DISCRIMINATION 28S RIBOSOMAL GENE OF TREMATODE CERCARIAE IN SNAILS FROM CHIANG MAI PROVINCE, THAILAND

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Abstract. Trematode cercariae are commonly found in many freshwater gastropods. These cercariae can serve to identify the occurrence of such trematodes as *Centrocestus formosanus*, *Haplorchis taichui*, *Haplorchoides* sp, and *Stellantchasmus falcatus*, which are important parasites in Chiang Mai Province, Thailand. As the species of these cercariae cannot be identified accurately based on morphology, this study employed sequencing of a fragment of 28S ribosomal DNA and phylogenetic analysis to identify the trematode cercariae found in freshwater gastropods in Chiang Mai Province. Eight types of trematode cercariae were identified, namely, distome cercaria (grouped with *Philophthalmus* spp clade), echinostome cercaria (grouped with *Echinostoma* spp clade), furcocercous cercaria (grouped with *Posthodiplostomum* sp/*Alaria taxideae*/*Hysteromorpha triloba* clade), monostome cercaria (grouped with *Catatropis indicus* clade), parapleurolophocercous cercaria (grouped with *Haplorchoides* sp clade), pleurolophocercous cercaria (grouped with *Centrocestus formosanus* clade), transversotrema cercaria (grouped with *Transversotrema* spp clade), and xiphidiocercaria (grouped with *Prosthodendrium* spp clade). These results provide important information that can be used for identifying these parasites in epidemiological surveys.

Keywords: trematode cercaria, 28S ribosomal gene, snail, Chiang Mai Province, Thailand

INTRODUCTION

Trematode cercariae commonly are found in many freshwater gastropods, such as *Melanoides tuberculata, Tarebia granifera*, and *Thiara scabra* (Chontananarth and Wongsawad, 2010). They are highly abundant in the northern region of Thailand. Cercariae discovered in snails from Chiang Mai Province, northern Thailand are furcocercous cercaria, gymnocephalus cercaria, megalurous cercaria, monostome cercaria, parapleurolophocercous cercaria, pleurolophocercous

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cercaria, strigea cercaria, xiphidiocercaria, and virgulate cercaria (Chontananarth and Wongsawad, 2013). Snails collected from Erawan waterfall, Erawan National Park, northeastern Thailand are infected with furcocercous cercaria, pleurolophocercous cercaria and xiphidiocercaria (Ukong *et al*, 2007), whereas in northeastern Thailand and Lao PDR over 20 types of cercariae infecting *Bithynia goniomphalos* have been identified including amphistome cercaria, armatae cercaria and virgulate cercaria, with the latter being the most prevalent (Nithiuthai *et al*, 2002; Kiatsopit *et al*, 2015). The identity of cercariae can serve to indicate the occurrence of trematode species, which have been acknowledged to be parasites of significant prevalence in Chiang Mai Province, *viz*, *Centrocestus formosanus*, *Haplorchis taichui*, *Haplorchoides* sp, and *Stellantchasmus falcatus* (Kumchoo *et al*, 2005; Nithikathkul and Wongsawad, 2008; Wongsawad and Wongsawad, 2010; Wongsawad *et al*, 2013).

Species identification using morphological characteristics is difficult to achieve at the larval stages of these trematodes. Several molecular techniques, such as sequences of genomic internal transcribed spacer 1 and internal transcribed spacer 2 (ITS1 and ITS2) and mitochondrial nicotinamide adenine dinucleotide dehydrogenase subunit 1 (ND1) and cytochrome c oxidase subunit 1 (CO1) genes, have been developed for identification of *Fasciola gigantica*, echinostome metacercariae, nuclear ribosomal (r)genes and ITS2 region of Haplorchiinae subfamily (Amer *et al*, 2011; Noikong *et al*, 2014), and high annealing temperature-random amplified polymorphic DNA (HAT-RAPD) method of *Stellantchasmus falcatus* (Wongsawad and Wongsawad, 2010).

Sequences of 28S rDNA have been employed for the classification and phylogeny studies of flukes, Polystome Monogeneans from the oral cavity and bladder of freshwater turtles in Australia, parasites in the genus *Udonella*, digenea and Plagiorchiida (Littlewood *et al*, 1998; Olsen *et al*, 2003). This approach was applied for the identification and phylogeny study of trematode cercariae in snails from Chiang Mai Province, Thailand.

MATERIALS AND METHODS

Sample collection

Snails (*n*=1,256), collected from eight districts in Chiang Mai Province, Thailand (Fig 1), during October 2014 to January 2015 and classified in 14 species (*Bithynia siamensis, Brotia costula costula, Brotia citrina, Brotia baccata, Tarebia granifera, Physa acuta, Filopaludina martensi martensi, F. dorliaris, F. polygamma, Thiara scabra, Melanoides tuberculata, M. jugicostis, Indoplanorbis* sp and *Lymnia auricularia rubiginosa*) based on morphological characteristic (Brandt, 1974). The number of snails from each sampling site were as follow: Chiang Dao (184 snails), Mae Taeng (210 snails), Mae Rim (158 snails), Mueang (242 snails), San Kam-phaeng (135 snails), Doi Lo (112 snails), Chom Thong (118 snails), and Hot (97 snails) districts. Snail specimens were crushed, and cercariae were compressed and fixed in 5% formalin, stained with Delafield's hematoxylin, dehydrated in an alcohol series, cleared in xylol, and mounted in permount. Cercariae were examined by basing on morphology under a stereomicroscope. Individually classified specimens were stored at -20^{o}C until used.

Sequencing of cercarial 28S rDNA

Cercarial DNA of each specimen was extracted using GF-1 Tissue DNA extraction kit (Vivantis, Subang Jaya, Malaysia) according to the manufacturer's instructions and made up as a stock solution of

Fig 1–Map of Chiang Mai Province, Thailand showing districts where snails were collected: Chiang Dao (S1), Mae Taeng (S2), Mae Rim (S3), Mueang (S4), San Kamphaeng (S5), Doi Lo (S6), Chom Thong (S7), Hot (S8).

50 ng/µl in Tris-EDTA buffer and stored at -20°C. PCR amplification of a 1,400 bp fragment of 28S rDNA was performed using primers LSU-5 (5′-TAGGTCGACCC-GCTGAAYTTAAGCA-3′) and 1500R (5′-GCTATCCTGAGGGAAACTTCG-3′), where Y is C or T (Olsen *et al*, 2003) in a 20 µl reaction mixture containing 2 mM of dNTPs, 2 µl of 10X reaction buffer, 2 µM of primer, 20 ng of DNA template and 1 U *Taq* DNA polymerase (Vivantis, Malaysia). Thermocycling (carried out in a My Cycler™ Thermal Cycler; Bio-Rad, Hercules, CA) conditions were as follows: 94°C for 2 minutes; 35 cycle of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds; and a final step at 72°C for 7 minutes. Amplicons were separated by 1.4% agarose gel-electrophoresis, stained with ethidium bromide and recorded using a Kodak digital camera (Gel Logic 100). Amplicons were purified using the GF-1 PCR Kit (Vivantis, Malaysia) and sequenced (BioDesign Pathum Thani, Thailand). Sequences are deposited with GenBank, accession numbers shown in Table 2.

Phylogenetic analysis

Sequence data were assembled using Chromas Pro software (Technelysium, South Brisbane, Australia) and Bio Edit software 5.0.6 (Hall, 1999) for sequence alignments available from GenBank database. Phylogenetic tree was generated from the 1,400 bp 28S rDNA fragment using Maximum Likelihood method. Branches of the phylogenetic tree were tested for all inferred trees using bootstrap analysis and 1,000 random trees were assessed with MEGA version 5.0 (Tamura *et al*, 2011). The 4x rule/K/θ ratio species criterion was applied to determine the likelihood of cryptic speciation (Birky, 2013).

RESULTS

Morphology of the trematode cercariae collected from snails in Chiang Mai Province, Thailand identified them as belonging to seven types, namely,

Table 1
Speciation of trematode parasites.

Cercaria type	Parasite species	GenBank accession number	Identity[a]
Xiphidiocercaria	*Prosthodendrium longiforme*	AF151921.1	95%
	Prosthodendrium chilostomum	AF151920.1	93%
	Prosthodendrium hurkovaae	AF151922.1	91%
Monostome	*Catatropis indicus*	AY222220.1	96%
	Notocotylus intestinalis	JQ890563.2	95%
	Notocotylus intestinalis	JQ890562.2	95%
Distome	*Philophthalmus* sp	JQ627832.1	97%
	Philophthalmus gralli	JQ246435.1	97%
	Cloacitrema narrabeenensis	AY222248.1	94%
Pleurolophocercous	*Centrocestus formosanus*	HQ874609.1	97%
	Metagonimus miyatai	HQ832635.1	91%
	Metagonimus yokogawai	HQ832640.1	91%
Parapleurolophocercous	*Procerovum varium*	HM004184.1	90%
	Procerovum cheni	HM004180.1	90%
	Haplorchis yokogawai	HM004177.1	90%
	Haplorchis pumilio	HM004173.1	90%
	Haplorchis taichui	HM004187.1	90%
	Haplorchoides sp	AY222226.1	89%
Echinostome	*Echinostoma revolutum*	AY222246.1	96%
	Echinoparyphium cinctum	AF184260.1	95%
	Echinostoma paraensei	EU025867.1	96%
	Echinoparyphium rubrum	JX262943.1	95%
Furcocercous	*Posthodiplostomum* sp	AB693170.1	86%
	Alaria taxideae	JF820609.1	86%
	Hysteromorpha triloba	HM114365.1	86%
Transversotremacercaria	*Transversotrema haasi*	AY222186.1	91%
	Transversotrema chevrarum	GQ180857.1	88%
	Transversotrema tragorum	GQ180854.1	88%

[a]Ribosomal gene.

Table 2
Cercariae types used for generation of the 28S rDNA sequences.

Cercaria type	GenBank accession number
Xiphidiocercaria	KU820962
Tranversotremacercaria	KU820963
Pleurolophocercouscercaria	KU820964
Parapleurolophocercouscercaria	KU820965
Echinostomecercaria	KU820966
Furcocercouscercaria	KU820967
Monostomecercaria	KU820968
Distomecercaria	KU820969

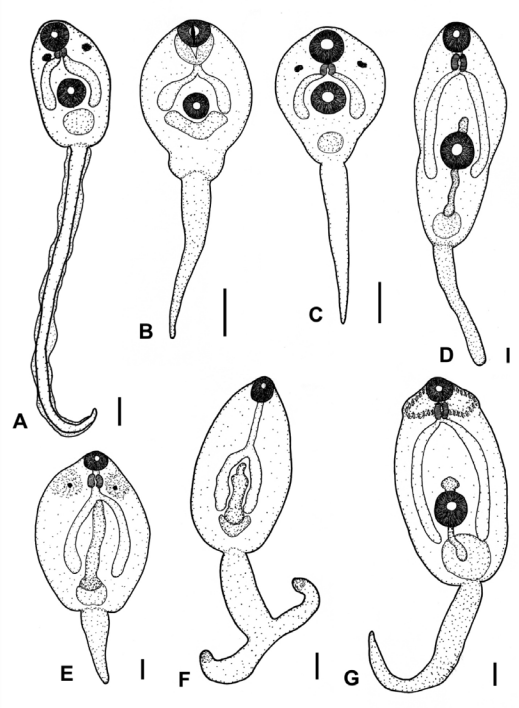

Fig 2–Illustration of trematode cercariae morphologies: A, parapleurolophocercous cercaria; B, xiphidiocercaria; C, pleurolophocercous cercariae; D, distome cercariae; E, monostome cercariae; F, furcocercous cercariae; G, echinostome cercaria. Vertical bar = 30 μm.

distome cercaria, echinostome cercaria, furcocercous cercaria, monostome cercaria, parapleurolophocercous cercaria, pleurolophocercous cercaria, and xiphidiocercaria (Fig 2).

The 1,400 bp fragments of 28S cercarial rDNA (data not shown) were sequenced and compared with those available in GenBank. The variable speciation of parasites compared is demonstrated in Table 1. The data obtained from eight unique sequences revealed strong support (>70%) for monophyletic clades of *Catatropis indicus*, *Centrocestus formosanus*, *Echinostoma* spp, *Haplorchoides* sp, *Philophthalmus* spp, *Posthodiplostomus* sp/*Alaria taxideae*/*Hysteromorepha triloba*, *Prosthodendrium* spp, and *Transversotrema* spp (Fig 3); and application of the K>4θ test revealed that these clades could be grouped with monostome cercaria, pleurolophocercous cercaria, echinostome cercaria, parapleurolophocercous cercaria, distome cercaria, furcocercous cercaria, xiphidiocercaria, and transversotrema cercaria, respectively (Table 3).

DISCUSSION

This study confirms the usefulness of 28S rDNA sequences (albeit fragment) in species discrimination of trematode cercariae, and allowing evolutionary relationship studies as their high sequence variability can be compared across organisms (Littlewood *et al*, 1998).

In previous epidemiological studies in Chiang Mai Province, a high prevalence of *H. taichui* in *Haplorchoides* sp was reported (Kumchoo *et al*, 2005; Nithikathkul and Wongsawad, 2008). Using ITS2 sequences, pleurolophocercous cercaria has been indicated to be the larva of *H. taichui* (Chontananarth and Wongsawad, 2013; Chuboon *et al*, 2013). Based on morphological characteristic, Dechruksa *et al* (2007) reported that parapleurolophocercous cercariae are grouped with *Haplorchis pumilio* and *Centrocestus formosanus*, xiphidiocercariae with *Acanthatrium hitaense*, *Loxogenoides bicolor* and *Haematoloechus similis*. Discrimination and identification of cercariae species should use several techniques to confirm the results.

The flukes, *A. taxideae*, *Catatropis indicus*, *H. triloba*, *Philophthalmus* spp, *Posthodiplostomum* spp, *T. chevrarum*, *T. haasi*, and *T. tragorum*, have not previously been discovered in Chiang Mai Province. An epidemic of *E. revolutum* has been reported recently in Chiang Mai Province (Chantima *et al*, 2013).

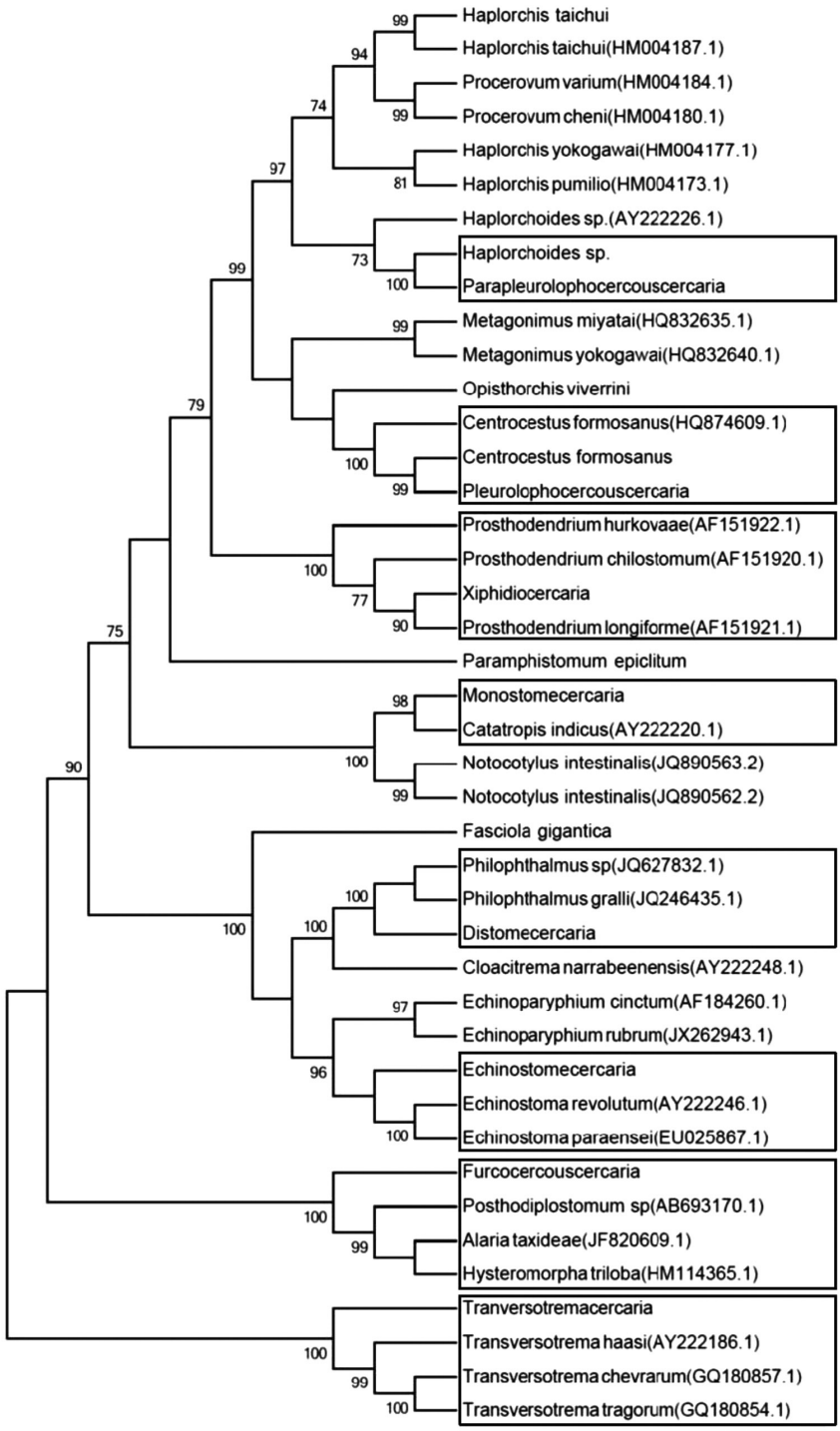

Fig 3–Phylogenetic tree demonstrating the eight trematodes groups. The tree was generated using Maximum Likelihood method with 1000 bootstraps analysis. Number at branch junction indicates percent identity.

Table 3
Variables associated with populations used to test compliance with ''4x rule'' for speciation.

Population	Nucleotide diversity[a] (π)	4 θ	Sequence[a] divergence between clades (K)	$K \geq 4\theta$
Haplorchoides sp-like	0.09091	0.36364	0.05541	No
Centrocestus formosanus-like	0.02461	0.09844	0.02618	No
Prosthodendrium sp-like	0.04695	0.19092	0.03771	No
Philophthalmus sp-like	0.02658	0.10632	0.01457	No
Echinostoma sp-like	0.02492	0.08468	0.03835	No
Transversotrema sp-like	0.11779	0.47116	0.28584	No
Posthodiplostomum sp/*Alaria taxideae*/ *Hysteromorpha triloba*-like	0.04773	0.19244	0.24505	Yes

[a]Based on partial 28S rDNA sequence.

The information obtained from this study can be used in a more accurate identification of these parasites, not only in the northern parts of Thailand, but elsewhere in the country and neighboring regions.

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