: 6C47EC447980
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Nocardia seriolae, a causative agent of systematic granuloma in spotted butterfish, *Scatophagus argus*, Linn

Pei-Chi Wang[1], Ming-An Tsai[1], Yu-Chi Liang[1], Yanting Chan[2] and Shih-Chu Chen[1]*

[1]Department of Veterinary Medicine, College of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, Taiwan, ROC.
[2]Diagnostic and Prevention Center of Livestock Diseases, Ilan, Taiwan, ROC.

Received 3 May, 2014; Accepted 5 September, 2014

A *Nocardia seriolae* was isolated from diseased spotted butterfish (*Scatophagus argus*, Linn). The cumulative mortality within one month was 30% (660 out of 2200). The diseased fish were two years old with lengths from 18 to 25 cm. Most fish suffered from haemorrhages and ulcers of the skin. The most significant gross pathological change was enlargement of the spleen, kidney and liver. White nodules, varying in size, were found in these organs. The isolated bacteria were either coccoid or filamentous in appearance, with bead-like staining. The identification of NS128 was verified by polymerase chain reaction (PCR) assay for *N. seriolae* that gave the expected specific amplicon of 432 bp of the 16S rDNA sequence with a 100% sequence identity with *N. seriolae* (GenBank accession number AF380937). A partial sequence of the 16S rRNA gene (GenBank accession number EU147501), the RNA polymerase B subunit (rpo B) gene (GenBank accession number DQ119300) and the heat shock protein gene (GenBank accession number DQ431437) of the organism, NS128 and the type strain of *N. seriolae* BCRC 13745 formed a monophyletic clade with a high sequence similarity and a bootstrap of 100%. White nodules that were induced in experimental fish, spotted butterfish and amberjack (*Seriola dumerili*, Risso) were similar to those in naturally infected fish cases and *N. seriolae* were re-isolated using brain heart infusion agar. These finding provides evidence that *N. seriolae* caused systemic granulomas in spotted butterfish. Based on the growth characteristics, and biochemical properties of the bacterium, its histopathological changes, PCR and the phylogenetic analysis, the pathogenic organism was identified as *N. seriolae*. This investigation is the first published on *N. seriolae* infection in spotted butterfish in aquaculture. The results reveal that the *N. seriolae* isolated in the field was pathogenic to spotted butterfish and amberjack.

Key words: Pathogenicity, *Nocardia seriolae*, spotted butterfish, *Scatophagus argus*, polymerase chain reaction (PCR).

INTRODUCTION

Nocardiosis in fish is a disease characterized by systemic nodulation, manifested both on the skin and internal organs. Three species of *Nocardia* have been isolated from diseased fish. They are *Nocardia salmonicida*, *N. seriolae* (formerly *N. kampachi*), and *N. asteroides* (Chen et al., 1989; Isik et al., 1999; Kudo et al., 1988).

N. asteroides in Formosa snakehead, *Channa maculata* and largemouth bass, and *Micropterus salmoides,* in

Taiwan was first described by Chen et al. (1989) and Chen and Tung (1991) in Taiwan. These outbreaks have caused substantial commercial losses. Notably, *N. seriolae* is the causative agent of nocardiosis in cultured yellowtail, *Seriola quinqueradiata* (Kariya et al., 1968; Kumamoto et al., 1985; Kusuda et al., 1974), fingerling or yearling rainbow trout, *Oncorhynchus mykiss*, which develop lesions within one to three months of experimental injection with *N*. *asteroides* (Snieszko et al., 1964). Elkesh et al. (2013) reported systemic nocardiosis in cultured meagre, *Argyrosomus regius* Asso with a low to variable morbidity and 1–4% total mortality. Recently, Japanese sea-perch, *Lateolabrax japonicum*, striped mullet, *Mugil cephalus*, large yellow croaker, *Larimichthys crocea* (Richardson), three striped tigerfish, *Terapon jarbua,* weakfish, *Cynoscion regalis* (Bloch and Schneider) and yellowtail have all been infected with *N. seriolae* (Chen et al., 2000; Wang et al., 2005; Shimahara et al., 2006; Shimahara et al., 2008; Wang et al., 2009; Cornwell et al., 2011). Although isolation and bacteriological analysis are complex and time-consuming, *N. seriolae* has been identified as one of the major nocardial pathogens in fish. Therefore, molecular detection methods of *Nocardia* species have been developed, including polymerase chain reaction (PCR) (Miyoshi and Suzuki, 2003), real-time PCR (Carrasco et al., 2013) and loop-mediated isothermal amplification (Itano et al., 2006). Miyoshi and Suzuki (2003) developed a PCR assay based on unique regions of the *N. seriolae* 16S rRNA gene that allows specific identification. In the present paper, we describe the first isolation and characterization of pathogenic *Nocardia* species obtained from diseased spotted butterfish, *Scatophagus argus* with systematic granuloma during an outbreak of nocardiosis in the summer of 2005 in Taiwan.

MATERIALS AND METHODS

Collection of fish sample

The farm that was sampled in this investigation has two ponds, one of which was affected. The disease developed between June and July, 2005 in a pond (with salinity 5‰ and water temperature 26°C) that contained 2200 spotted butterfish (*Scatophagus argus*, Linn), that were approximately two year old. The fish had an average body weight of 1.2–1.4 kg and an average length of 21–27 cm. They had been fed with commercial pellets without any trash fish. The moribund eight fish from affected pond were subjected to histopathological and bacteriological examination.

Bacteriology

The inoculating loops were taken from the kidney, heart, spleen, and liver and streaked on tryptic soy agar (TSA), blood agar (BA), brain heart infusion (BHI) agar and Lowenstein–Jensen medium (LJM). Plates were then incubated at 25°C for 30 days.

Histopathology

The kidney, spleen, liver and other internal organs with lesions were fixed in 10% buffered formalin and processed for paraffin sectioning. Sections were stained using haematoxylin and eosin (H&E) and the Ziehl-Neelsen's (ZN) methods.

Bacterial strains

The following bacterial strains were employed as controls for microbiological tests and polymerase chain reaction (PCR) assays: *N. farcinica* (Bioresource Collection & Research Center, Hsinchu, Taiwan, BCRC 13380), *N. seriolae* (BCRC 13745), *N. seriolae* NS127, and *N. salmonicida* (BCRC 12441). The purified bacterial isolate derived from infected fish was labeled NS128. These strains were routinely grown on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Minch.) at 25℃ for five days.

API ZYM test

The API ZYM test was applied to identify the enzymatic profile of NS128 and reference strains. The test kit has 20 cupules: 19 substrates and a control. A representative bacterial colony on BHI agar was suspended in 3 mL of sterile saline (0.85% NaCl) that contained sterile glass beads (2 mm in diameter). The suspension was then shaken using a mixer for 10 min to disperse the bacteria. The turbidity of each suspension was adjusted to a number 6 McFarland standard and 65 µL of bacterial suspension was added to each cupule of the strip following the manufacturer's instructions. Plates were incubated at 25°C for NS128 and reference strains*, N. seriolae* (BCRC 13745), *N. seriolae* (NS127), *N. salmonicida* (BCRC 12441) and at 35°C for reference strain for *N. farcinica* (BCRC 13380). Following incubation for 5 h, ZYM A and B reagents were added and plate reactions were terminated at 5 min.

Specific primer for *N. seriolae* 16S analysis (432 bp)

The DNA sample preparation was modified from the methods developed by Telenti et al. (1993). The PCR amplification was performed by using the oligonucleotide primers NS1: 5'-ACTCACAGCTCAACTGTG-3' and NG1: 5'-ACCGACCACAAGGGGG-3' according to the manufacturer's protocol of Laurent et al. (1999 and 2000) and Miyoshi and Suzuki (2003). The specific primer set NS1-NG1 targets 16S rRNA gene (16S rDNA) of *N. seriolae* and yields the 432 bp amplicons.

16S rDNA analysis (596 bp)

The PCR amplification of the 16S rDNA gene from *Nocardia* spp. was conducted using a purified DNA template with the forward primer NG1 (5'-ACCGACCACAAGGGGG-3') and the reverse primer NG2 (5'-GGTTGTAAACCTCTTTCGA-3') and amplified a 596 bp fragment using a method modified from that of Laurent et al. (1999, 2000).

*Corresponding author. E-mail: scchen@mail.npust.edu.tw. Tel: 886 8 7703202 ext 5076 or 5095. Fax: 886 8 7700447.

Heat shock protein gene analysis (439bp)

The PCR method was performed according to Telenti et al. (1993). Primers Tb11 (5'-ACCAACGATGGTGTGTCCAT-3') and Tb12 (5'-CTTGTCGAACCGCATACCCT-3') amplified a 439-bp fragment between positions 398 and 836 of the published gene sequence (Schinnick, 1987).

RNA polymerase B subunit (rpo B) gene analysis (342bp)

Kim et al. (1999), adopted a set of primers (MF, 5'-CGACCACTTCGGCAACCG-3'; MR, 5'-TCGATCGGGCACATCCGG-3') which was used to amplify rpo B DNA (342 bp) in this study. The rpo B gene encompasse the Rif^r region, which is associated with rifampicin resistance in *M. tuberculosis*. The PCR products were electrophoresed on a 2% agarose gel.

Phylogenetic analysis

The PCR amplicons from the 16S rRNA gene, the heat shock protein gene, and the RNA polymerase beta subunit (rpo B) gene of *N. seriolae* strain NS127, NS128 and reference strain*, N. seriolae* (BCRC 13745) used a model 377 automatic DNA sequencer (performed by Tri-I Biotech, Taipei,. Taiwan). The DNA sequences thus obtained were aligned with representative sequences from the GenBank, DDBJ, and EMBL databases. To prepare phylogenetic trees, CLUSTAL X software version. 1.81 was utilized with the minimum evolution (Rzhetsky and Nei, 1993), maximum parsimony (Fitch, 1971), and neighbor-joining NJ, (Saitou and Nei, 1987) treeing algorithms. Evolutionary distance matrices for the minimum evolution, MP, and NJ methods were generated using the methods developed by Jukes and Cantor (1969). The MEGA2 computer program (Kumar et al., 2001) was utilized for all analyses. The resulting tree topologies were investigated using bootstrap analyses (Felsenstein, 1985) according to the NJ method with 1000 repetitions.

Sensitivity to drug

For antibiotic sensitivity tests, isolates were spread on Mueller-Hinton agar plates and exposed to antibiotic discs that contained erythromycin (15 µg), doxycycline (30 µg), spiramycin (100 µg), tetracycline (30 µg), oxytetracycline (30 µg), streptomycin (10 µg), and neomycin (30µg). The plates were incubated at 25°C for 18 h and the inhibition of the bacteria by chemotherapeutic agents was evaluated.

Experimental infection

Healthy, spotted butterfish *Scatophagus argus*, with body weights of 50-55 g, and amberjack *Seriola dumerili* with body weights of 100-125 g, were obtained from a fish farm in Pingtung, Taiwan, and maintained in continuously aerated 500 L aquaria that contained 450 L of water at approximately 25°C for seven days until they were acclimatized to the laboratory. The fish were fed twice daily with commercial fish pellets and waste was removed daily. Spotted butterfish and amberjack were assigned to two equal treatment groups (with eight in each group of spotted butterfish and four in each group of amberjack) in separate tanks and fed daily using a commercial diet until they were starved one day before the inoculation to avoid fish stress. *N. seriole* (NS128) was grown on BHI at 25°C for five days and then harvested using normal saline. A bacterial suspension was prepared in saline solution to a final concentration of $4.7x10^7$ CFU mL^{-1}. The 0.1 mL^{-1} ($4.7x10^6$ CFU) bacteria suspension was injected intraperitoneally (IP) into eight spotted butterfish and four amberjack in each treatment group, respectively. The fish in the control group were inoculated with sterile saline. Following the injections, each group was kept separately in a 150 L aquarium under the same conditions as described above for the acclimatization period. The fish were continuously monitored for morbidity and mortality and sampled for histopathological and bacteriological analyses. The experiment was terminated at fifteen days after inoculation. Organ smear and re-isolation were also performed from survivors.

RESULTS

Clinical signs and pathology

The naturally infected cultured spotted butterfish had skin ulcers and necrosis. They had numerous marked yellowish-white nodules, ranging from 0.1 to 0.4 cm in diameter on the serosal surface, and on many internal organs, especially the liver, gills, heart, spleen, and kidney (Figure 1). Cumulative mortality within 30 days was 30% (660 out of 2200). Histopathologically, most nodules were typically granulomas. Some were multifocal, while others were relatively more diffusely distributed. The granulomatous foci, varied in size and comprised necrotic tissue debris with some bacterial clumps in their centres, surrounded by epithelioid cells, particularly in the liver, spleen and kidney (Figure 2). Macrophages and epithelioid cells were present in the young nodules, but no multinucleated giant cells or connective tissue was observed. Acid-fast, filament or bead-like bacteria were identified in the nodules using ZN staining (Figure 3), especially in the centres of the granulomas and within macrophages. Liver, kidney, and spleen were consistently affected.

Bacteriology

The bacteria from lesions of internal organs were Gram and ZN-positive. The bacterial shapes ranged from coccoid to short rods and some appeared as long, filament-tous, multiseptate rods. Bacterial colonies from the spleen, liver and heart appeared three days after culturing on BA, TSA, BHI and LJM at 25°C. The bacteria were Gram and ZN-positive. The bacteria grew at 25°C, but not at 37°C or 41°C; they did not survived for 8 h at 50°C (Table 1). The organisms could neither reduce nitrate nor degrade starch, xanthine, tyrosine or casein. The biochemical characteristics of NS128 were identical to those of reference strain *N. seriolae* BCRC 13745 and *N. seriolae* NS127, but differences were evident among the urea utilizations, temperatures and salinity tolerances between NS128 and other reference strains, BCRC 13364 *N. asteroides,* BCRC 12441 *N. salmonicida* and BCRC 13380 *N. farcinica*.

API ZYM test

Table 2 shows the enzymatic profiles of the *N. seriolae*

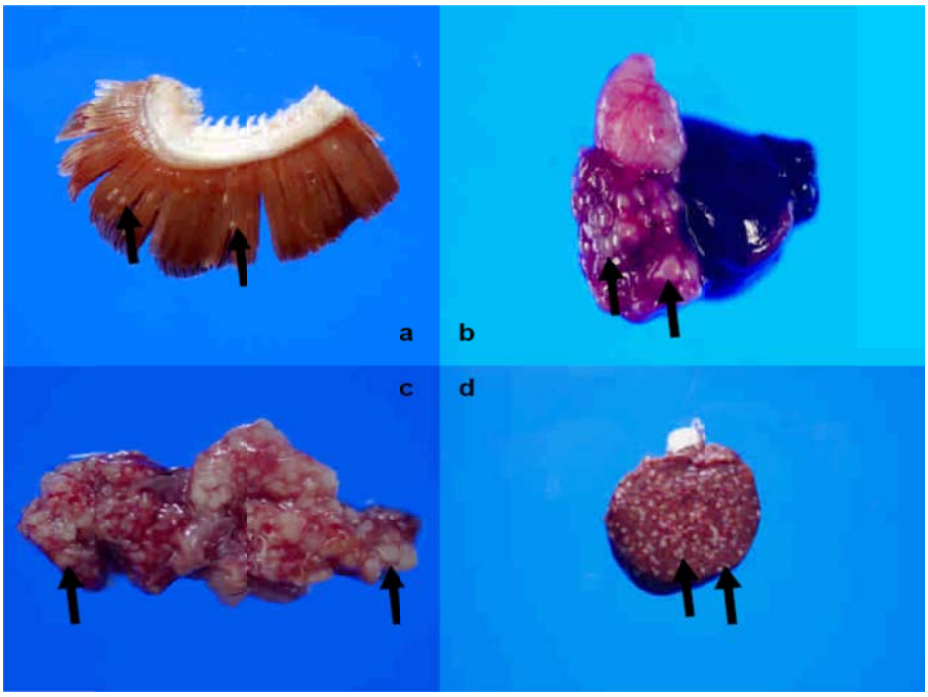

Figure 1. Diseased spotted butterfish *Scatophagus argus* by natural infection showing numerous nodular structures in gill (a), heart (b), kidney (c) and spleen (d) (arrows).

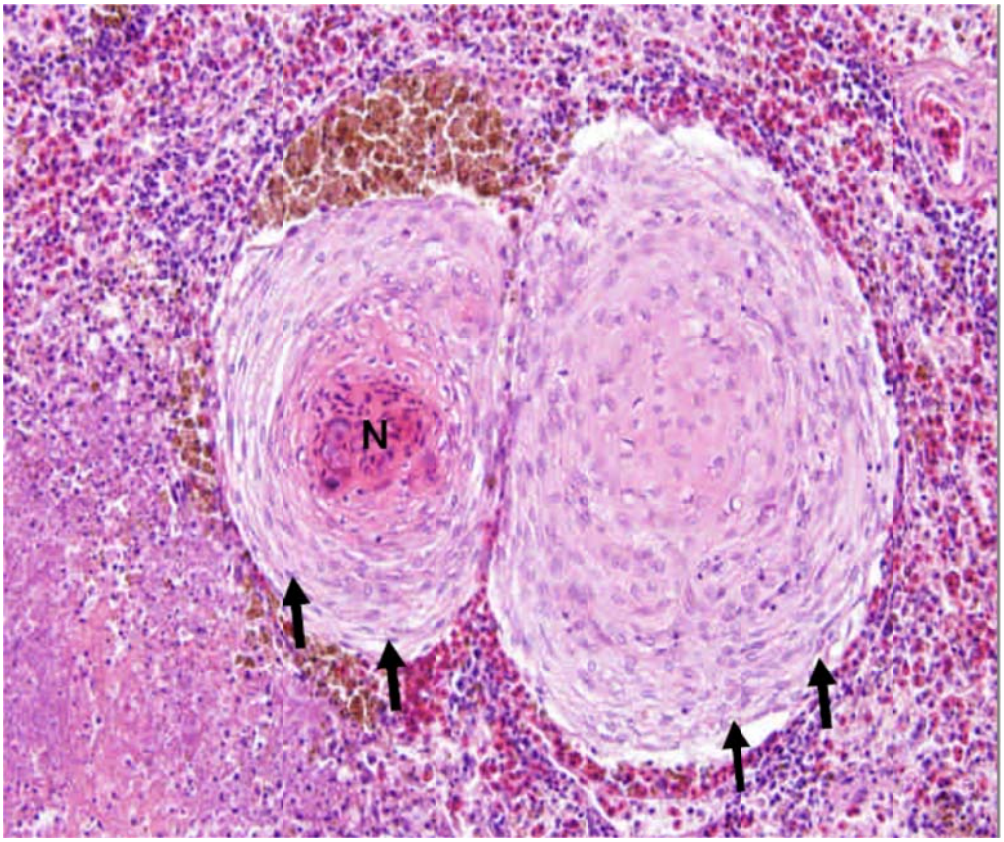

Figure 2. Diseased spotted butterfish *Scatophagus argus* by natural infection. Granuloma (arrows) with marked central necrosis (N) in the spleen of spotted butterfish. (H & E stain methods, X 400).

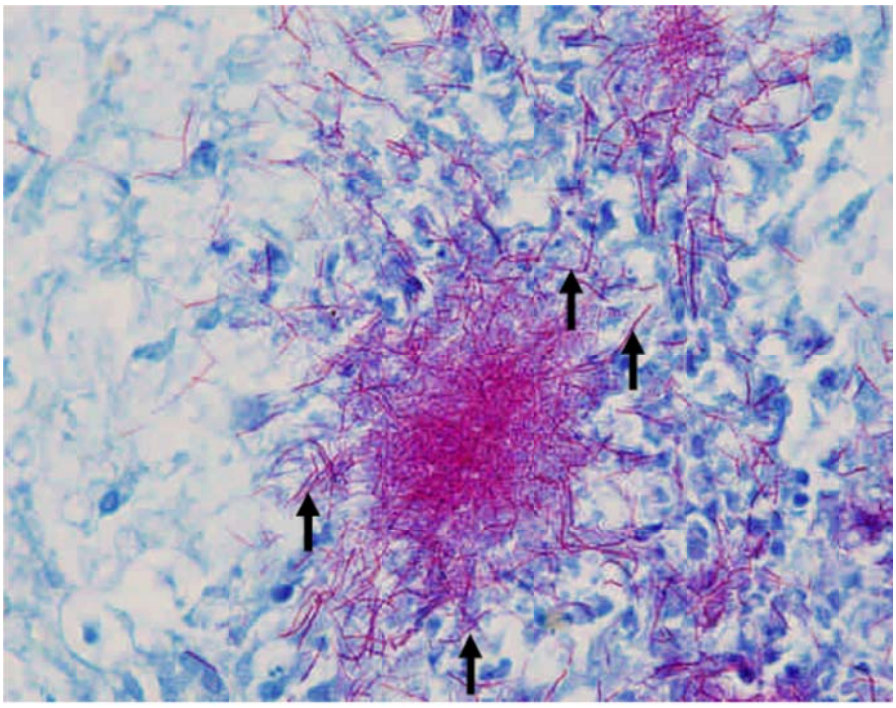

Figure 3. Spleen of the spotted butterfish *Scatophagus argus*, Many beaded filamentous bacteria (arrows) are seen in the necrotic area of granuloma in the spleen (Z-N staining method, X1000).

Table 1. Physiological and biochemical characteristics of NS128 isolates and reference strains of *Nocardia.*

Parameter	**NS 128**	***Nocardia seriolae* NS127**	***Nocardia seriolae*** BCRC 13745	***Nocardia asteroides*** BCRC 13364	***Nocardia salmonicida*** BCRC 12441	***Nocardia farcinica*** BCRC 13380
Urea	–	–	–	+	+	+
Starch	–	–	–	–	–	–
Xanthine	–	–	–	–	–	–
Tyrosine	–	–	–	–	–	–
Casein	–	–	–	–	–	–
Gelatin	–	–	–	–	–	–
Growth at						
25°C	+	+	+	+	+	+
37°C	–	–	–	+	–	+
40°C	–	–	–	+	–	–
Survival at 50°C						
2h	–	–	–	+	–	+
4h	–	–	–	+	–	+
8h	–	–	–	+	–	+
Catalase	+	+	+	+	+	+
Oxidase	–	–	–	–	–	–
Acid form						
Mannose	–	–	–	–	–	–
Rhamnose	–	–	–	–	–	–
Trehalose	–	–	–	–	–	–

Table 1. Contd

Erythritol	−	−	−	−	−	−
Maltose	−	−	−	−	−	−
Arabinose	−	−	−	−	−	−
PH9.0	−	−	−	+	+	+
Growth with						
1%NaCl	+	+	+	+	+	+
2%NaCl	−	−	−	+	+	+
3%NaCl	−	−	−	−	−	−

isolates and reference strains that were obtained by using the API ZYM test. There were minor differences between NS128 and reference strain *N. seriolae* BCRC 13745 in some assimilation tests, such as with cystine arylamidase, valine arylamidase chymotrypsin, and β-galactosidase. Based on the results of morphological, physiological and biochemical tests, the NS128 that was isolated from diseased spotted butterfish, was classified in the genus *Nocardia*. However, from the growth characteristics, and the biochemical properties of the bacteria, determined by the API ZYM test, determining whether NS128 was conspecific with *N. seriolae* was difficult. The isolate from spotted butterfish was cultured and identified as *N. seriolae* using PCR methods.

Specific primer for *N. seriolae* (432 bp)

The PCR assay amplified a band of 432 bp of 16S rDNA gene from NS128 and the reference strain, *N. seriolae* (BCRC 13745) (Figure 4). These amplicons were not obtained from *N. farcinica,* BCRC 13380, *N. salmonicida* (BCRC 12441), or *Mycobacterium fortuitum* (BCRC 15320).

16S rDNA analysis (596bp)

Isolate NS128 bacteria from infected fish yielded an expected PCR product of 596 bp using NG1 and NG2 primers. The sequence had 100% identity with *N. seriolae* (GenBank accession number AF380937), 97.3% similarity to *N. nova* (GenBank accession number Z36930), 95.8% similarity to *N. asteroides* (GenBank accession number X84851), 94% similarity to *N. salmonicida* Z46750 (GenBank accession number X84851), and 93.6% similarity to *N. otitidiscaviarum* (GenBank accession number X80611).

RNA polymerase gene (rpo B) analysis (342 bp)

The bacterial isolate of NS128 from infected spotted butterfish yielded an expected PCR product of 342 bp using MF and MR primers. The sequence exhibited a 100% identity with *N. seriolae* (GenBank accession number AY017474 and BCRC 13745), 92.9% similarity to *M. fortuitum* AY147156, and 92.2% similarity to *M. mucogenicum* (GenBank accession number AY147174).

Heat shock protein gene (439 bp)

Sequencing the heat shock protein gene yielded an expected PCR product of 439 bp using Tb11 and TB12 primers. The sequence had 100% identity with *N. seriolae* (GenBank accession number AY756533), 95.4% similarity to *N. asteroides* (GenBank accession number AY756513), 94.9% similarity to *N. otitidiscaviarum* (GenBank accession number AY756528), 93.8% similarity to *N. farcinica* (GenBank accession number AY756523), 93.2% similarity to *N. brevicatena* (GenBank accession number Z36930), and 92.7% similarity to *N. nova* (GenBank accession number AY756527).

Phylogenetic analysis

The PCR sequences of the 16S rDNA gene, heat shock protein gene (439 bp), and RNA polymerase B subunit (rpoB) gene analysis (342 bp) of NS128 from diseased spotted butterfish, were deposited in GenBank (Accession Nos.: EU147501 for 16S rDNA, DQ119300 for RNA polymerase B subunit (rpoB) gene, DQ431437 for heat shock protein, respectively). The sequences of 16S rDNA gene, heat shock protein gene, and RNA polymerase B subunit (rpoB) gene of NS128 from diseased spotted butterfish, exhibited identities of 100% with reference strains (*N. seriolae* GenBank accession Nos.: AY380937, AY756553 and BCRC 13745), respectively. CLUSTAL X software version 1.81 was used to determine an evolutionary tree (Figures 5, 6 and 7) that indicates that these strains form a unique clade with the reference strain, *N. seriolae*, at a distance from other *Nocardia* and *Mycobacteria*. This relationship was emphasized by the relatively high nucleotide similarity

Table 2. API-ZYM Kit results of isolates NS128 and reference strains of *Nocardia.*

Characteristics	NS128	*Nocardia seriolae* NS 127	*Nocardia seriolae* BCRC 13745	*Nocardia asteroides* BCRC 13364	*Nocardia salmonicida* BCRC 12441	*Nocardia farcinica* BCRC 13380
Control	–	–	–	–	–	–
Alkaline Phosphatase	+	+	+	+	+	+
Butyrate esterase	–	+	–	–	+	–
Caprylate esterase lipase	+	+	+	+	+	–
Myristate lipase	–	–	–	–	+	–
Leucine arylamidase	+	+	+	+	+	+
Valine arylamidase	+	+	–	–	–	+
Cystine arylamidas	+	–	–	+	–	–
Trypsin	+	+	+	–	+	+
Chymotrypsin	+	–	–	–	–	–
Acid Phosphatase	+	+	+	+	+	+
Naphthol- AS-BI-phosphohydrolase	+	+	+	+	+	+
α-Galactosidase	–	–	–	–	–	–
β- Galactosidase	+	–	–	–	–	–
β- Glucuronidase	–	–	–	–	–	+
α-Glucosidase	+	+	+	+	+	+
β- Glucosidase	+	+	+	+	+	+
N-acrtyl-β-Glucosaminidase	–	–	–	–	–	–
α-Mannosidase	–	–	–	–	–	–
α-Fucosidase	–	–	–	–	–	–

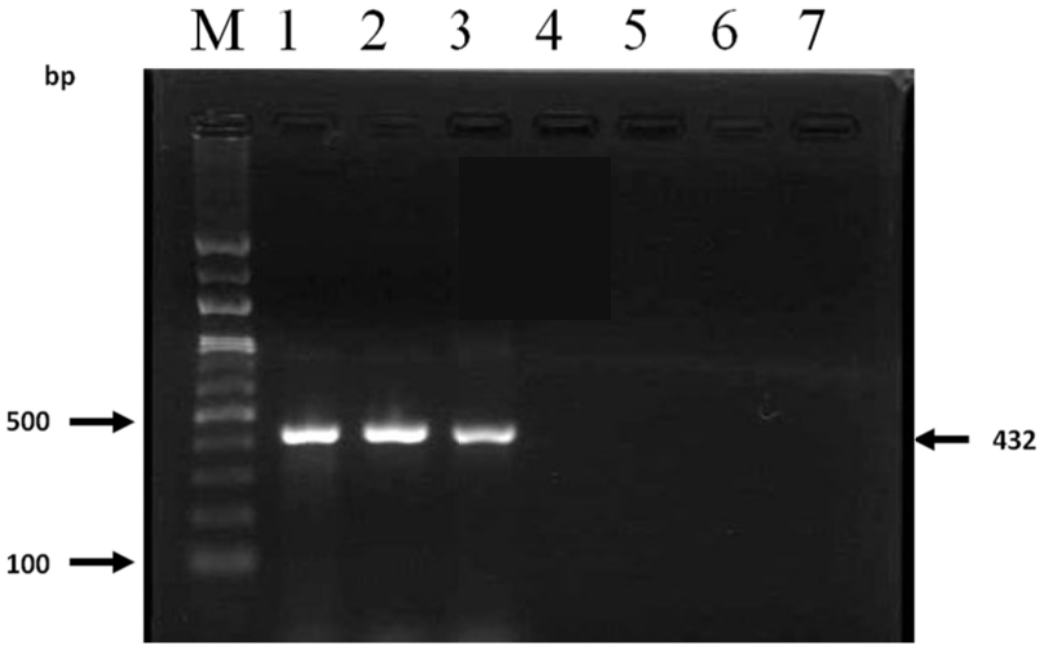

Figure 4. Electrophoretic analysis of 16S rDNA gene of *N. seriolae* isolates by primers NS1 and NG1. M, marker; 1, NS128; 2, *N. seriolae* NS127; *N. seriolae* BCRC13745; 4, *N. salmonicida* BCRC12441; 5, *N. asteroides* BCRC13364; 6, *N. farcinica* BCRC13722; 7, negative control (without template DNA).

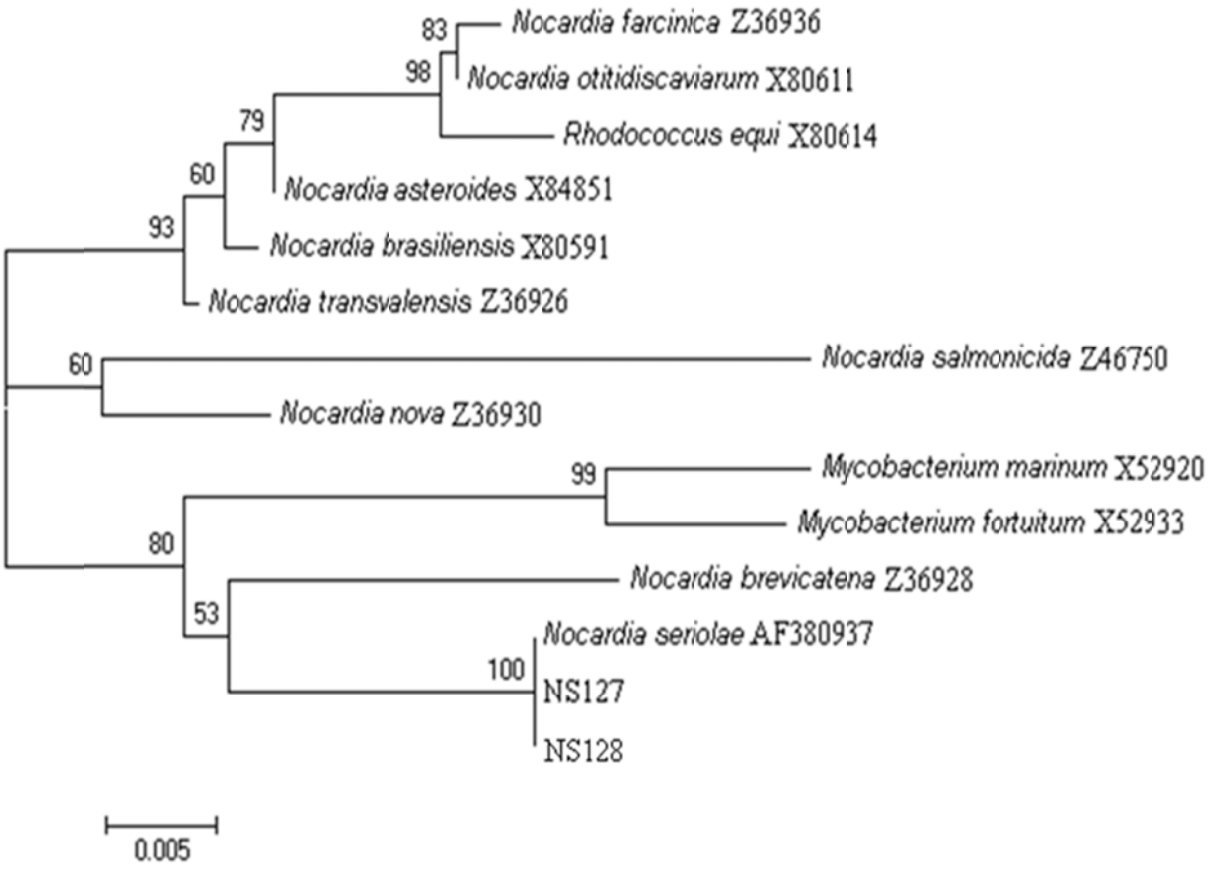

Figure 5. 16S rDNA sequence-based phylogenetic tree of NS128 isolates from our study and from GenBank. Bars indicate genetic distance. Numbers at each node indicate percent bootstrap values. Scale represents 0.005 nucleotide substitutions per position.

value and the high bootstrap-support value (100 % for the 16S rDNA, the heat shock protein gene, and rpoB gene) of NS128 determined by the neighbor-joining, maximum-parsimony and minimum-evolution methods.

Sensitivity to drugs

The isolated and reference strains were sensitive to erythromycin, doxycycline, spiramycin, neomycin and streptomycin. However, they were resistant to tetracycline (Table 3).

Experimental infection

Table 4 presents the cumulative mortalities of both spotted butterfish and amberjack that had been inoculated with 4.7 X 10^6 bacteria of *N. seriolae*. A bacterial concentration of 4.7 x 10^6 CFU per fish caused 100% mortality of the spotted butterfish within ten days of the inoculation. The amberjack showed 100% mortality at 15 days post-inoculation. The bacteria were reisolated from the kidney and liver of all dead fishes. No bacteria were isolated from the liver or kidney of fish selected randomly prior to the experimental infection or from the control group. The infected spotted butterfish had yellowish-white nodules on the serosal surface, mesentery and in many internal organs, especially the kidney, spleen, liver, muscle and skin. The granulomatous nodules found in the spotted butterfish were observed in the muscle, heart, kidney, spleen and liver, and especially in the kidney, and liver. No lesion was formed in either control group. Granulomas and other histopathological changes that resembled those in naturally infected spotted butterfish were exhibited.

DISCUSSION

The isolate, NS128 which is Gram-positive and acid-fast, generated a mycelium that is fragmented into irregular rod-like elements. The phenotypical characteristics of the bacterial isolate, NS128 from diseased spotted butterfish, in Taiwan were almost identical to those of the reference strain, *N. seriolae* BCRC 13745 and *N. seriolae*

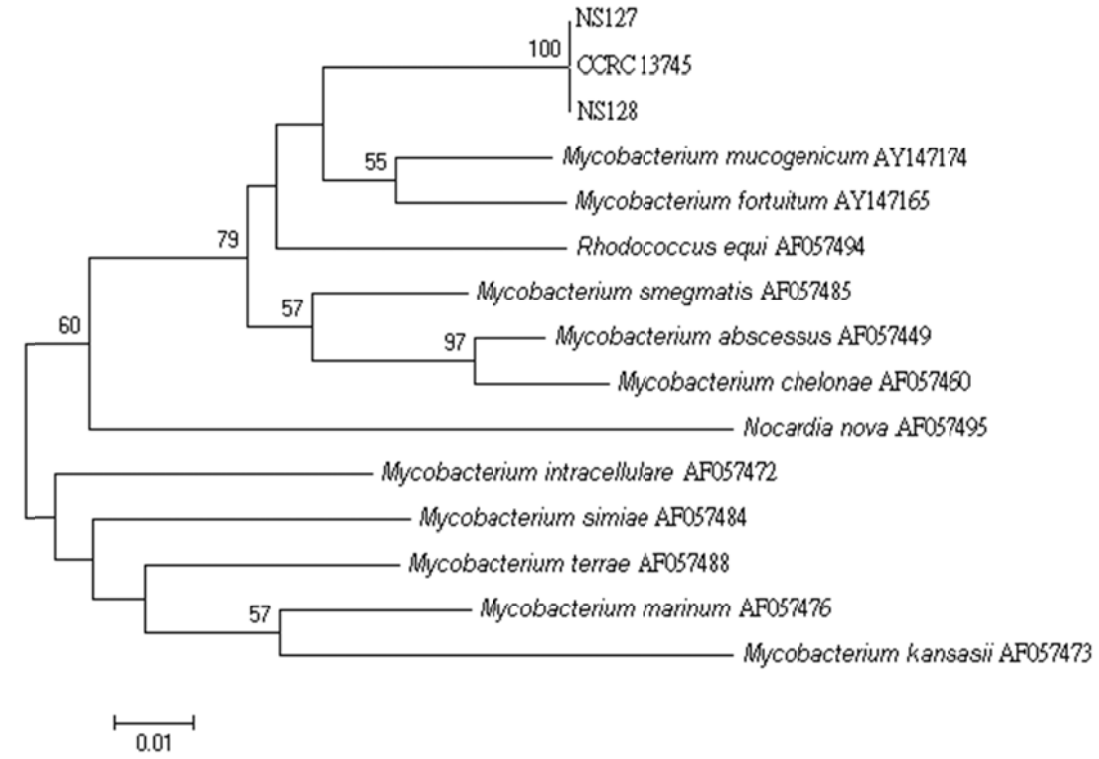

Figure 6. RNA polymerase B subunit gene sequence-based phylogenetic tree of NS128 isolates from our study and from GenBank. Bars indicate genetic distance. Numbers at each node indicate percent bootstrap values. Scale represents 0.01 nucleotide substitutions per position.

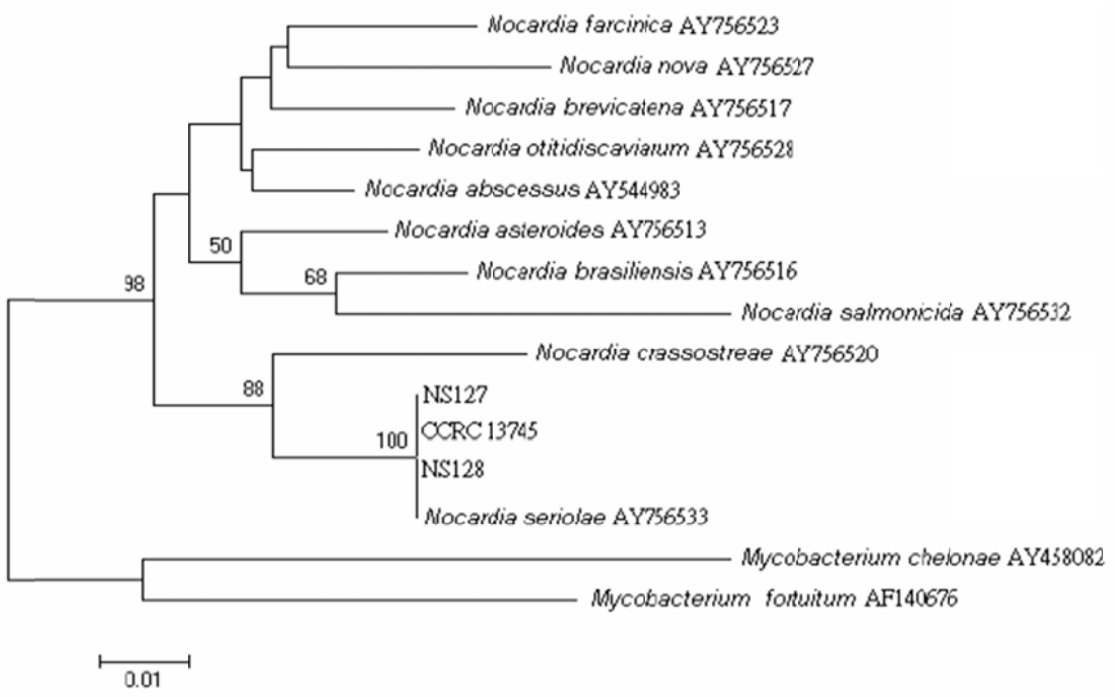

Figure 7. Heat shock protein sequence-based phylogenetic tree of NS128 isolates from our study and from GenBank. Bars indicate genetic distance. Numbers at each node indicate percent bootstrap values. Scale represents 0.01 nucleotide substitutions per position.

Table 3. Sensitivity to antibiotic disks for strains isolated from diseased spotted butterfish *Scatophagus argus* in comparison to the reference strains of the *Nocardia seriolae.*

Antimicrobial class	Disc potency (µg)	Susceptible (mm)	NS128	*Nocardia seriolae* NS127
Erythromycin (E15)	15	≧18	S	S
Doxycycline (DO30)	30	≧16	S	S
Spiramycin (SP100)	100	≧22	S	S
Tetracycline (TE30)	30	≧19	R	R
Oxytetracycline (OT30)	30	≧14	MS	S
Streptomycin (S10)	10	≧15	S	S
Neomycin (N30)	30	≧17	S	S

R, resistance; S, sensitive; MS, moderately.

Table 4. Cumulative mortality (%) of spotted butterfish and amberjack inoculated with *Nocardia seriolae* NS128.

Fish	Group	Route of inoculated	No. of fish used	Cumulative mortality (%) days after injection		
				5	10	15
Spotted butterfish	$4.7x10^6$	IP	8	12.5	100	
	PBS control	IP	8	0	0	0
Amberjack	$4.7x10^6$	IP	4	0	25	100
	PBS control	IP	4	0	0	0

NS127. All properties are consistent with the classification of the isolates in the genus *Nocardia* (Chen et al., 2000; Wang et al., 2005). *N. seriolae* which is an emerging pathogen in Taiwan, has been isolated from various fish species, including red snapper *Lutjanus erythropterus*, grey mullet *Mugil cephalus*, and three striped tigerfish *Terapon jarbua* in freshwater (Wang et al., 2009) and brackish water culture. Spotted butterfish when infected with *N. seriolae* exhibit gross lesions and histopathological changes that are similar to those observed in three striped tigerfish (Wang et al., 2009). Notably, *N. seriolae* dominates the nocardia infection of cultured fish in Taiwan.

The experimental investigation in this study demonstrated that NS128 isolated from the field case was pathogenic to spotted butterfish. The lesions from experimentally infected fish were highly similar to those found in naturally infected fish, suggesting that NS128 is probably the pathogen responsible for the losses of cultured spotted butterfish in Taiwan. In this study, the pathogen was reisolated in pure culture only from challenged fish. These experimental results verify that NS128 is the aetiological agent of this disease in cultured spotted butterfish. Most experimental fish exhibited various skin ulcers and granulomas in their internal organs. The multiple focal granulomas caused by nocardiosis have been described as predominant lesions in several fish (Campbell and Mackelvie, 1968; Ribelin and Migaki, 1975; Chen, 1992; Chen et al., 2000). In this investigation, affected fish that had anorexia, were emaciated and had distended abdomens with granulomas that were commonly diffused throughout the visceral organs. More acute lesions, such as those associated with muscle necrosis are characterized by hemorrhage and Zenker's necrosis were associated with a marked inflammatory response at the centre of mass of the organism (Kubota et al., 1968; Chen, 1992).

The experimental results of the IP injection study suggests that NS128 in bacterial suspension (4.7×10^6 CFU per fish) caused 100% mortality of spotted butterfish group within ten days of the inoculation and of the amberjack given a same concentration of bacterial suspension also exhibited 100% mortality at fifteen days post-inoculation. In this experiment, the NS128 isolated from the spotted butterfish was pathogenic for spotted butterfish and amberjack. Outbreaks of nocardiosis in largemouth bass in Taiwan have been associated with heavy rainfall, hot weather and handling (Chen et al., 2000). Handling is a major causal factor of the recrudescence of nocardiosis at the fish farms.

Miyoshi and Suzuki (2003) presented the PCR assay results for a 432 bp amplicon from *N. seriolae*, including *N. seriolae* JCM3360, and eight clinical strains. If that PCR assay is regarded as definitive for *N. seriolae*, then the bacterial isolate, NS128 obtained from diseased spotted butterfish must be considered a strain of *N. seriolae*. To support this argument, and increase the probably that NS128 corresponded to *N. seriolae*, the

PCR product from NS128 was sequenced and compared to sequences for *N. seriolae* in public databases.

The PCR method that was employed in investigation can be conducted directly using clinical samples such as infected fish liver, kidney, and muscle (Miyoshi and Suzuki, 2003). This approach was developed to facilitate the rapid identification of isolates from pure cultures and infected fish tissues. The detection of nocardial infections in fish using PCR combined with sequencing has considerable advantages over conventional histopathological and bacteriological diagnosis: speed, specificity and sensitivity for purified *Nocardia* spp. isolated from infected fish (Miyoshi and Suzuki, 2003). The extreme speed, and specificity of PCR may facilitate future work in elucidation of the mode of disease outbreak and propagation of nocardia in fish, whether through contaminated feed, water, or soil distribution of these bacteria in cultured ponds, or from parent to offspring.

Notably, 16S rDNA, heat shock protein gene, and RNA polymerase gene (rpo B) phylogenetic analyses also verified that the organism, NS128 was a member of the genus *Nocardia*. The evolutionary trees (s 5, 6 and 7) reveal that NS128 forms a monophyletic clade with reference strain *N. seriolae* BCRC 13745, and with GenBank accession Nos.: AF380937 and AY756533, respectively. This relationship was verified by the high nucleotide similarity value (100%) and the high bootstrap value (100%). The results together constitute the first verification that *N. seriolae* can cause disease outbreaks in the spotted butterfish.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This study was supported by the National Science Council, Taiwan, ROC (NSC101-2313-B-020 -015 -MY3 and NSC102-2811-B-020 -004).

REFERENCES

Campbell G, Mackelvie RM (1968). Infection of brook trout (*Salvelinus fontinalis*) by Nocardiae. J. Fish. Res. Board Can. 25:423-42.

Carrasco N, Roozenburg I, Voorbergen-Laarman M, Itoh N, Engelsma MY (2013). Development of a real-time PCR for detection of the oyster pathogen Nocardia crassostreae based on its homogeneous 16S–23S rRNA intergenic spacer region. J. Invertebr. Pathol. 114:120-127.

Chen SC (1992). Study on the pathogenicity of *Nocardia asteroides* to formosa snakehead, *Channna maculata*, (Lacepede) and Largemouth bass, *Micropterus salmoides* (Lacepede). J. Fish Dis. 15:47-53.

Chen SC, Lee JL, Lai JC, Gu YW, Wang CT, Chang HY, Tsai KH (2000). Nocardiosis in seabass, *Lateolabrax japonicus* in Taiwan. J. Fish Dis. 23:299-307.

Chen SC, Tung MC (1991). An epizootic in large mouth bass, *Micropterus salmoides*, Lacepede caused by *Nocardia asteroides* in freshwater pond in southern Taiwan. J. Chi. Soc. Vet. Sci. 17:15-22.

Chen SC, Tung MC, Tsai WC (1989). An epizootic in Formosa snakehead fish, *Channa maculata* Lacepede, caused by *Nocardia asteroides* in fresh water pond in southern Taiwan. COA Fisheries. Series No. 15 Reports Fish Dis. Res. (IX) ROC, 95-105.

Cornwell ER, Cinelli MJ, McIntosh DM, Blank GS, Wooster GA, Groocock GH, Getchell RG, Bowser PR (2011). Epizootic *Nocardia* infection in cultured weakfish, *Cynoscion regalis* (Bloch and Schneider). J. Fish Dis. 34:567-571.

Elkesh A, Kantham KP, Shinn AP, Crumlish M, Richards RH (2013). Systemic nocardiosis in a Mediterranean population of cultured meagre, *Argyrosomus regius* Asso (Perciformes: Sciaenidae). J. Fish Dis. 36:141-149.

Felsenstein J (1985). Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783-791.

Fitch WM (1971). Towards defining the course of evolution: minimum change for a specific tree topology. Syst. Biol. 20:406-416.

Isik K, Chun J, Hah YC, Goodfellow M (1999). *Nocardia salmonicida* nom. rev., a fish pathogen. Int. J. Syst. Bacteriol. 2:833-837.

Itano T, Kawakami H, Kono T, Sakai M (2006). Detection of fish nocardiosis by loop-mediated isothermal amplification. J. Appl. Microbiol. 100:1381-1387.

Jukes TH, Cantor CR (1969). Evolution of protein molecules. In: Munro HN (ed) Mammalian protein metabolism. Academic Press, New York, NY, pp. 21-132.

Kariya T, Kubota S, Nakamura Y, Kira K (1968). Nocardial infection in cultured yellow tail, *Seriola quinqueradiata*and *S. purpurascens*. I. Bacteriological study. Fish Pathol. 3:16-23.

Kim BJ, Lee SH, Lyu MA, Kim SJ, Bai GH, Chae GT, Kim EC, Cha CY, Kook YH (1999). Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase gene (rpoB). J. Clin. Microbiol. 37:1714-1720.

Kubota S, Kariya T, Nakamura Y, Kira K (1968). Nocardial infection in cultured yellow tail, *Seriola quinqueradiata* and *S. purpurascens*,. II. Histological study. Fish Pathol. 3:24-33.

Kudo T, Hatai K, Seino A (1988). A *Nocardia seriolae* sp. nov. causing nocardiosis of cultured fish. Int. J. Syst. Bacteriol. 38:173-178.

Kumamoto H, Horita Y, Kubota S, Isoea M, Yasumoto S, Yasunaga N (1985). Studies on fish nocardiosis. I. Comparison in the histopathology of both yellow tails. *Seriola quinqueradiata* experimentally inoculated and those naturally infected with *Nocardia kampachi*. Bull. Nippon Vet. Zootechnical College 34:100-118.

Kumar S, Tamura K, Jakobsen IB, Nei M (2001). MEGA2: Molecular Evolutionary Genetics Analysis software. Bioinformatics 17:1244-1245.

Kusuda R, Taki H, Takeuchi T (1974). Studies on a nocardial infection of cultured yellow tail. II. Characteristics of *Nocardia kampachi* isolated from a gill tuberculosis of yellow tail. Bull. Jap. Soc. Sci. Fish. 10:369-373.

Laurent FJ, Provost F, Boiron P (1999). Rapid identification of clinically relevant *Nocardia* species to genus level by 16S rRNA gene PCR. J. Clin. Microbiol. 37:99-102.

Laurent FJ, Provost F, Boiron P (2000). Rapid identification of clinically relevant *Nocardia* species to genus level by 16S rRNA gene PCR. J. Clin. Microbiol. 38:2805.

Miyoshi Y, Suzuki S (2003). A PCR method to detect *Nocardia seriolae* in fish samples. Fish Pathol. 38:93-97.

Ribelin WE, Migaki G (eds) (1975). Fish Pathology. The University of Wisconsin Press ltd. pp. 82-84.

Rzhetsky A, Nei M (1993). Theoretical foundation of the minimum-evolution method of phylogenetic inference. Mol. Biol. Evol. 10:1073-1095.

Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.

Schinnick T (1987). The 65-kilodalton antigen of *Mycobacterium tuberculosis*. J. Bacteriol. 169:1915-1920.

Shimahara Y, Nakamura A, Nomoto R, Itami T, Chen SC, Yoshida T (2008). Genetic and phenotypic comparison of *Nocardia seriolae* isolated from fish in Japan. J. Fish Dis. 31:481-488.

Shimahara Y, Nakamura A, Yasuda H, Itami T, Yoshida T (2006). A study on the enzymatic profiles of the fish pathogen, *Nocardia*

seriolae isolates. Bull. Eur. Ass. Fish Pathol. 26:260-266.
Snieszko SF, Bullock GL, Dunbar CE, Pettijohn LL (1964). Nocardial infection in hatchery-reared fingerling rainbow trout, *Salmo gairdneri*. J. Bacteriol. 88:1909-1810.
Telenti A, Marchesi F, Balz M, Bally F, Bottger EC, Bodmer T (1993). Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J. Clin. Microbiol. 31:175-178.
Wang GL, Yuan SP, Jin S (2005). Nocardiosis in large yellow croaker, *Larimichthys crocea* (Richardson). J. Fish Dis. 28:339-345.
Wang PC, Chen SD, Tsai MA, Weng YJ, Chu SY, Chern RS, Chen SC (2009). *Nocardia seriolae* infection in the three striped tigerfish, *Terapon jarbua* (Forsska° l). J. Fish Dis. 32:301-310.