

GENETIC CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF ECHINOSTOMES FROM FREE-GRAZING DUCKS IN THAILAND USING RIBOSOMAL DNA SEQUENCES

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Abstract. We conducted this study to identify species and determine the phylogenetic relationships using ribosomal DNA (rDNA) sequences [partial sequences of 28S rDNA and second internal transcribed spacer (ITS2)] of echinostomes collected from free-grazing ducks in Phitsanulok Province, Thailand. Four adult echinostomes were morphologically identified as *Echinostoma revolutum*, 4 as *Hypodermaeum conoideum* and 2 unidentified. Sequences of other species/isolates of echinostomes retrieved from the GenBank database were employed to compare and construct the phylogenetic tree. Three major lineages were found, namely, genus *Echinostoma*, genus *Echinoparyphium* and genus *Hypodermaeum*. One of the unidentified echinostome specimen was 99% identical to and clustered with genus *Echinoparyphium*, whereas the other was located in the "*revolutum*" group, but was closely related to the geographical isolates from America rather than from Thailand. This study indicates that 28S rDNA and ITS2 regions are suitable molecular markers for genetic characterization and phylogenetic analysis of echinostomes.

Keywords: echinostomes, domestic duck, genetic characterization, phylogenetic analysis, ITS2, 28S ribosomal DNA

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INTRODUCTION

Echinostomes are zoonotic, intestinal parasitic helminthes commonly found in wild and domestic animals (Kanev, 1994). They have a complex life cycle with three hosts: the first and second intermediate host

is generally snails, while the final hosts are a wide range of aquatic birds, poultry, and mammals. Echinostomes include many species and species complexes, such as the "37-collar spines" group (Kostadinova *et al*, 2003; Detwiler *et al*, 2010; Georgieva *et al*, 2013). The life cycle of echinostomes involves eggs, which are passed through host feces into water where they develop into the fully developed miracidial stage. When the miracidia hatch, they seek out and penetrate into their first intermediate snail hosts. They then develop from sporocysts to rediae and finally into cercariae, which emerge to seek a second intermediate host, *eg*, other gastropods, bivalves, fish or tadpoles. Infection of the definitive hosts occurs through eating raw or partially cooked second intermediate hosts containing the infectious metacercariae (Huffman and Fried, 1990).

There are many metropolitan species of echinostomes. Some species are taxonomically confusing with much synonymy due to their wide geographical distribution. For example, there are many synonyms of *Echinoparyphium cinctum* reported worldwide, *eg*, *E. skrjabini* in the Ukraine, *E. borneonense* and *E. dunnii* in Malaysia, *E. querquedulae* in India and *E. oshmarini* in Russia (Kanav, 1994). Although morphological examination is the standard method for species identification, however in cases of the very similar morphology found in species complexes, identification based on this method requires expertise. Thus, species identification using genetic markers provides a more reliable alternative method for species determination.

There are many molecular markers available for genetic characterization, identification, and differentiation at the genus/species level for echinostomes, *eg*, allozymes, and nuclear and mitochondrial

genes (Saijuntha *et al*, 2010; Saijuntha *et al*, 2011a, b). However, a suitable genetic marker for interspecific differentiation should be polymorphic between genus/species level but conserved within species. The ribosomal DNA (rDNA) gene consists of 5.8S, 18S, 28S and two internal transcribed spacer (ITS1 and ITS2) regions, which have been proven to be useful genetic markers to differentiate at the genus/species level for many organisms, including parasitic helminthes (Coleman, 2003).

In this study, the partial sequences of the 28S rDNA and ITS2 were used as genetic markers for the characterization of adult echinostomes recovered from domestic ducks in Phitsanulok Province, northern Thailand.

MATERIALS AND METHODS

Sample collection and morphological identification

Two carcasses of free-grazing ducks were provided by a farmer from Mueang District, Phitsanulok Province, Thailand. Worms were extracted from the intestines by extensive washing with sterile normal saline and identified under a light microscopy using standard identification keys (Kostadinova, 2005). Parasite samples were kept in 80% alcohol and transferred to Kochi Medical School, Japan for molecular analysis.

PCR and DNA sequencing

Genomic DNA was extracted from each adult echinostome using Eazy DNA™ kit (Invitrogen, Carlsbad, CA) after evaporating off the alcohol. Primers 3S (5'-CGG TGGATCACTCGGCTCGT-3') and A28 (5'-CCTGGTTAGTTTCTTTTCCTCCGC-3') were used to amplify the ITS2 region (Bowles *et al*, 1995) and primers TSD2 (5'-GTACCGTGAGGGAAAGTTG-3') and D4AR (5'-GTCCGTGTTTCAAGAC-

GGG-3') for 28S rDNA (Littlewood and Johnston, 1995). PCR assays were performed using reaction volumes of 25 µl containing 20 ng of genomic DNA, deionized water, 1X *ExTaq* buffer, 2.0 mM MgCl₂, 0.2 mM dNTPs, 0.6 µM each primer, and 1 U *ExTaq* polymerase (TaKaRa, Shiga, Japan). Negative controls containing no genomic DNA were included in all experiments. Thermocycling conditions (conducted in MyCycler Thermal Cycler, Bio-Rad, Hercules, CA) were as follows: 94°C for 5 minutes; 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 45 seconds; and a final step at 72°C for 5 minutes. Amplicons were purified using GeneClean®II kit (Q-BIO Gene, Carlsbad, CA) and sequenced with a Big Dye kit v3.1 in an ABI PRISM 377 automated sequencer (Perkin-Elmer, Santa Clara, CA).

DNA sequence analysis

All sequences were submitted to GenBank: the 28S rDNA sequences under accession no. KF894680 for *E. revolutum* and KF894681 for *H. conoideum*, and ITS2 sequences under accession no. KF894682 for *E. revolutum* and KF894683 for *H. conoideum*. Multiple alignments of sequences were performed using BioEdit version 5.0.6 (Hall, 1999). Sequence similarity search was performed using NCBI nucleotide BLAST program (Altschul *et al*, 1990). Phylogenetic relationships were constructed based on neighbor-joining (NJ) analysis using Phylip program version 3.6 (Felsenstein, 2005) and the relative support for clades in the NJ analyses was determined using 1,000 bootstrap replicates. The sequence of *Fasciolopsis buski* was included as an out group in the phylogenetic tree construction.

RESULTS

Ten worms were recovered from the

two free-grazing domestic ducks. Eight specimens could be morphologically identified, *ie*, four adult *E. revolutum* and four adult *H. conoideum*, and two worms were unidentified. Comparison within the nucleotide sequences of 554 bp 28S rDNA (Table 1) and 588 bp ITS2 (Table 2) revealed no intraspecific variation within the identified samples of both *E. revolutum* and *H. conoideum*. Multiple alignments of 28S rDNA and ITS2 sequences for all samples of this study and those retrieved from GenBank showed differences ranging from 6 to 28 (1.08%-5.05%) and 10 to 62 (1.70%-10.54%) in nucleotide positions of 28S rDNA and ITS2 sequence, respectively.

The unidentified-1 sample showed no identity to either any identified samples of this study or the available sequences in GenBank database of both DNA regions (Tables 1 and 2). However, alignments to sequences in the NCBI database revealed that unidentified-1 sample is most similar to the American isolates of *E. revolutum*, amounting to 98% of 28S rDNA (Table 1) and 99% of ITS2 sequences (Table 2). This result suggests that unidentified-1 sample belongs to the genus *Echinostoma* and there is a high possibility of it being a sibling species or a species within *E. revolutum* complex, as it clustered and closely aligned with *E. revolutum* in the phylogenetic tree (Fig 1). In the case of the unidentified-2 sample, the 28S rDNA sequence is 99% identical to *E. cinctum* (Table 1) and ITS2 sequence 100% identical to *E. recurvatum* (Table 2), indicating that it belongs to the species complex in the genus *Echinoparyphium*.

DISCUSSION

Many echinostomes that are important zoonotic intestinal trematodes,

Table 1
Variations in nucleotide positions of 554 bp 28S rDNA sequence of *Echinostoma* species.

| | | Nucleotide sequences | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
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| <i>E. revolutum</i> ^b | | C A A G A T G G C A G T T G A C T T T T C C G T C G A G T T G T C G A A T | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| <i>E. revolutum</i> | DQ471888 ^c | G G T | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| <i>E. revolutum</i> | EF470908 | G G C T A | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| <i>E. revolutum</i> | AY222246 | G G T A A | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| <i>E. revolutum</i> | EF470905 | G G T A | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| <u>Unidentified-1</u> ^b | | . G . T T C C . C . T . . T T . . | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| <i>H. conoideum</i> ^b | | T . T . G . T T G G A G C A . T C T A C . . G A . . . C T . . C . | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| <u>Unidentified-2</u> ^b | | T . T . G . T T A G A G C A . T C . C . . T . C . A G A A A . C . . . C . | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| <i>E. cinctum</i> | EF470907 | T . T . G . T T A G A G C A . T C . C . . T . C . A G A A A . C T . . C . | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| <i>E. rubrum</i> | JF820594 | T . T . G . T T A G A G C A . T C . C . . T . C . A G A A A . C T . . C . | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

^aNumber of nucleotide changes among the listed *Echinostoma* sp.
^bFrom this study. ^cGenBank accession number. Dot indicates identical nucleotide with that of *E. revolutum*.

especially in birds and poultry are morphologically similar and represent species complexes, leading to problems in species identification, in particular for the genera *Echinostoma* and *Echinoparyphium* (Huffman and Fried, 2012). Molecular analysis, as performed in this study, were used to solve this problem. Two morphologically unidentified echinostome samples could be classified as members of the *E.*

revolutum and *Echinoparyphium* species complexes, respectively, based on nucleotide sequence similarity of their rDNA sequences, as well as their genetic clustering in the ITS2 phylogenetic tree. *Echinostoma revolutum* has been unexpectedly found infecting a tadpole in north-eastern USA, where another sibling species (*E. trivolvis*) had previously been recognized and characterized using the 28S rDNA sequence

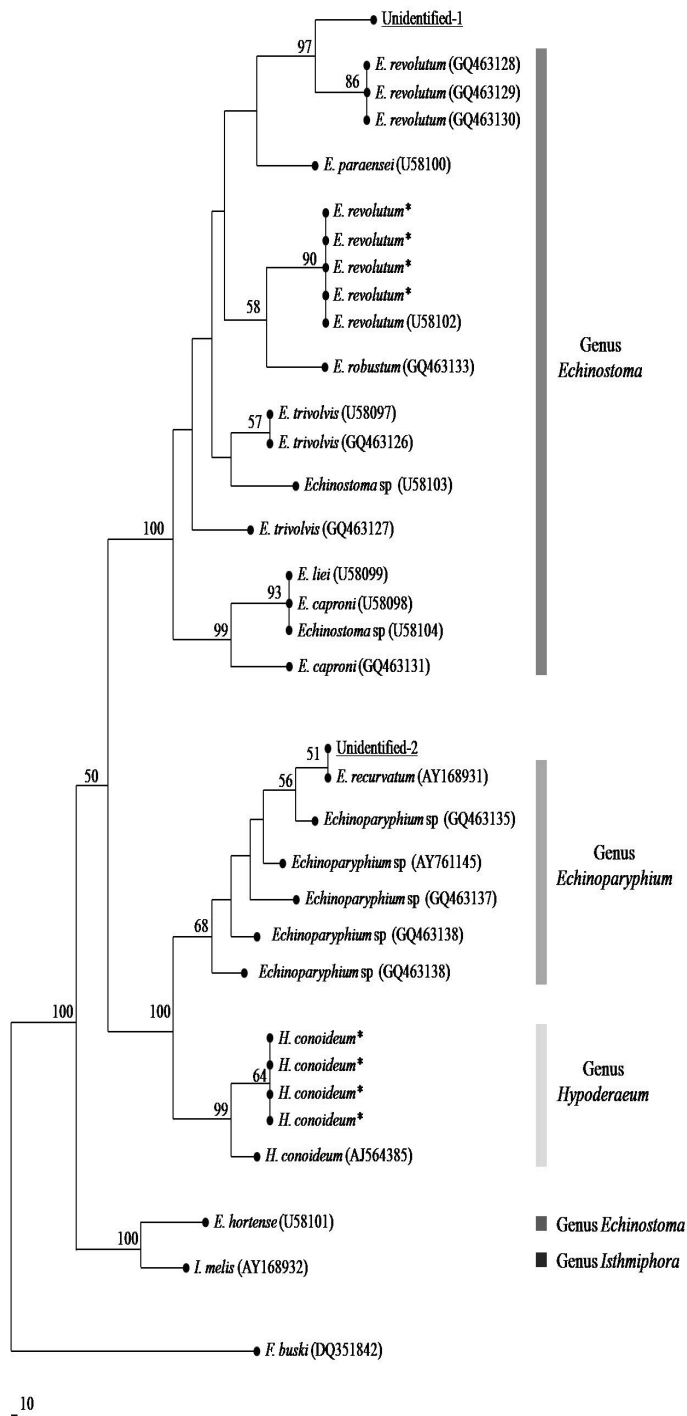


Fig 1—Consensus tree depicting the relationships of two unidentified echinostome samples with other echinostome species. The tree was inferred from 1,000 replicates of ITS2 sequences data based on neighbor-joining using *Fasciolopsis buski* as an out group. Bootstrap values (> 50%) are indicated above branches. *Samples from this study.

data (Holland *et al*, 2007). However, the genus *Echinoparyphium* has been reported worldwide with an estimate of at least 151 species (Huffman and Fried, 2012). At least one species, *E. recurvatum* from ducks in Thailand, has been characterized by allozymes and mitochondrial DNA markers (Saijuntha *et al*, 2010; Saijuntha *et al*, 2011a, b). This study has demonstrated that 28S rDNA and ITS2 regions could also serve as genetic markers for the characterization, identification, and differentiation of the genera and species of echinostomes.

Many echinostomes species are infective to mammals and birds (both migratory and non-migratory) which exhibit different degrees of dispersal (Detwiler *et al*, 2010). Thus, it is possible to associate the migration of these echinostomes with zoonotic animal hosts, as well as the migratory pathways of infected birds. In Phitsanulok Province a large number of migratory birds occur together with free-grazing ducks. Our study has shown that one echinostome specimen was genetically very similar to the American strain of *E. revolutum* (accession no. GQ463128 to GQ463130), and it is possible that this sample have been introduced into Phitsanulok Province by migratory birds. Thus, the observation of intraspecific genetic variation in the rDNA sequences shown in this study may be due to the following reasons: (i) adaptation of the parasite to survive in a new environment and/or host, which usually occurs in parasitic trematodes (Gandon and Michalakis, 2002), (ii) cross fertilization between species or strains, which has been reported in an intermediate form of *Fasciola* sp probably caused by hybridization between *F. gigantica* and *F. hepatica* in Japan, Vietnam and Myanmar (Agatsuma *et al*, 2000; Le *et al*, 2008; Ichikawa *et al*, 2011), or (iii) it is a valid species/subspecies within the

E. revolutum complex group that is more closely related to the American *E. revolutum* than to the Thai and other strains.

In conclusion, this study has demonstrated that the rDNA sequences can provide useful genetic markers for species identification of zoonotic echinostomes, as well as in the construction of phylogenetic relationships of these echinostomes at species or genus level. The availability of such tools should encourage a program of a comprehensive analysis of the genetic and morphological variations of echinostomes in Thailand, as well as other regions in Southeast Asia.

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