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Mitochondrial DNA sequences of 37 collar-spined echinostomes (Digenea: Echinostomatidae) in Thailand and Lao PDR reveals presence of two species: *Echinostoma revolutum* and *E. miyagawai*

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ABSTRACT

The "37 collar-spined" or "*revolutum*" group of echinostomes is recognized as a species complex. The identification of members of this complex by morphological taxonomic characters is difficult and confusing, and hence, molecular analyses are a useful alternative method for molecular systematic studies. The current study examined the genetic diversity of those 37 collar-spined echinostomes which are recognized morphologically as *Echinostoma revolutum* in Thailand and Lao PDR using the cytochrome c oxidase subunit 1 (CO1) and the NADH dehydrogenase subunit 1 (ND1) sequences. On the basis of molecular investigations, at least two species of 37 collar-spined echinostomes exist in Southeast Asia, namely *E. revolutum* and *Echinostoma miyagawai*. The specimens examined in this study, coming from ducks in Thailand and Lao PDR, were compared to isolates from America, Europe and Australia for which DNA sequences are available in public databases. Haplotype analysis detected 6 and 26 haplotypes when comparing the CO1 sequences of *E. revolutum* and *E. miyagawai*, respectively, from different geographical isolates from Thailand and Lao PDR. The phylogenetic trees, ND1 haplotype network and genetic differentiation (ϕ_{ST}) analyses showed that *E. revolutum* were genetically different on a continental scale, i.e. Eurasian and American lineages.

1. Introduction

Echinostomes of the "37 collar-spined" or the "*revolutum*" complex are recognized as zoonotic, food-borne intestinal trematodes that are causative agents of echinostomiasis worldwide. Species of this complex have three-host life cycles, with the first intermediate hosts being freshwater lymnaeid, planorbid, viviparid or physid snails (Kostadinova and Gibson, 2000). A variety of second intermediate hosts have been reported including pulmonate and prosobranch snails, mussels, tadpoles and freshwater turtles. The final hosts are birds, rodents, and mammals, including humans, which are infected by eating raw or partially cooked aquatic hosts containing metacercariae (Huffman and Fried, 1990). There are currently numerous species defined as members of this group, i.e. *Echinostoma caproni*, *Echinostoma cinetorchis*, *Echinostoma echinatum*, *Echinostoma friedi*, *Echinostoma jurini*, *Echinostoma miyagawai*, *Echinostoma nasincovae*, *Echinostoma paraensei*, *Echinostoma parvocirrus*, *Echinostoma revolutum* and *Echinostoma trivolvis* (Fried et al., 2004; Georgieva et al., 2013; Faltynkova et al., 2015).

E. revolutum is the most widely distributed species from this group, occurring from Asia and Oceania to Europe, the Americas and Australia (Fried et al., 2004; Sohn et al., 2011). In Southeast Asia, *E. revolutum* has been reported infecting humans from Thailand (Bhaibulaya et al., 1966), Lao PDR (Chai et al., 2012) and Cambodia (Sohn et al., 2011), especially in areas where local people

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consume raw or partially cooked aquatic animal hosts such as golden apple snails (*Pomacea canaliculata* and *Pila* spp.). In other Asian countries, the discovery of *E. revolutum* in rodents and snails hosts, e.g. house rats in Korea (Lee et al., 1990) and *Filopaludina* spp. (snails) in Vietnam (Chai et al., 2011), has been reported. Moreover, other sibling species have also been reported, e.g. *E. cinetorchis* infecting dogs, ducks and chickens in Vietnam (Anh et al., 2010; Lan-Anh et al., 2009).

The taxonomy and species identification of these morphospecies has long been confused because there are no unique characteristics to discriminate between adults of the species. Previously, only one consistently different morphological character, i.e. the number of pores on the para-esophageal gland cells in the cercariae was recognized (Kanev, 1994; Morgan and Blair, 2000). More recently, Faltynkova et al. (2015) provides keys to identify the European species of '*revolutum*' group based on morphological characters of cercariae and adult worm. However, some species have a large number of synonyms due to their wide geographical distribution and sympatric distributions with two or more sibling species, e.g. *E. revolutum*, *E. paraensei*, *Echinostoma robustum* and *E. trivolvis* in the Americas, *E. revolutum*, *E. robustum* and *E. friedi* in Europe or *E. revolutum* and *E. cinetorchis* in Asia (Fried and Graczyk, 2004).

To avoid taxonomic confusion, molecular markers/techniques can be used to advance our understanding of the systematics and genetic differentiation between these echinostomes (Kostadinova et al., 2003), including the 37 collar-spined group. Some molecular markers have already been established (Morgan and Blair, 1995). Evidence of two cryptic species, one in Europe and one in North America, was recently demonstrated based on molecular as well as morphological analyses (Georgieva et al., 2013).

Substantial genetic variation was reported in *E. revolutum* based on isolates from Europe (Kostadinova et al., 2003), North America (Detwiler et al., 2010; Georgieva et al., 2013), Australia (Morgan and Blair, 1998a) and Southeast Asia (Saijuntha et al., 2011a). Saijuntha et al. (2011b, 2011c) reported intraspecific variation in *E. revolutum* based on spatial and temporal factors in Thailand and Lao PDR using allozyme and ITS1 sequence markers. More recently, Noikong et al. (2014) reported evidence that echinostome metacercariae, molecularly classified as an *E. revolutum*-like group and recovered from snail hosts in northern Thailand using ITS and ND1 sequences, belonged to several species. Based on these reports, the taxonomic status and systematics of the 37 collar-spined group, including *E. revolutum* and other related species, in Southeast Asia is still unclear.

Many previous publications suggest that the mitochondrial ND1 gene provides the best resolution for investigating relationships within the 37 collar-spined complex (Morgan and Blair, 1995, 1998b). Additionally, the mitochondrial CO1 gene reveals high sequence variability in Southeast Asian echinostomes (Saijuntha et al., 2011a). Therefore, this study aims to examine the genetic diversity of the Southeast Asian 37 collar-spined echinostomes recovered from free-grazing ducks originating from different locations in Thailand and Lao PDR. Haplotype network and phylogenetic data will be analyzed using both CO1 and ND1 sequences, which will be compared with the other isolates from North America and Europe, as well as other closely related species.

2. Materials and methods

2.1. Sample collection and DNA extraction

One hundred and one adult echinostomes with 37 collar-spines were collected from the intestines of domestic ducks from abattoirs. Of these, 92 were collected from Thailand and 9 from Lao PDR (Table 1). The specimens were identified under a light microscope based on the number of collar-spines, the shape of the collar head (well developed) and the testicles (multi-lobed or oval) (Radomyos et al., 2004) and the morphological key to the genera of the Echinostomatidae (Kostadinova, 2005). Once removed, adult worms were washed several times in NSS before being fixed in molecular grade ethanol and stored at −20 °C until used. To extract DNA, a single worm was placed into a 1.5 ml vial and the alcohol removed by vacuum centrifuge. Next an E.Z.N.A.® Tissue DNA kit (Omega bio-tek, USA) was used following the manufacturer's instructions.

2.2. Polymerase Chain Reaction and DNA sequencing

To amplify the mitochondrial genes, Polymerase Chain Reaction (PCR) was performed using primer pairs of FH3 (5′-TTT TTT GGG CAT CCT GAG GTT TA-3′) and FH5 (5′-TAA AGA AAG AAC ATA ATG AAA ATA ATC-3′) for the CO1 fragment (Bowles et al., 1993), whereas JB11 (5′-AGA TTC GTA AGG GGC CTA ATA-3′) and JB12 (5′-ACC ACT AAC TAA TTC ACT TTC-3′) were used for ND1 fragment amplification (Morgan and Blair, 1998b). PCR comprised initial denaturation at 95 °C for 5 min, followed by 35 cycles with 30 s denaturation at 95 °C, 40 s for primer annealing at 50 °C, and 1 min for primer extension at 72 °C, with a final extension step at 72 °C for 8 min. All PCR products were gel-purified using Gene Clean II Kit (Q-BIO Gene, Carlsbad, CA, USA). The purified PCR products were cycle-sequenced by using the forward primer, i.e. primer FH3 for CO1 and primer JB11 for ND1 as sequencing primer and sequenced using ABI BigDye v3.1 (Warrington, UK) and run on an ABI Prism 377 automated sequencer (Perkin-Elmer Corp., Foster City, CA, USA).

2.3. Data analyses

All CO1 and ND1 sequences from Thailand and Lao PDR examined in this study were deposited in GenBank under the accession numbers KP455511–KP455633. The sequences of *E. revolutum* from the isolates of North America and/or Europe, *E. miyagawai* from Europe and Australia, as well as other related species used for comparative analysis, were retrieved from the GenBank database. The sequences were aligned using the ClustalW program (Larkin et al., 2007). Haplotype data was calculated in the DnaSp v5 program (Librado and Rozas, 2009) and Arlequin ver 3.5.1.3 (Excoffier and Lischer, 2010). A maximum parsimony haplotype network was generated using the Network 4.6.1.3 program based on median-joining network (Bandelt et al., 1999). The

Table 1
Collection localities of adult worms of 37-collar-spined echinostomes from domestic ducks in Thailand and Lao PDR.

Code	n*	District	Province	Region	Country
ST	6	Kong Krailat	Sukhothai	North	Thailand
PL	6	Mueang	Phitsanulok	North	Thailand
PJ	1	Bueng Na Rang	Phichit	North	Thailand
NW	3	Takhli	Nakhon Sawan	Central	Thailand
LB	4	Ban Mi	Lop Buri	Central	Thailand
AY	14	Bang Ban	Ayutthaya	Central	Thailand
PT	5	Khlong Laung	Pathum Thani	Central	Thailand
NM	2	Chum Phuang	Nakhon Ratchasima	Northeast	Thailand
KK	35	Mueang	Khon Kaen	Northeast	Thailand
MS	6	Mueang	Maha Sarakham	Northeast	Thailand
RE	12	Changhan	Roi Et	Northeast	Thailand
VT	9	Kampang Nakhon	Vientiane	North	Lao PDR

* Number of adult worms collected.

Neighbor-Joining (NJ) and Maximum Likelihood (ML) trees analyses of the Kimura-2-parameter model were constructed by using the MEGA v6.06 program (Tamura et al., 2011) with nodal support estimated using 1000 bootstrap re-sampling. We used the program MrModeltest ver. 2.3 (Nylander, 2008) to determine the most appropriate model for molecular evolution that can be utilized in Bayesian inference (BI) analyses using corrected Akaike information criterion (AIC). The model selected was GTR + I + G for CO1 and GTR + G for ND1 analyses. BI analyses were performed in MrBayes software package 3.1.2 (Ronquist and Huelsenbeck, 2003). The number of generations used in these analyses was 2,000,000 for the CO1 alignment and 12,000,000 for the ND1 alignment, sampling every 100th generation. To calculate the posterior probabilities, a number of trees were sampled at 5000 for the CO1 alignment and 100,000 for the ND1 alignment after the standard deviation values of the runs dipped below 0.01.

3. Results

3.1. Haplotype diversity and network analyses

The partial sequence of the 382 bp of CO1 from all 101 specimens was determined, while the partial sequence of the 448 bp of ND1 of 22 specimens was determined. We found that 9 and 92 specimens could be molecularly identified as *E. revolutum* and *E. miyagawai*, respectively. Intra-specific genetic variation among the Southeast Asian specimens was analyzed based on the variation of the CO1 sequence of which 6 and 26 haplotypes of *E. revolutum* and *E. miyagawai* were classified, respectively. The most common haplotype of the CO1 sequence was H3. Haplotype H3 of *E. miyagawai* was found in 27 specimens from 8 different localities; the other common haplotypes were H2, H5 and H11 (Fig. 1). The haplotype frequency distribution was not significantly related to geographical isolates in Southeast Asia. However, there were several singletons of the CO1 haplotype, which is a unique genotype of *E. miyagawai* in particular localities in Thailand and Lao PDR, e.g. H1 and H4 were singletons of the VT isolate, H8 of ST, H10 of PL, H13 of LB, H15 and H16 of AY, H19–H21 of RE, and H22–H26 of KK isolates (see more details in Fig. 1).

To compare the isolates from Southeast Asia with the other isolates from America, Europe and Australia, the ND1 gene was partially sequenced for 8 and 14 specimens of *E. revolutum* and *E. miyagawai*, respectively. The haplotype network of ND1 sequence showed that the group of the isolates of *E. revolutum* from America differed from the group of the isolates from Europe and Southeast Asia at 20 mutational steps. The group of isolates of *E. miyagawai* from Eurasia and Australia differed at 4 mutational steps (Fig. 2).

3.2. Genetic differentiation analysis

Genetic differentiation (ϕ_{ST}) between the isolates from different continents was calculated. The isolates of *E. miyagawai* from the three isolates, i.e. Southeast Asia, Europe and Australia showed no significant genetic difference ($P > 0.05$) based on ND1 sequence with ϕ_{ST} ranging from 0.021 to 0.028. In the case of *E. revolutum*, the isolates from Southeast Asia showed no significant genetic difference from the isolates from Europe based on ND1 sequence with $\phi_{ST} = 0.033$ ($P = 0.153$). The isolates from America were highly significantly genetically different from the isolates from Southeast Asia based on CO1 and ND1 sequences with $\phi_{ST} = 0.797$ ($P < 0.001$) and 0.862 ($P < 0.001$), respectively. They also differed from the isolates from Europe based on comparison of the ND1 sequence with $\phi_{ST} = 0.856$ ($P < 0.001$) (Table 2).

3.3. Phylogenetic analyses

The phylogenetic analyses of the CO1 (188 bp after trimming) sequences constructed from NJ and BI analyses showed similar topologies as did the ND1 (448 bp) sequences based on ML and BI analyses. The sequences of CO1 of three specimens, two of *E. revolutum* (GU324943 and GU324944) and one of *Echinoparyphium recurvatum* (GU324945), published by Saijuntha et al. (2011a) were included. The sequences of *E. revolutum* clustered within the Eurasian lineage of *E. miyagawai*, whereas the *E. recurvatum* sequence clustered within the Southeast Asian lineage of *E. revolutum* examined in the current study (Fig. 3). In addition, one sequence of *E. miyagawai* from Europe clustered together with 92 specimens from Southeast Asian isolates, which appeared as sister to *E. robustum*. The nine specimens from Southeast Asian isolates were closely clustered and could be depicted as a sister group to the isolates from America, which were identified as *E. revolutum* based on morphological and molecular analyses (Fig. 3).

The ML tree of ND1 sequences showed that the *E. revolutum* and *E. miyagawai* from Southeast Asia examined in this study clustered together with the European isolates of both species. The 14 and eight specimens from Thailand and Lao PDR clustered together with numerous isolates from Europe identified based on morphological and molecular evidence as *E. miyagawai* and *E. revolutum*, respectively (see Georgieva et al., 2014; Faltynkova et al., 2015). The *E. revolutum* sequence of Morgan and Blair (1998a,b) from Australia clustered within the *E. miyagawai* lineage, but was slightly divergent from the other Eurasian specimens. The lineages of *E. miyagawai* appeared as a sister group to *E. robustum* and *Echinostoma paraulum*. Moreover, we also recovered two separated lineages of *E. revolutum*, namely the Eurasian and American lineages (Fig. 4).

4. Discussion

Previous molecular analyses of the echinostomes in "37 collar-spined" or the "*revolutum*" group have demonstrated that several cryptic species/lineages exist in this complex group in central and northern Europe and North America (Kostadinova et al., 2003). The current investigation using DNA sequence variation, the first intensive investigation of 37 collar-spined echinostomes in Southeast Asia, has provided us with strong evidence that cryptic species exist in free-grazing ducks in Thailand and Lao PDR. These cryptic species belong to two currently morphologically defined species, namely *E. revolutum* and *E. miyagawai*. Of the several reports of human infection by 37 collar-spined echinostomes in Southeast Asia, only *E. revolutum* has been identified, mainly based on the morphology of adult worms or eggs (Chai et al., 2012; Sohn et al., 2011). A sibling species, *E. cinetorchis* infecting poultry and dogs, has been reported in Vietnam (Anh et al., 2010; Lan-Anh et al., 2009) for which, unfortunately, no DNA sequences were available for comparison in the current study. *E. miyagawai* was previously reported to infect animals in Asia and Europe (Kostadinova et al., 2000; Toledo et al., 2009), and our present molecular data suggest that both species of 37 collar-spined echinostomes are present in free-grazing ducks in Thailand and Lao PDR, with *E. miyagawai* being more wide spread in our study areas.

Saijuntha et al. (2011a) might have confused *E. revolutum* with *E. recurvatum* (Georgieva et al., 2014) due to a lack of CO1 data on the 37 collar-spined echinostomes at that time. Our reanalysis and newly publishing data shows that *E. revolutum* (GU324943 and GU324944) (Saijuntha et al., 2011a) is in fact *E. miyagawai*, whereas *E. recurvatum* (GU324945) is *E. revolutum* as confirmed by CO1 sequences. Thus molecular diagnosis to differentiate the

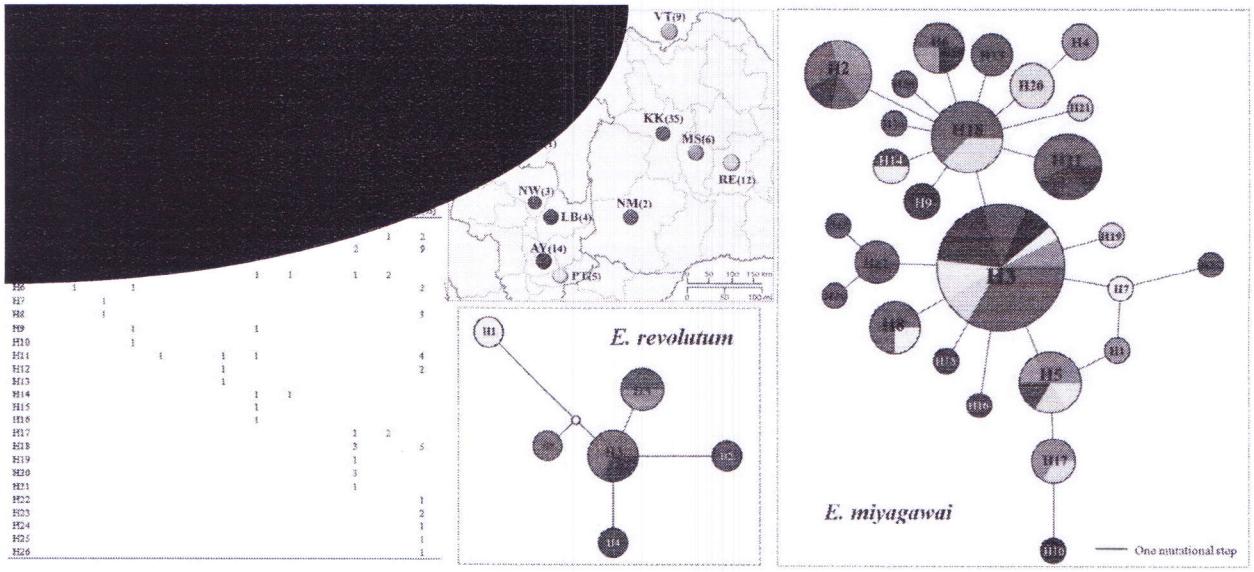

Fig. 1. Haplotype frequency and haplotype network generated based on 382 bp of CO1 sequence of *E. revolutum* and *E. miyagawai* from difference isolates in Thailand and Lao PDR. Number of analyzed specimens from each locality is indicated in the brackets after localities code in a map and table.

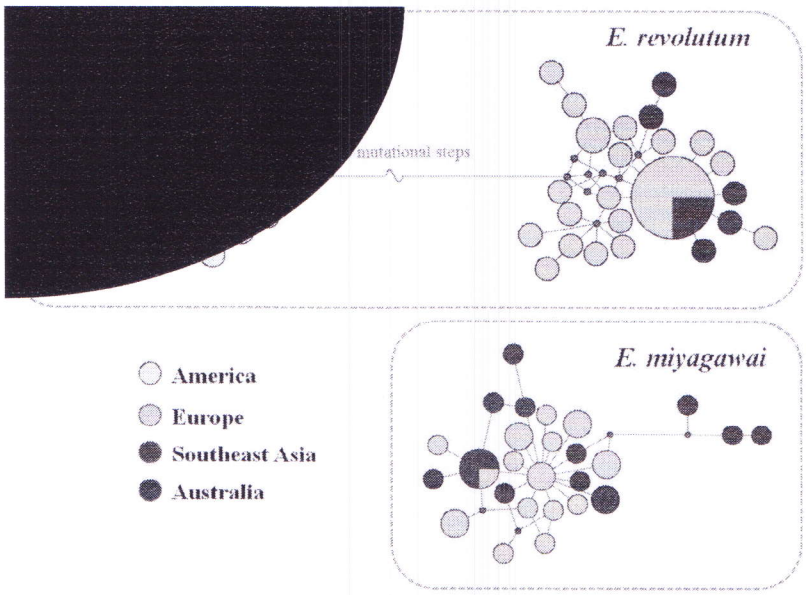

Fig. 2. Haplotype network generated based on 448 bp of ND1 sequence of *E. revolutum* and *E. miyagawai* from Southeast Asia, Europe, America and Australia.

Table 2
Genetic differentiation (ϕ_{ST}) (lower triangle) and *P*-value (upper triangle) among the different isolates of *E. revolutum* and *E. miyagawai* from Southeast Asia (SEA), Europe (EU), America (US) and Australia (AUS) examined by CO1 and ND1 sequence.

	CO1			ND1			
	Isolates	US	SEA	Isolates	US	EU	SEA
E. revolutum	US	–	<0.001	US	–	<0.001	<0.001
	SEA	0.797	–	EU	0.856	–	0.153
				SEA	0.862	0.033	–
				Isolates	AUS	EU	SEA
		E. miyagawai		AUS	–	0.171	0.054
				EU	0.021	–	0.504
				SEA	0.028	0.026	–

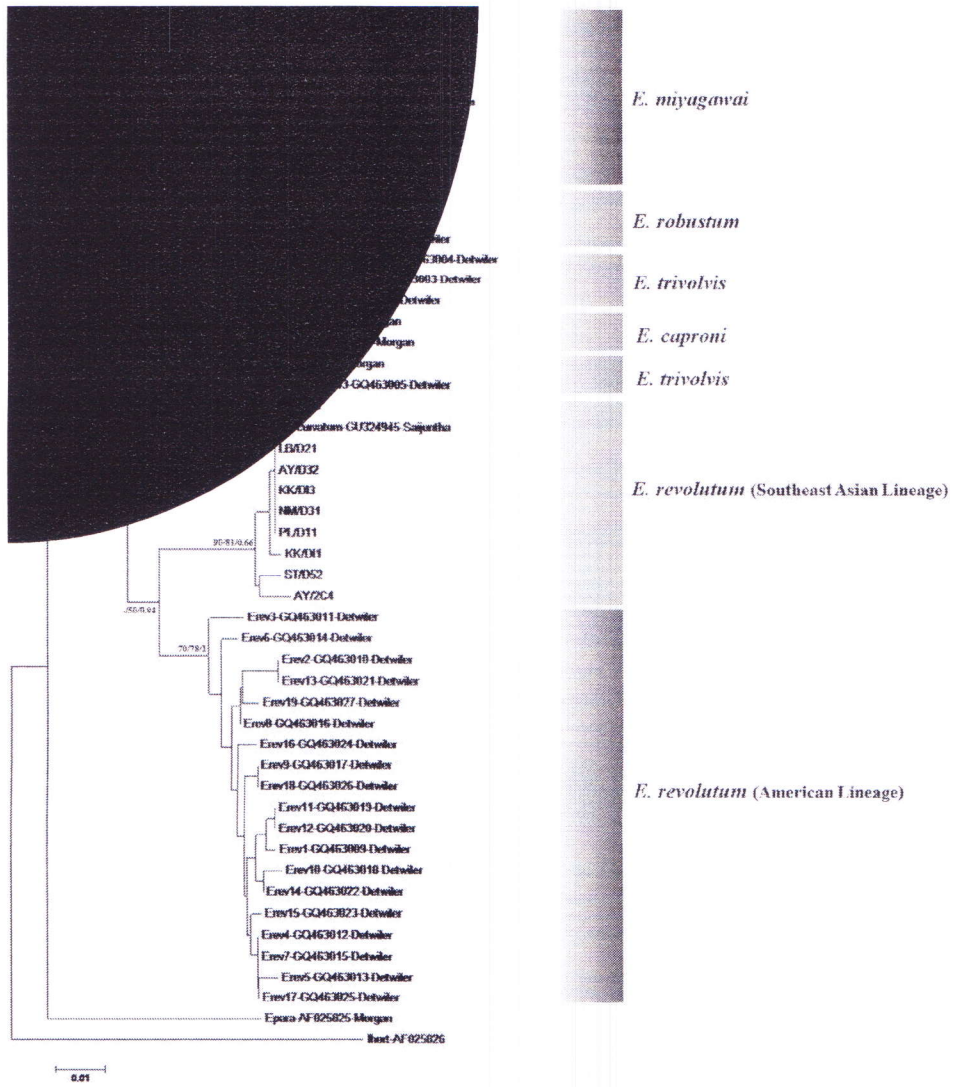

Fig. 3. A Neighbor-Joining (NJ) tree constructed based on 188 bp of CO1 sequence of *E. revolutum* and *E. miyagawai* from Thailand and Lao PDR, the accession number of which is not provided. The other isolates as well as related species available in GenBank were also included. Each sequence was submitted/published by "Detwiler" are Detwiler et al. (2010, 2012), "Morgan" are Morgan and Blair (1998a, 1998b), "Saijuntha" is Saijuntha et al. (2011a). Nodal supports are of bootstrap values obtained by NJ and ML analyses and Bayesian posterior probability, respectively. The scale-bar indicates the expected number of substitutions per site. A sequence of *Isthmiophora hortensis* (Ihort) was used as out-group.

cryptic species of this group in Southeast Asia can be used to resolve problems associated with the morphological similarity of species. There is still high prevalence of echinostome infection in humans in some areas in Southeast Asia, in particular Lao PDR and Cambodia (Chai et al., 2012; Sohn et al., 2011). All of these previous reports have identified echinostomes specimens based on morphology alone. It would be worth aiming at molecularly identifying future human cases of echinostomiasis in Southeast Asia. It is interesting that only *E. revolutum* of the 37 collar-spined group infects humans in Southeast Asia.

A previous study of mitochondrial ND1 sequence variation among the "*revolutum*" group indicated that *E. revolutum* haplotypes from Europe form a monophyletic group which clusters closely with a monophyletic group of isolates from North America, however, isolates from Southeast Asia were not included in this study due to the lack of data (Georgieva et al., 2013). Therefore, the Southeast Asian isolates were analyzed and included in this study. Interestingly, our results show that *E. revolutum* from Southeast Asia clustered as a monophyletic clade with the European isolates, named as the "Eurasian lineage", which is distinct from the American isolates. In addition, the ND1 haplotype network showed that the *E. revolutum* isolates from Eurasia and America were very genetically distinct from one another. There is currently no DNA sequence of *E. miyagawai* from America available in any database, thus only Southeast Asian, European and Australian isolates could be compared. We found that *E. miyagawai* from Southeast Asia was monophyletic with the European isolates, named as the "Eurasian lineage" for this species, which was slightly different from the Australian lineage with no significant genetic differentiation (ϕ_{ST}) being observed between those lineages. These results strongly suggested that genetic differentiation within *E. revolutum* may be influenced geographically at the continent scale, except for the isolates from Europe and Southeast Asia, which are defined as the "Eurasian population".

Interestingly, these echinostomes can probably be carried in snails ingested by water birds but not digested (Fried and

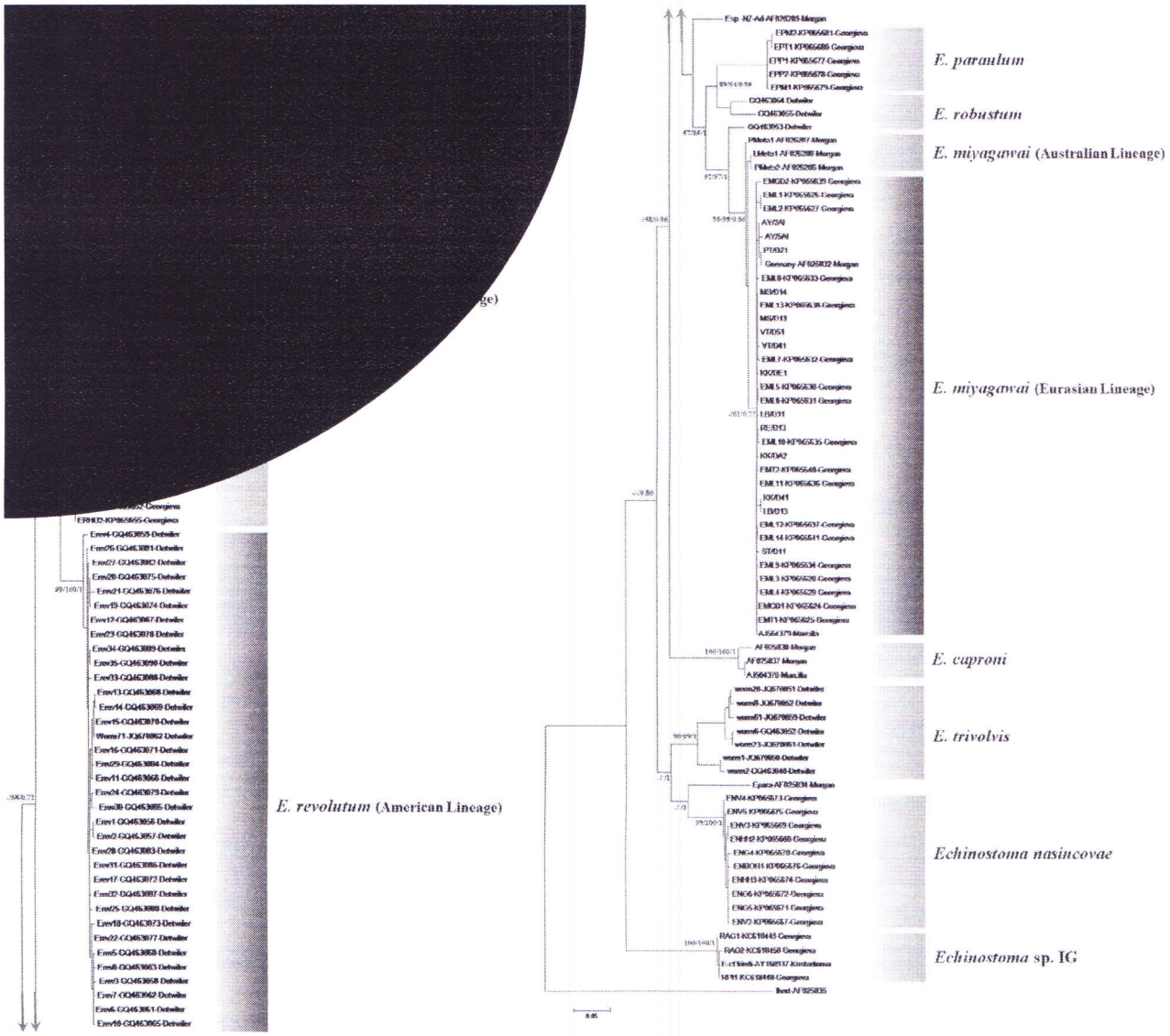

Fig. 4. A Maximum Likelihood (ML) tree constructed based on 448 bp of ND1 sequence of *E. revolutum* and *E. miyagawai* from Thailand and Lao PDR for which the accession number is not provided. The other isolates as well as related species available in GenBank were also included. Each sequence was submitted/published by "Detwiler" are Detwiler et al. (2010, 2012), "Georgieva" are Georgieva et al. (2013, 2014), "Kostadinova" is Kostadinova et al. (2003), "Marcilla" is Marcilla et al. (unpublished), "Morgan" are Morgan and Blair (1998a, 1998b). Nodal supports are of bootstrap values obtained by NJ and ML analyses and Bayesian posterior probability, respectively. The scale-bar indicates the expected number of substitutions per site. A sequence of *Isthmiophora hortensis* (Ihort) was used as out-group.

Graczyk, 2004). The nine main flyways are quite separate between the American and Eurasian routes (Boere and Stroud, 2006). This is potentially the route of gene flow of *E. revolutum* and *E. miyagawai* between continents, resulting in a European and Southeast Asian (Eurasia) lineage, and Australian and American lineages.

Our results show that the cryptic lineages of *E. revolutum* exist at a continental scale. Another potential causes of genetic differentiation among the 37 collar-spined echinostomes is specialization of infecting different species of intermediate and final hosts, i.e. the various aquatic animals which act as intermediate hosts and the final hosts which could be birds, rodents or other mammals, including humans. The various species or cryptic species of snail intermediate hosts located in a particular area may contain differently adapted genotypes/haplotypes. As previously reported a unique haplotype based on the ND1 sequence was observed in *Artyfechinostomum malayanum* (syn. *Echinostoma malayanum*) recovered from *P. canaliculata* snails in Thailand, which were introduced to Southeast Asia several years ago. This haplotype, however, has not yet been found in native snail species (Tantrawatpan et al., 2013).

However, we found many common or shared haplotypes of *E. miyagawai* in Thailand and Lao PDR. This indicates that there has been gene flow among the populations in this region. This finding may be related to the farmers feeding their free-grazing ducks on naturally occurring snails by rotating them among rice paddies in Thailand (Saijuntha et al., 2013).

In conclusion, there are two species of 37 collar-spined echinostomes infecting ducks in Thailand and Lao PDR with *E. miyagawai* being more wide spread. Moreover, our specimens were clustered together with European isolates. However, there are still some other cryptic species of this group distributed in Southeast Asia, e.g. *E. cinetorchis*, *E. jurini* and *E. echinatum*, that have not been

reported by molecular genotyping. Thus, comprehensive investigations of the genetic variation and the relationships between populations and species of 37 collar-spined echinostomes in Southeast Asia need to be continued using larger sample sizes covering the full extent of species across their whole range in endemic regions including different species of animal host. The biology and morphology of each life cycle stage should also be investigated to confirm the number of species that make up the species complex of the 37 collar-spined echinostomes in Southeast Asia.

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Date: Jul 18, 2015
To: "Weerachai Saijuntha" weerachai.s@msu.ac.th
cc: serge.morand@univ-montp2.fr;michel.tibayrenc@ird.fr
From: "MEEGID - Infection, Genetics and Evolution" meegid@elsevier.com
Subject: Your Submission

Ms. Ref. No.: MEEGID-D-15-00224R2
Title: Mitochondrial DNA sequences of 37 collar-spined echinostomes (Digenea: Echinostomatidae) in Thailand and Lao PDR reveals presence of two species: Echinostoma revolutum and E. miyagawai.
Infection, Genetics and Evolution

Dear Dr. Saijuntha,

I am pleased to confirm that your paper "Mitochondrial DNA sequences of 37 collar-spined echinostomes (Digenea: Echinostomatidae) in Thailand and Lao PDR reveals presence of two species: Echinostoma revolutum and E. miyagawai." has been accepted for publication in Infection, Genetics and Evolution.

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