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SHORT COMMUNICATION

Genetic differentiation of *Artyfechinostomum malayanum* and *A. sufrartyfex* (Trematoda: Echinostomatidae) based on internal transcribed spacer sequences

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Abstract Genetic differentiation between two synonymous echinostomes species, *Artyfechinostomum malayanum* and *Artyfechinostomum sufrartyfex* was determined by using the first and second internal transcribed spacers (ITS1 and ITS2), the non-coding region of rDNA as genetic makers. Of the 699 bp of combined ITS1 and ITS2 sequences examined, 18 variable nucleotide positions (2.58 %) were observed. Of these, 17 positions could be used as diagnostic position between these two sibling species, whereas the other one variation was intraspecific variation of *A. malayanum*. A clade of *A. malayanum* was closely aligned with *A. sufrartyfex* and clearly distance from the cluster of other echinostomes. Our results may sufficiently suggest that the current synonymy of these species is not valid.

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Introduction

The genus *Artyfechinostomum* comprises intestinal and zoonotic food-borne trematodes. *Artyfechinostomum* spp. have a small head collar ranging between 39 and 45 collar spines arranged in two alternating rows along the dorsal side, two deeply lobed testes, and a large, long cirrus sac reaching beyond the ventral sucker (Premvati and Pande 1974). In Thailand, the first intermediate hosts of these parasites are the freshwater snails *Indoplanorbis exustus* and *Gyraulus convexiusculus* (Waikagul 1991; Radomyos et al. 1994). There is a broader second intermediate host range including a wide variety of aquatic animals, such as lymnaeid snails, *Pila* spp., *Pomacea* spp., and tadpoles (Huffman and Fried 1990; Waikagul 1991; Radomyos et al. 1994; Saijuntha et al. 2011a). Reservoirs of this parasite include many domesticated and wild mammals, and essentially all aquatic organisms that are the first and the second intermediate hosts in a particular endemic area (Huffman and Fried 1990).

A. malayanum was first described from a human in Malaysia under the name *Echinostoma malayanum* Leiper 1911 (Beaver et al. 1984), subsequently being reported from Singapore, Thailand, Indonesia, India, and the Philippines (Chai et al. 2009). More recently, *E. malayanum* is now considered to be a synonym of *A. malayanum* (Chai et al. 2009). A closely related species, *Artyfechinostomum sufrartyfex* is endemic in India (Chai et al. 2009) and has recently been reported from southern Vietnam (Ha Duy et al. 2011). There are several previous reports suggesting that *A.*

malayanum Leiper 1911 is synonymous with *A. sufrartyfex* Lane 1915 (Lie 1963; Mukherjee and Ghosh 1968; Chai et al. 2009). However, more recent work shows that *A. malayanum* fits more closely to the generic features and is possibly conspecific with *A. sufrartyfex* (Kostadinova et al. 2002). Moreover, *A. malayanum* (as *E. malayanum*) does not form a monophyletic clade with other species in the genus *Echinostoma*, i.e., *Echinostoma revolutum*, conforming to the new generic placement (Saijuntha et al. 2011a).

Several previous reports have shown that the genetic variation in echinostomes is correlated not only with geographical localities, but also that intermediate and/or reservoir hosts may influence the biological and genetic variation of these parasites (Maldonado et al. 2005; Saijuntha et al. 2011a). Moreover, Saijuntha et al. (2011a) showed that intraspecific variation in *A. malayanum* is associated with the snail and rodent host species based on mitochondrial cytochrome c oxidase subunit 1 (CO1) sequences. However, additional molecular genetic analysis is required to confirm whether snail and rodent hosts influence the intraspecific and/or sympatric variation of *A. malayanum*.

Then, genetic status of these two sibling species needs to be confirmed. In this study, the first and second internal transcribed spacer sequences, the non-coding region of rDNA are used as markers to differentiate the sibling species, *A. malayanum* and *A. sufrartyfex*. In addition, the first and second internal transcribed spacers (ITS1 and ITS2) sequences of other echinostomes were retrieved from GenBank to construct a phylogenetic tree depicting their genetic relationships. Furthermore, sequences of *A. malayanum* derived from snails and rodent hosts were compared with those of *A. sufrartyfex* from India.

Materials and methods

Sample collection

Metacercarial cysts of *A. malayanum* were obtained from three species of naturally infected freshwater snails, *I. exustus*, *Lymnaea rubiginosa*, and *Pomacea canaliculata*, which were collected from a pond in the Muang district, Khon Kaen Province, Thailand. Twenty to 100 snails of each species were pooled and digested by pepsin solution (Srisawangwong et al. 2004). Fifty to 100 metacercarial cysts from each snail species were then separately fed to hamsters by intragastric intubation. Thirty days after infection, the hamsters were euthanatized and intestines were removed to collect adult worms. The experimental protocol was approved by the Animal Ethics Committee of Khon Kaen University (AEKKU 25/2552). Adult *A. malayanum* were also collected from the small intestine of rice field rats, *Rattus* sp., trapped in the Muang district, Khon Kaen Province. All adult worms were examined by light microscopy and identified to species based on their morphological characteristics, i.e., small circumoral disk deep lobed and branched testes and 43 collar spines (Yamaguti 1958; Miliotis and Bier 2003). All specimens were washed thoroughly in physiological saline and stored at –80 °C until required.

PCR and DNA sequencing

Five to 10 adult worms derived originally from the snails or the rats were separately pooled for genomic DNA (gDNA) extraction by using the DNA extraction kit (QIAGEN, Hilden, Germany) for adult worms that had first been crushed using a tissue grinder and lysed with lysis buffer, proteinase K (200 μg/ml). The ITS1 and ITS2 sequences were amplified by using primers BD1 (5′-GTCGTAACAAGGTTTCCGTA-3′) and BD2 (5′-TATGCTTAAATTCAGCGGGT-3′; Morgan and Blair 1995). PCRs were performed using a PTC-200 thermal cycler (MJ Research, Watertown, MA, USA) and reaction volumes of 25 μl containing 200 ng of genomic DNA, 50 mM $MgSO_4$, 1× buffer, 2.5 mM dNTPs, and 0.25 U of *Taq* DNA polymerase (iNtRON Biotechnology, Korea). No gDNA was included in each run as negative control. The PCR condition used for ITS amplification was 35 cycles of 96 °C for 30 s, 54 °C for 1 min, 72 °C for 1 min. Amplicons were sequenced by the dideoxynucleotide chain termination method using Dye Primer and Dye Terminator Cycle sequencing kits (Applied Biosystem Inc., Foster City, CA, USA) and an ABI DNA sequencer 373A.

Sequencing data and phylogeny analysis

The nucleotide sequences obtained have been deposited in GenBank under accession numbers JF412723–JF412726 for 301 bp of partial ITS1 sequences, JF412727–JF412730 for 398 bp of partial ITS2 sequences. The DNA sequences of *A. sufrartyfex* (Indian isolate) and other echinostome species/isolates were retrieved from the GenBank database. Sequence alignment was conducted using BioEdit version 5.0.6. Phylogenetic trees were constructed based on neighbor-joining (NJ) analysis using Phylip program version 3.6 (Felsenstein 2005). 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 and discussion

Variation within the 699 bp ITS sequence was observed at 18 positions (2.58 %) when comparing *A. malayanum* and *A. sufrartyfex*. Of these, two (0.29 %) and 16 (2.29 %) positions were observed in ITS1 and ITS2 sequences, respectively (Table 1). These variable positions could be used to

Table 1 Variable nucleotide positions in the nucleotide sequence alignments of the partial ITS1 (301 bp) and ITS2 (398 bp) of *A. malayanum* collected from different hosts

	Nucleotide positions																	
	ITS1		ITS2															
	141	100	900	101	123	152	199	219	239	241	269	275	277	280	308	313	339	399
A. malayanum–Lr	T	G	G	T	A	G	G	G	G	G	T	G	A	T	A	G	G	C
A. malayanum–Ie	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. malayanum–Pc	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. malayanum–Ra	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A. sufrartyfex[a]	C	T	C	.	G	A	T	T	C	C	G	T	G	G	G	T	A	A

The sequences of a related species, *A. sufrartyfex* were retrieved from GenBank database. A dot represents an identical nucleotide to that in *A. malayanum* from *L. rubiginosa*

Lr, *L. rubiginosa*; *Ie*, *I. exustus*; *Pc*, *P. canaliculata*; *Ra*, *Rattus* sp

[a] *A. sufrartyfex* retrieved from GenBank database under accession no. EF027101 for ITS1 and EF027100 for ITS2 sequences

differentiate *A. malayanum* and *A. sufrartyfex* except at position 101, which showed only intraspecific variation (0.25 %) in the ITS2 sequence for *A. malayanum* (Table 1). However, a previous study by Morgan and Blair (1995) found that intraspecific variation in the *Echinostoma* (37-collar-spine group) isolates is problematic when considering the utility of the ITS

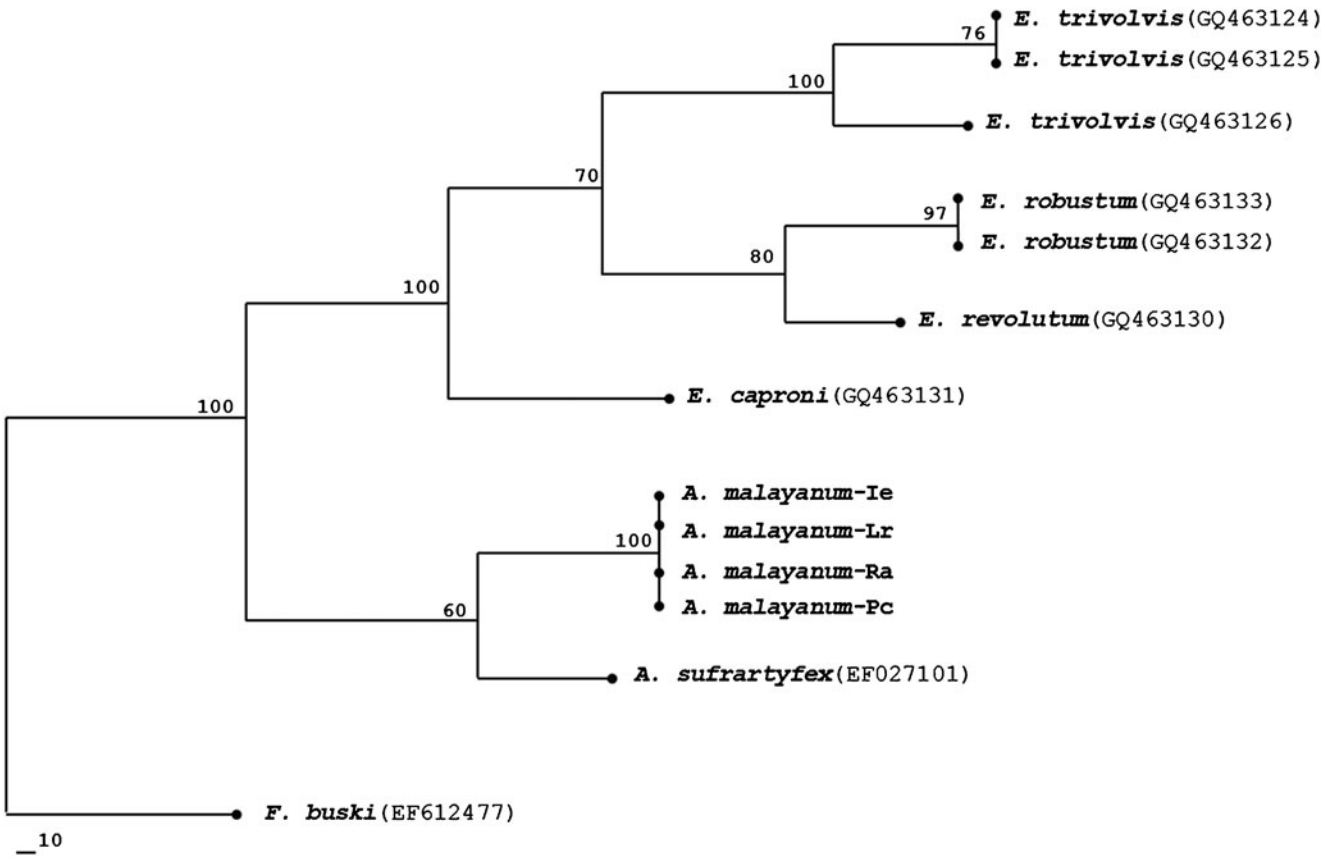

Fig. 1 Strict consensus tree depicting relationships between *A. malayanum* and other related echinostomes inferred from 1,000 replicates of combined ITS1 and ITS2 sequences data based on neighbor-joining by using *F. buski* as an out group. Bootstrap values (>50 %) are indicated above branches. *Lr*, *L. rubiginosa*; *Ie*, *I. exustus*; *Pc*, *P. canaliculata*; *Ra*, *Rattus* sp

sequences for taxonomic diagnosis. Our results are in concordance to the previous study that ITS sequences could be used to differentiate echinostome species, i.e., *E. revolutum* and *Echinostoma trivolvis* (Sorensen et al. 1998).

Our previous report (Saijuntha et al. 2011a) found intraspecific variation in the mitochondrial CO1 sequence within a population of *A. malayanum* (as *E. malayanum*) at 1.95 % of positions, which is higher than in the present study. This result supports of the proposition that the mitochondrial DNA mutates at a much faster rate than the nuclear DNA. The phylogenetic tree for echinostomes based on combined the ITS1 and ITS2 sequences in this study showed that the clustering at the genus/species level is in concordance with that reported previously (Saijuntha et al. 2011b). Moreover, a phylogenetic tree showed that *A. sufrartyfex* is clustered as a monophyletic group with a clade of *A. malayanum*, and clearly separated from other clusters of other echinostomes with high bootstrap value (Fig. 1).

In this case, the results of our study suggest that the synonymy of *A. sufrartyfex* with *A. malayanum* (Mukherjee and Ghosh 1968) is probably incorrect and that both are valid species. This would fit with the disjunct distribution of the two species, with *A. sufrartyfex* being described from India (Lane 1915; Chai 2009) and *A. malayanum* from Southeast Asia (Leiper 1911; Chai 2009) with no records from the intervening Myanmar. However, *A. sufrartyfex* has recently been reported infect mammals in southern Vietnam. These specimens were identified by morphology (Ha Duy et al. 2011). Thus, the genetic characterization of these parasites may be needed to confirm the species taxonomy in Southeast Asian countries.

Previous research indicates that morphological characteristics are insufficient to identify digenean trematode species, including echinostomes, due to the lack of adequate distinguishing characteristics, and that, therefore, genetically distinct cryptic species may be common (Saijuntha et al. 2007; Leung et al. 2009; Saijuntha et al. 2011a). The variable nucleotide positions between *A. malayanum* and *A. sufrartyfex* in their ITS1 and ITS2 sequences found in this study could provide genetic markers for solving the taxonomy status of these closely related echinostomes. Our results indicate that the two sibling species, *A. malayanum* and *A. sufrartyfex* are not synonymous, and that the further comprehensive analysis of their biology, morphology and genetics is needed.

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