Acanthocephalan Fauna of Marine Fish in Taiwan and the Differentiation of Three Species by Ribosomal DNA Sequences

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ABSTRACT: Three Acanthocephala species were recovered and identified from three species of fish host. They are *Neoechinorhynchus agilis* collected from grey mullet, (*Mugil cephalus*), *Neorhadinorhynchus macrospinosus* from rabbit fish (*Siganus fuscescens*), and *Rhadinorhynchus pristis* from spotted mackerel (*Scomber australasicus*). All are new locality records. Scanning electron microscopy and light microscopy were used to describe the morphological characters. In addition, these three acanthocephalans were characterized genetically using a molecular approach. The nuclear ribosomal DNA region spanning the first internal transcribed spacer (ITS-1), the 5.8S gene and the second internal transcribed spacer (ITS-2) was amplified and the sizes of PCR products derived were different in length. They are 450 bp for *N. agilis*, 800 bp for *N. macrospinosus*, and 600 bp for *R. pristis*.

KEY WORDS: Acanthocephalans, marine fish, *Neoechinorhynchus*, *Neorhadinorhynchus*, *Rhadinorhynchus.*

INTRODUCTION

Acanthocephalans, known as thorny-headed worms or spiny-headed worms, characterized by the presence of an evertable proboscis, armed with spines, which they use to pierce and hold the gut wall of their gnathostome definitive host. Acanthocephalans are obligate endoparasites which infected in hosts' intestines (Taraschewski, 1989a, b; 2000). They typically have complex life cycles, involving a number of hosts, including arthropods as the intermediated hosts, fishes, amphibians, birds, and mammals as the definitive hosts. Roughly 1150 species have been described. Acanthocephalans have received little attention in the fields of human and veterinary medicine. Human cases of acanthocephalosis are only common in certain parts of Mainland China and remain sporadic elsewhere (Taraschewski, 2000). Cases of serious illness or high mortality induced by acanthocephalan infections in fish were seldom reported due to the much lower infection intensity compared with other helminth parasites.

The role of Acanthocephala within the parasitism with its fish host is more complicated than an intestinal parasite. Sures et al. (1999) stated that certain parasites, particularly intestinal acanthocephalans and cestodes of fish, can accumulate heavy metals at concentrations that are orders of magnitude higher than those in the host tissues or the environment. And such phenomenon of conspicuous metal accumulation makes fish acanthocephalans could be applied to environmental monitoring. Acanthocephalans have thus gained attention from ecologists and environmental toxicologists within the last decade (Taraschewski, 2000).

Adult acanthocephalans that infect fish as definitive hosts belong to two Classes, Eoacanthocephala and Palaeacanthocephala. The former includes two Orders: Cyracanthocephala and Neoacanthocephala; and the latter consists of two Orders: Echinorhynchidea and Polymorphida (Amin, 1987). Key exclusively based on the morphological characters to the Classes, Orders, Families, and Subfamilies of Acanthocephala was described previously (Amin, 1987).

For accurate and effective identification of helminthes, various studies have recently demonstrated that the internal transcribed spacers (ITS) and 5.8S of nuclear ribosomal DNA (rDNA) provide distinguishing genetic markers in parasitic nematodes (Zhu et al., 1998; Shih, 2004) and acanthocephalans (García-Varela et al., 2000; 2005). The list of parasitic helminth candidates whose identifications were contributed by polymerase chain reactions (PCR) and advanced assays derived, such as PCR-RFLP (restriction fragment length polymorphism) and PCR-SSCP (single-strand conformation polymorphism), is growing rapidly.

The aim of this study is to inventory the Acanthocephala fauna from a few marine fish which are either common or economically important in Taiwan. This is the first report dealing with this topic. Beside of the morphological identification, a molecular delineating method based on the difference of ITS and 5.8S rRNA sequences to differential three species is reported herein.

MATERIALS AND METHODS

Sample collection

Marine fishes including rabbit fish (*Siganus fuscescens*), spotted mackerel (*Scomber australasicus*),

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and grey mullet (*Mugil cephalus*) were purchased from fishermen in Keelung and Ilan, Taiwan, and then transported to the laboratory. After identification, their abdomens were opened, and acanthocephalans were washed out and collected from the gastrointestinal tracts in general and the lower part of intestine in particular with tap water. Acanthocephalans were repeatedly washed to remove any mucus or debris from their surfaces and dipped in 70% ethanol for fixation and storage until further use.

Morphology identification of acanthocephalans

Acanthocephalans were selected and immersed into a mixture of glycerin and 70% ethanol (1:1=v/v). After clearing, they were temporally mounted and observed under a stereomicroscope for identification based on morphological characteristics and followed the key to the families and subfamilies of Acanthocephala (Amin, 1987).

To reveal the ultrastructures of their proboscis and spines on their body surfaces, scanning electron microscopy was applied in parallel. For scanning electron microscopy, acanthocephalans were fixed with 1% glutaraldehyde for 12 h and post-fixed with OsO_4 for 1 h. They were then dehydrated, critical point dried, and ion sputter-coated. Coated worms were observed under a scanning electron microscope (Hitachi-S2500, Japan) at 15 kV.

Molecular identification of acanthocephalans

Acanthocephalan specimens used for DNA extraction were picked out of the extracted samples using a sterile needle under a stereomicroscope. Each specimen was placed into a mixture of 5% glycerol and 10% ethanol for clearing and then mounted in glycerol on a separate slide covered by a cover slip. After taxonomic identification, specimens were individually placed in 0.2 mL PCR tubes containing 500 μL of lysis buffer and 7 mg/mL proteinase K and incubated at 55℃ until lysed totally. DNA was extracted by adding 135 μL of Protein precipitation solution, 500 μL of isopropanol and ethanol mixture, and 150 μL of Hydration Solution.

To amplify the DNA fragments, including complete ITS-1, ITS-2, and 5.8S rRNA, the previously described (Luton et al., 1992) primers (forward 5' GTC GTA ACA AGG TTT CCG TA 3' and reverse 5' ATA CGA ATT TAA GTC GCC CA 3') were used. Routine PCRs reported before (Garcia-Varela et al., 2005) were conducted with 5 μL of the extracted DNA, 5 μL 10x buffer with $MgCl_2$ and BSA, 6 μL of 2 mM dNTPs, 1 μL of each primer (100 μM), 4.6 μL of enzyme diluent, 0.4 μL DNA polymerase (5 U/μL, BIOTAQ) and double-distilled water to make a total volume of 50 μL for each sample. The thermal cycler parameters were 95℃ for 3 min, 35 cycles of 94℃ for 1 min, 54℃ for 1 min and 72℃ for 1 min, and finally one cycle of 10 min at 72℃ followed by a holding temperature of 4℃. PCR products were loaded into a 2% agarose gel buffered with TBE. Electrophoresis was normally at a constant voltage of 200 V.

RESULTS

Three Acanthocephala species were recovered and identified from three species of fish host. All are new locality records. Their classifications and morphological characters were described in the following: *Neoechinorhynchus agilis* (Figs. 1A & 1B) collected from grey mullet belongs to Class Eoacanthocephala, Order Neoacanthocephala, Family Neoechinorhynchidae. The worm is small and the male and female adults are both approximately 15 mm in length. The worm is cylindrical with sparse macronuclei which were generally 4 to 5 in the dorsal part and 1 to 2 in ventrally part of the syncitial tegument. The short and bulbous proboscis is covered with 6 spiral hook rows and each with 3 hooks, so that there are totally 18 hooks on each proboscis (Figs. 2A & 2B). Besides, the anterior hooks are always longer and tougher than the posterior ones. Lemniscus contains a few macronuclei, and the cement glands are syncytium with many nuclei. The sexuality of adult acanthocephalans could be easily distinguished based on the structures of their posterior extremities, in which male adult has a cap-like burse (Fig. 2C) and female has one genital opening (Fig. 2D).

Neorhadinorhynchus macrospinosus (Figs. 1C & 1D) collected from rabbit fish belongs to Class Palacacanthocephala, Order Echinorhynchidea. Family Echinorhynchidae. There are many but smaller amoeboid nuclei within the syncytium. The cylinder proboscis is covered with 14 longitudinal hook lines and each with 8-16 hooks that gradually decrease in length posteriorly. Its lemniscus is claviform and is about as long as jroboscis receptacle. Four cement glands distribute into two lines.

Rhadinorhynchus pristis (Figs. 1E & 1F) collected from spotted mackerel belongs to Class Palacacanthocephala, Order Polymorphida, Family Rhadinorhynchidae. The cylinder proboscis is covered with 8 to 26 longitudinal rows of hooks and each with 8 to 37 hooks. The lemniscus is long but shorter than proboscis receptacle. The testes arrange in series which located at the anterior body. Four cement glands are present.

The sizes of PCR products derived from the ITS and 5.8S sequences are different in length among three acanthocephalans identified in this study. They are 450 bp for *N. agilis*, 800 bp for *E. macrospinosus*, and 600 bp for *R. pristis* (Fig. 3).

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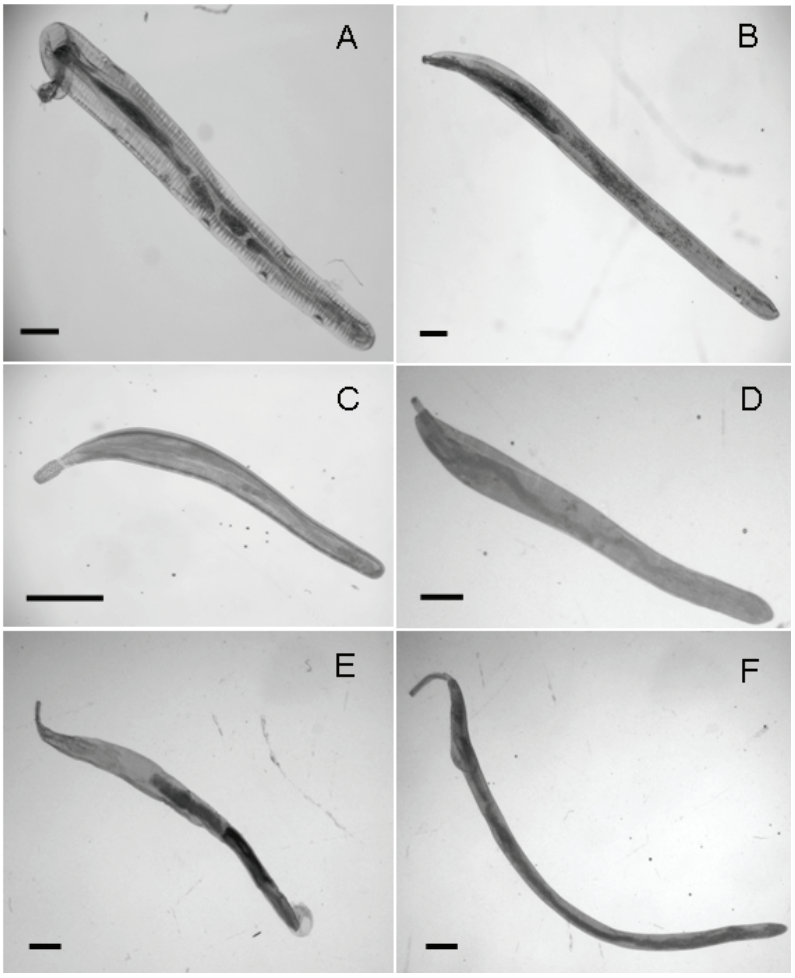


Fig. 1. Morphology of three adult acanthocephalans illustrated by light microscopy. A: Male *Neoechinorhynchus agilis*. B: Female *N. agilis*. C: Male *Neorhadinorhynchus macrospinosus*. D: Female *N. macrospinosus*. E: Male *Rhadinorhynchus pristis*. F: Female *R. pristis*. Scale bars = 1 mm.

DISCUSSION

This study provides the first report of the Acanthocephala fauna from marine fish in Taiwan. There are three acanthocephalan species, e.g. *Neoechinorhynchus agilis* (Amin, 2002), *Neorhadinorhynchus macrospinosus* (Amin and Nahhas, 1994), and *Rhadinorhynchus pristis* (Rego, 1987), collected and identified from three fish species, respectively. The phenomenon might be mainly contributed by the previous observations rather than host specificity. Acanthocephala may have more of an impact upon intestinal parasite communities than other kinds of helminthes. And they are more likely to exhibit negative nteractions with their own and other species, under both field and experimental conditions (Byrne et al., 2003).

In addition to morphological characters, these worms could be unambiguously distinguished from one another by PCR analysis amplifying ITS and 5.8S rDNA sequences. More detailed comparisons between the differences of acanthocephalan genes should be included as other Acanthocephala were recovered from marine fish in Taiwan. Ribosomal RNA (rRNA) has been used as a molecular marker for inferring phylogenetic relationships among species of helminthes with few morphological differences, including the ITS spacers 1 and 2, which separate the small subunit (18S rRNA) from the 5.8S and the large subunit (28S) rRNA (García-Varela et al., 2005). The ITS regions can exhibit considerable differentiation between closely related species. The relatively fast rate of evolution of ITS sequences makes it useful for inferring relationship among closely related species, and the highly conserved regions contiguous to the spacer sequences facilitate amplification by PCR (García-Varela et al., 2005).

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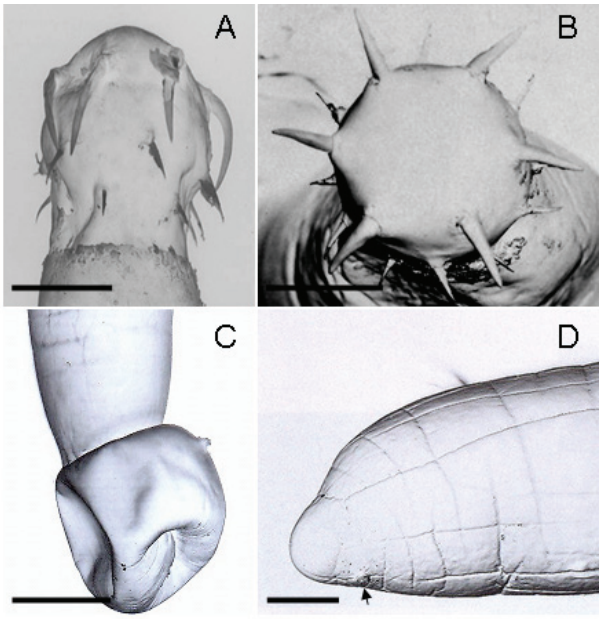


Fig. 2. Morphology of two adult *Neoechinorhynchus agilis* illustrated by scanning electron microscopy. A: Proboscis, side view. B: Proboscis, top view. C: The posterior extremity of male showing the bursa. D: The posterior extremity of female, the genital opening is indicated by an arrow. Scale bars = 75 μm in A, 8 mm in B, 250 μm in C, 13 mm in D.

Identification and differentiation of the species of acanthocephalans based on their morphological characters is not always feasible or reliable. In practical, samples collected from fish GI tracts could be either incomplete or not obtainable fixation and preservation procedures which could reduce the clarity of their internal structures too substantially for them to be recognized. This is the reason why molecular classification protocols are generally tested and applied for parasitic helminthes.

Acanthocephala have been used as indicators to show limited movements between host fish populations on scale as fine as less than 1 km in coral reef environments (Cribb et al., 2000). However, adult acanthocephalans are not long-lived parasites compared with nematode larvae and cestodes, and their life spans in definitive host are usually less than 1 year (Brattey, 1988). Hence the utility of the acanthocephalans as a parasite marker may be limited to examination dynamics of short-term (within-season) migrations, rather than long-term differences between fish populations. The significant differences in abundance of Acanthocephala between two blue mackerel groups which were collected from two localities at the same time period in New Zealand indicated a lack of short-term movement between these areas (Smith et al., 2005). Thus *R. pristis* identified from the same fish species herein does not seem to be an ideal

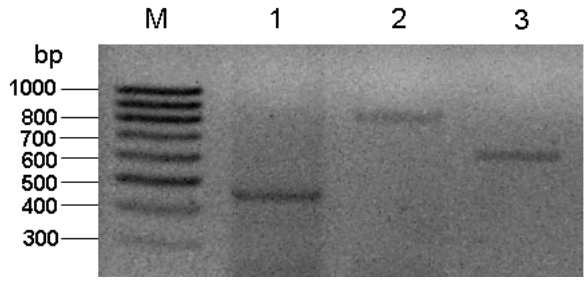


Fig. 3. Molecular delineation of three adult acanthocephalans by PCR. Products representing ITS-1, 5.8S gene and ITS-2 were revealed by electrophoresis through a 2% agarose gel and staining with ethidium bromide. DNA templates used were extracted from adult *Neoechinorhynchus agilis* (lane 1), *Neorhadinorhynchus macrospinosus* (lane 2), and *Rhadinorhynchus pristis* (lane 3). M: 100-bp markers.

biological marker to study its population biology around Taiwanese waters. The potential utility of *N. agilis* together with other nematodes as tags to investigate grey mullet, an economically important fish species in Taiwan, is evaluated and will be published elsewhere.

Acanthocephalans resident in fish intestine were found to accumulate heavy metals at concentrations that are orders of magnitude higher than those in the host tissues or the environment (Sures et al., 1999; Taraschewski, 2000). Although this parasite had been applied to environmental monitoring recently, there are still many questions concerning its role within parasitism remain unsolved. Whether it is advantageous to a fish to be infected by acanthocephalans if its aquatic environment is contaminated by heavy metals? May these parasites display a sanitary function in the fish by absorbing large quantities of the heavy metals passing through the gut of the fish (Taraschewski, 2000)? The answers should be helpful in revealing the interactions between acanthocephalans and their fish hosts, and might thus be useful for environmental monitoring and bioremediation of heavy metals.

Nevertheless, acanthocephalans are obligate parasites, while they are also interesting helminthes which impact on their gnathostome definitive hosts in many aspects as discussed above. The species richness of marine fish, one main definitive host of this worm, is more than 2000 described species in Taiwan. For fisheries and environmental monitoring, both acanthocephalan fauna parasitized in marine fish and the parasitism between these parasites and their hosts are suggested to be deeply concerned.

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臺灣海魚棘頭蟲相並以核糖體 DNA 序列鑑別三種棘頭蟲

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摘要：本研究從三種海魚腸道中分離與鑑別出三種屬於棘頭蟲動物門之蠕蟲，分別為來自烏魚之活動新棘吻蟲、臭肚魚之巨棘新長棘吻蟲以及澳洲鯖(俗稱花腹鯖)之鋸長棘吻蟲。三種棘頭蟲皆為發現地點新紀錄。同時以光學顯微術和掃描式電子顯微術解析此三種棘頭蟲之型態特徵，此外亦以分子生物技術區別三者遺傳上之差異。藉由聚合酶連鎖反應增幅其 ITS-1、5.8S 以及 ITS-2 基因片段，獲得之產物長度不同而足以清楚區別，其長度分別為：活動新棘吻蟲 450 bp、巨棘新長棘吻蟲 800 bp 以及鋸長棘吻蟲 600 bp。

關鍵詞：棘頭蟲、海魚、新棘吻蟲屬、新長棘吻蟲屬、長棘吻蟲屬。

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