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GENETICS, EVOLUTION, AND PHYLOGENY - ORIGINAL PAPER

Intron sequence variation of the echinostomes (Trematoda; Echinostomatidae): implications for genetic investigations of the 37 collar-spined, *Echinostoma miyagawai* Ischii, 1932 and *E. revolutum* (Fröelich, 1802)

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Abstract
Echinostomes are a diverse group of digenetic trematodes that are difficult to classify by predominantly traditional techniques and contain many cryptic species. Application of contemporary genetic/molecular markers can provide an alternative choice for comprehensive classification or systematic analysis. In this study, we successfully characterized the intron 5 of domain 1 of the taurocyamine kinase gene (TkD1Int5) of *Artyfechinostomum malayanum* and the other two species of the 37 collar-spined group, *Echinostoma revolutum* and *Echinostoma miyagawai*, whereas TkD1Int5 of *Hypoderaeum conoideum* cannot be amplified. High levels of nucleotide polymorphism were detected in TkD1Int5 within *E. revolutum* and *E. miyagawai*, but not in *A. malayanum*. Thus, TkD1Int5 can be potentially used as genetic marker for genetic investigation of *E. miyagawai* and *E. revolutum*. We therefore used TkD1Int5 to explore genetic variation within and genetic differentiation between 58 samples of *E. miyagawai* and five samples of *E. revolutum*. Heterozygosity was observed in 17 and two samples with 16 and three insertion/deletion (indel) patterns in *E. miyagawai* and *E. revolutum*, respectively. Heterozygous samples were then cloned and nucleotide sequence was performed revealing the combined haplotypes in a particular sample. Based on nucleotide variable sites (excluding indels), the 72 *E. miyagawai* and seven *E. revolutum* haplotypes were subsequently classified. The haplotype network revealed clear genetic differentiation between *E. miyagawai* and *E. revolutum* haplogroups, but no genetic structure correlated with geographical localities was detected. High polymorphism and heterogeneity of the TkD1Int5 sequence found in our study suggest that it can be used in subsequent studies as an alternate independent potential genetic marker to investigate the population genetics, genetic structure, and possible hybridization of the other echinostomes, especially the 37 collar-spined group distributed worldwide.

Keywords Genetic variation · Genetic differentiation · Heterozygosity · Intestinal fluke · Indel · Taurocyamine kinase

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Introduction

Echinostomes are intestinal trematodes which have a global distribution (Fried and Graczyk 2004; Sohn et al. 2011). At least 20 species belonging to the family Echinostomatidae have been currently recognized as causative agents of human echinostomiasis (Toledo et al. 2019). These echinostomes have various aquatic animals as their first and second intermediate hosts with a broad spectrum of final and reservoir hosts (Huffman and Fried 1990; Kostadinova and Gibson 2000). Several species of echinostomes, such as *Echinostoma revolutum* (Fröelich, 1802), *Artyfechinostomum malayanum* (Leiper, 1911) Mendheim, 1943 (syn. *Echinostoma malayanum*) and *Hypoderaeum conoideum* (Bloch, 1872) Dietz, 1909, have been reported infecting humans in Southeast Asia (Bhaibulaya et al. 1966; Sohn et al. 2011; Chai et al. 2012). This is especially the case in areas where local people consume raw or partially cooked aquatic animal hosts such as golden apple snails, naiads, and tadpoles (Toledo et al. 2019). On the other hand, other members of the "37 collar-spined" or "*revolutum*" species complex group contain at least 12 currently defined valid species (Fried and Graczyk 2004; Chai 2019). Of these, *E. revolutum* and *Echinostoma miyagawai* Ischii, 1932 are the most widely distributed species of the complex, occurring worldwide (Fried and Graczyk 2004; Sohn et al. 2011). In addition, *E. revolutum* and *E. miyagawai* have been reported to coinfect ducks in Korea, Thailand, and China (Eom et al. 1984; Nagataki et al. 2015; Li et al. 2019).

Comprehensive genetic variation has been substantially reported in *E. revolutum* and *E. miyagawai* from isolates in Europe, North America, Australia, and Southeast Asia (Morgan and Blair 1998; Kostadinova et al. 2003; Detwiler et al. 2010; Saijuntha et al. 2011a; Georgieva et al. 2013; Nagataki et al. 2015). Comparisons of the complete mitochondrial genome of *E. miyagawai* with closely related species as well as phylogenetic analyses have been reported (Fu et al. 2019; Li et al. 2019). Intraspecific variation in the 37 collar-spined echinostomes based on spatial, temporal, and geographical factors in Thailand and Lao PDR using allozyme, nuclear DNA, and mitochondrial DNA markers has been previously reported and discussed (Saijuntha et al. 2011a, b; Nagataki et al. 2015). Noikong et al. (2014) also reported evidence that echinostome metacercariae, molecularly classified as an *E. revolutum*-like group and recovered from snail hosts in northern Thailand using internal transcribed spacer (ITS) and NADH dehydrogenase subunit 1 (ND1) sequences, belonged to different genetic groups. Mitochondrial DNA sequences revealed that the 37 collar-spined echinostomes found infecting free-grazing ducks consisted of two species, *E. revolutum* and *E. miyagawai* (Nagataki et al. 2015). Therefore, the taxonomic status and systematics of the 37 collar-spined group in Southeast Asia remain controversial.

Several previous reports suggested that the intron sequences of taurocyamine kinase gene (TK) could provide suitable resolution to investigate genetic variation, genetic differentiation, genetic relationships, and heterozygosity of parasitic trematodes (Jarilla et al. 2013; Saijuntha et al. 2016; Saijuntha et al. 2018). Moreover, the TK intron region could be applied to explore genetic recombination in a *Fasciola* species, in this case, evidence of hybridization between *F. gigantica* and *F. hepatica* (Saijuntha et al. 2018). Therefore, we aimed to characterize and use the intron 5 of domain 1 of taurocyamine kinase gene (TkD1Int5) as a molecular marker for the genetic investigations of the morphospecies 37 collar-spined echinostomes, i.e., *E. revolutum* and *E. miyagawai* collected from domestic ducks in Thailand and Lao PDR.

Materials and methods

DNA samples and primer design

Fifty-eight DNA samples of *E. miyagawai* and five *E. revolutum* from a study by Nagataki et al. (2015) were used for comparative analyses in the current study as detailed in Table 1. The same samples have previously been identified by morphology as 37 collar-spined echinostomes and subsequently by partial cytochrome c oxidase subunit 1 (CO1) and ND1 sequence analyses for species confirmation as *E. miyagawai* and *E. revolutum* (Nagataki et al. 2015). In addition, eight DNA samples of *A. malayanum* and two samples of *H. conoideum* from Saijuntha et al. (2011a) were also examined. The TkD1Int5 was amplified by using two sets of primers for nested PCR. The first PCR was performed by EcapPkInt5F1 (5′-TTA TTA ACC AGG CTC AAA AC-3′) and EcapPkInt5R1 (5′-TAG TGG AAC GCG TGC GTG CAC-3′), whereas EcapPkInt5F2 (5′-TTC CTT GTC TGG GTG AAT GA-3′) and EcapPkInt5R2 (5′-CGT AGT GCC CAA ATT AGA TG-3′) were used for the second PCR. Each primer was designed from a full length *Echinostoma caproni* taurocyamine kinase gene deposited in GenBank under accession number (UZAN01069901–UZAN01069904) for annealing to the conserved flanking exon regions to amplify a whole fragment of TkD1Int5 region.

Polymerase chain reaction

The PCR mixture contained 1× *TaKaRa Ex* PCR buffer, 0.2 mM dNTPs (each), 0.2 μM of each primer, and 1.0 U of *TaKaRa Ex Taq* polymerase (Takara Bio Inc., Japan). The thermal cycling conditions were the same in all PCR amplifications, namely, initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min, and a final extension

Table 1 Detail of sample localities, duck hosts, heterozygosity, haplotype classification, and insertion/deletion patterns detected in a particular sample

Country Clone	Region	Province	District	Duck host	Specimen code	Genus species	Homo/ Het	Clone	Haplotype	Indel pattern
Thailand	Central	Ayutthaya	Bang Ban	AY-1A	AY-1A1	*E. miyagawai*	Het	id	H55	nd
					AY-1A2	*E. miyagawai*	Het	C4	H49	nd
								C2	H48	433_435delCCT
					AY-1A4	*E. miyagawai*	Het	C4	H2	82delT, 326_327delGT
								C2	H1	326_327delGT
				AY-2A	AY-2AI	*E. miyagawai*	Homo	nc	H46	nd
				AY-2C	AY-2C1	*E. miyagawai*	Homo	nc	H51	nd
				AY-5A	AY-5AI	*E. miyagawai*	Het	C2	H63	nd
								C3	H62	433_435delCCT
				AY-D3	AY-D32	*E. revolutum*	Het	C4	H73	nd
								C2	H79	**167_171delCGCTG**
					AY-D33	*E. miyagawai*	Het	id	H31	nd
				AY-D4	AY-D42	*E. miyagawai*	Homo	nc	H65	527delA
					AY-D43	*E. miyagawai*	Het	C4	H7	326_327delGT
								C3	H13	527delA
		Lop Buri	Ban Mi	LB-D1	LB-D11	*E. miyagawai*	Homo	nc	H46	nd
					LB-D12	*E. miyagawai*	Het	nc	H53	146delT
								C2	H56	527delA, 799delA
					LB-D13	*E. miyagawai*	Het	C3	H60	nd
								C4	H5	82delT, 326_327delGT, 431_433delCTC
				LB-D3	LB-D31	*E. miyagawai*	Het	C2	H66	82delT
								C1	H35	82_83insTTT
		Nakhon Sawan	Takhli	NSw-D1	NSw-D11	*E. miyagawai*	Het	id	H33	nd
					NSw-D12	*E. miyagawai*	Homo	nc	H56	nd
				NSw-D2	NSw-D21	*E. miyagawai*	Het	C2	H51	nd
								C1	H53	527delA
		Pathumthani	Khlong Laung	PT-D1	PT-D11	*E. miyagawai*	Homo	nc	H39	691_692insC, 728_729insT
					PT-D12	*E. miyagawai*	Het	C1	H18	nd
								C2	H57	82_83insT
					PT-D13	*E. miyagawai*	Het	C3	H29	nd
								C1	H9	527delA
					PT-D14	*E. miyagawai*	Het	id	H9	527delA
				PT-D2	PT-D21	*E. miyagawai*	Het	C2	H50	nd
								C1	H21	82delT, 146delT

Table 1 (continued)

Country Clone	Region	Province	District	Duck host	Specimen code	Genus species	Homo/ Het	Clone	Haplotype	Indel pattern
	North	Phichit	Bueng Na Rang	PJ-D1	PJ-D11	*E. miyagawai*	Het	id	H9	527delA
		Phitsanulok	Mueang	PL-D1	PL-D11	*E. miyagawai*	Homo	nc	H51	nd
				PL-D2	PL-D21	*E. miyagawai*	Homo	nc	H47	728_729insT
					PL-D22	*E. miyagawai*	Het	C2	H28	nd
								C1	H9	527delA
					PL-D23	*E. miyagawai*	Het	C1	H36	nd
								C2	H67	82_83insTT
					PL-D24	*E. miyagawai*	Het	C1	H30	nd
								C2	H58	527delA
		Sukhothai	Kong Krailat	STk-D1	STk-D11	*E. miyagawai*	Het	C2	H26	82delT
								C1	H71	82_83insT
				STk-D3	STk-D31	*E. miyagawai*	Het	id	H22	82_83insT
				STk-D5	STk-D51	*E. miyagawai*	Het	C1	H9	nd
	Northeast	Khon Kaen	Mueang	KK-1	KK-11	*E. miyagawai*	Het	C1	H40	82delT
								C3	H8	326_327delGT
				KK-4	KK-4A	*E. miyagawai*	Homo	nc	H38	559_560insC, 683delA, 689delT, 691_692insC, 728_729insA
					KK-4C	*E. miyagawai*	Homo	nc	H38	559_560insC, 683delA, 689delT, 691_692insC, 728_729insA
				KK-D5	KK-D51	*E. miyagawai*	Het	C2	H68	nd
								C1	H66	82_83insTT
				KK-DA	KK-DA2-08	*E. miyagawai*	Het	C3	H27	82delT
								C1	H10	527delA
				KK-DF	KK-DF1	*E. miyagawai*	Het	C3	H64	nd
								C2	H11	82_83insTTT
				KK-DI	KK-DI1	*E. revolutum*	Homo	nc	H73	nd
					KK-DI2	*E. miyagawai*	Het	id	H25	82delT
					KK-DI3	*E. revolutum*	Homo	nc	H77	nd
				KK-G	KK-G1	*E. miyagawai*	Het	C1	H32	nd
								C2	H9	527delA
					KK-G2	*E. miyagawai*	Het	C1	H3	82delT, 326_327delGT
								C3	H24	82delT
					KK-G3	*E. miyagawai*	Het	C4	H69	nd
								C1	H70	82_83insTT
					KK-G4	*E. miyagawai*	Homo	nc	H37	689delT, 691_692insC, 728_729insA, 732delA, 829delA

Table 1 (continued)

Country	Clone	Region	Province	District	Duck host	Specimen code	Genus species	Homo/ Het	Clone	Haplotype	Indel pattern
						KK-G5	*E. miyagawai*	Homo	nc	H37	689delT, 691_692insC, 728_729insA, 732delA, 829delA
						KK-G6	*E. miyagawai*	Het	id	H4	82delT, 326_327delGT
			Maha Sarakham	Mueang	MS-D1	MS-D11	*E. miyagawai*	Het	C1	H15	527delA
									C3	H59	527delA, 799delA
						MS-D12	*E. miyagawai*	Het	C1	H72	82_83insT
									C2	H9	82_83insTT
						MS-D15	*E. revolutum*	Het	C3	H75	nd
									C4	H73	542delT
			Nakhon Ratchasima	Chum Phuang	NMc-D3	NMc-D31	*E. revolutum*	Het	C2	H74	nd
									C4	H78	264delT
					NMc-D4	NMc-D41	*E. miyagawai*	Het	C2	H16	nd
									C1	H12	527delA
			Roi Et	Changhan	RE-D1	RE-D12	*E. miyagawai*	Het	C3	H23	nd
									C2	H20	146delT
						RE-D13	*E. miyagawai*	Homo	nc	H41	829delA
					RE-D2	RE-D22	*E. miyagawai*	Het	C1	H19	nd
									C3	H17	326_327delGT
					RE-D1B	RE-D1B1	*E. miyagawai*	Homo	nc	H52	728_729insT, 829delA
						RE-D1B2	*E. miyagawai*	Homo	nc	H60	728_729insT
						RE-D1B5	*E. miyagawai*	Het	id	H9	527delA
					RE-D2A	RE-D2A6	*E. miyagawai*	Het	C2	H6	326_327delGT
									C3	H14	527delA
						RE-D2A7	*E. miyagawai*	Homo	nc	H42	829delA
Lao PDR		Central	Vientiane	Kampang Nakhon	VT-D1	VT-D13	*E. miyagawai*	Homo	nc	H45	nd
					VT-D2	VT-D21	*E. miyagawai*	Homo	nc	H43	nd
					VT-D5	VT-D51	*E. miyagawai*	Het	id	H34	82delT
						VT-D52	*E. miyagawai*	Het	id	H61	nd

C1 to C4 = clone number of a particular heterozygous sample

Indel insertion/deletion, *Het* heterozygous, *Homo* homozygous, *id* all clones were identical sequence, *nc* not clone, *nd* no indel was detected, indel patterns were described follow den Dumnen et al. (2001)

at 72 °C for 5 min. One microliter of the first PCR product was used as the DNA template for the second PCR. The second PCR products underwent electrophoresis in 1% agarose gel and visualized with GelRed™ Nucleic Acid Gel Stain (Biotium, Inc., Hayward, CA). The second PCR product (~ 1000 bp) was cut for gel purification using the E.Z.N.A.® Gel Extraction kit (Omega bio-tek, USA) and subsequently used for DNA sequencing and cloning.

DNA sequencing and cloning

The purified PCR products were cycle-sequenced using EcapPkInt5F2 primer as the sequencing primer then sequenced via a commercial service provider (Eurofins, Japan). When DNA sequence chromatograms showed heterozygous patterns and contained insertion/deletion (indel) (Fig. 1), the PCR product was cloned into a pGEM-T easy vector (Promega, Madison, WI), following the manufacturer's instructions. Subsequently, the recombinant plasmid was introduced and propagated in *Escherichia coli* JM109, and four white colonies were randomly picked and cultured in Luria-Bertani (LB) broth at 37 °C with continuous horizontal shaking for 12 h. Then, plasmid DNA was extracted using the FastGene® Plasmid Mini kit (Nippon Genetics Co., Ltd., Japan) following manufacture's protocol; subsequently, plasmid DNA was cycle-sequenced via a commercial service provider (Eurofins, Japan) using M13F and M13R as sequencing primers.

Data analysis

The sequences were checked and manually edited using the BioEdit program version 7.2.6 (Hall 1999). The sequences of each species were multiply aligned using the ClustalW program (Larkin et al. 2007) to find the variable sites, including indel regions. Haplotype data was calculated using the DnaSp v5 program (Librado and Rozas 2009) by excluding the indel positions. Molecular indices and a neutrality test were performed in Arlequin v.3.5.2.2 (Excoffier and Lischer 2010). A minimum spanning haplotype network was generated using the Network 5.0.1.1 program based on a median-joining network (Bandelt et al. 1999).

Results

Sequence analysis

We successfully amplified the TkD1Int5 region of *E. miyagawai*, *E. revolutum*, and *A. malayanum*, except *H. conoideum*. The TkD1Int5 sequences of *E. miyagawai*, *E. revolutum*, and *A. malayanum* examined in this study were deposited in GenBank under the accession numbers MN994887–MN994973, MN994879–MN994886, and MT175435–MT175442, respectively. The eight samples of *A. malayanum* were all homozygous and 100% identical (data not show). The TkD1Int5 sequence analysis of 58 *E. miyagawai* and five *E. revolutum* samples found that 17 *E. miyagawai* and two *E. revolutum* samples were

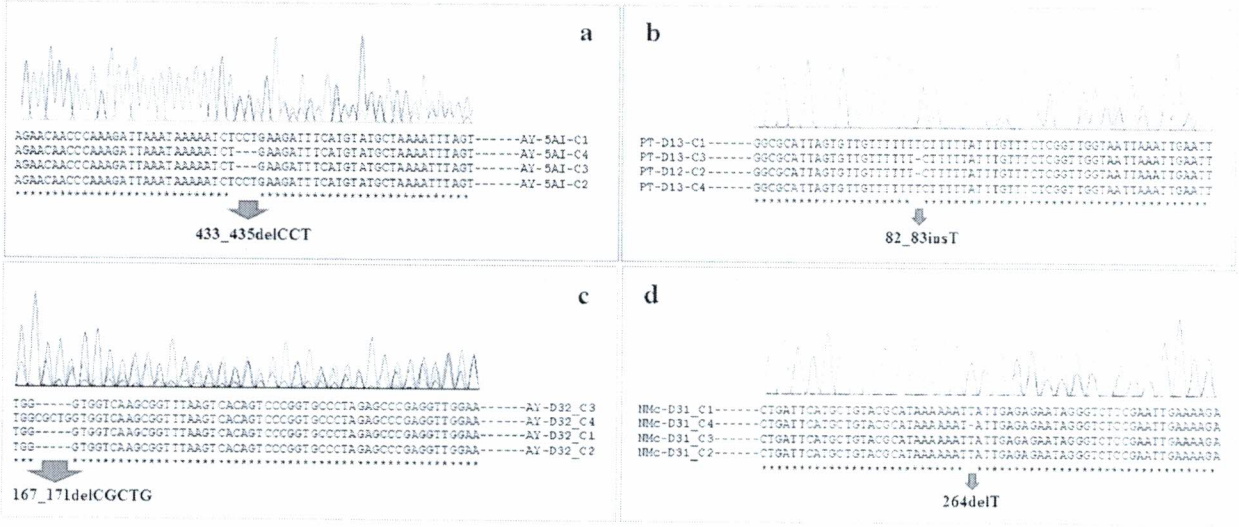

Fig. 1 An example of a DNA sequencing chromatogram showing heterozygosity of *Echinostoma miyagawai*, namely AY-5AI (**a**) and PT-D13 (**b**), including *E. revolutum*, namely AY-D32 (**c**) and NMc-D31 (**d**). Four inserted clones (C1–C4) of each heterozygous sample were picked for plasmid DNA sequencing. An example of insertion/deletion (indel) patterns, such as 433_435delCCT (**a**) and 82_83insT (**b**) were detected in *E. miyagawai*, whereas 167_171delCGCTG (**c**) and 264delT (**d**) were detected in *E. revolutum*

homozygous, whereas the rest were heterozygous. All heterozygous samples were cloned and plasmid DNA sequenced to explore the indel pattern combined in a particular sample. Of these nine samples of *E. miyagawai*, the heterozygous pattern could not be deciphered as all randomly picked clones had identical sequences (Table 1). The 16 and three indel patterns were found specifically in *E. miyagawai* and *E. revolutum*, respectively. The most common indel pattern which was detected in *E. miyagawai* was 527delA which was found in 17 samples, whereas the three indel patterns, namely 167_171delCGCTG, 542delT, and 264delT were found only in *E. revolutum* (Table 1).

Genetic diversity and haplotype analyses

High levels of polymorphism were observed in the TkD1Int5 sequences of *E. miyagawai* and *E. revolutum* where averages of haplotype diversity and nucleotide diversity were found at 0.9922 ± 0.0046 and 0.0105 ± 0.0054 for *E. miyagawai* and 0.9987 ± 0.0625 and 0.0087 ± 0.0051 for *E. revolutum*, respectively. The Tajima's D, Fu, and Li values were all negative, which signifies an excess of low-frequency polymorphisms relative to expectation, indicating population size expansion and/or purifying selection. Seventy-two and seven haplotypes of *E. miyagawai* and *E. revolutum*, respectively, were generated. Haplotype analysis revealed that almost all of the haplotypes were singleton and unique for a particular sample (Table 2; Fig. 2). Six haplotypes were shared between samples/isolates of *E. miyagawai*, whereas one haplotype was shared between *E. revolutum*, respectively (Fig. 2). The haplotype network showed that *E. revolutum* and *E. miyagawai* were clearly separated into two haplogroups with differences at 106 mutational steps (ms). The genetic differences within species were low which was reflected by mutational steps less than 10 ms (Fig. 2). Genetic structuring related to geographical localities was not observed.

Discussion

We successfully characterized and used the TkD1Int5 sequence as a genetic marker to elucidate the genetic variation and heterozygosity of the two morphospecies echinostomes, *E. miyagawai* and *E. revolutum*. *Artyfechinostomum malayanum* showed no variation in TkD1Int5 sequence, which may be due to low sample size from the one locality examined and/or low polymorphism of this intron region existing in this species. The primer designed in this study was unable to amplify TkD1Int5 region of *H. conoideum*, which revealed that the flanking region of primer annealing was not similar to that in the genera, *Echinostoma* and *Artyfechinostomum*.

Our results provide supporting evidence that *E. miyagawai* is a valid species by use of the TkD1Int5 sequence, demonstrating that there is clear differentiation of *E. miyagawai* from its synonymized species *E. revolutum*. Previous investigations of mitochondrial genome of *E. miyagawai* have shown that it is clearly different from the other closely related species (Fu et al. 2019; Li et al. 2019). Employing the TkD1Int5 sequence toward the genetic differentiation of *E. miyagawai* from its other synonymized species, namely *Echinostoma robustum/friedi*, needs further comprehensive investigation.

It is important to highlight that further species identification by morphology alone may not be sufficiently robust for definitive answers, and we suggest that morphological investigations be done in conjunction with nucleotide differences between these sibling species, especially in hypervariable genes, such as ITS1 or ITS2 (Heneberg 2020), as well as in the TkD1Int5 sequence currently examined. Thus, this intron can now be applied to differentiate the other members of the 37 collar-spined echinostomes, where their taxonomic and systematic status are still controversial (Kostadinova and Gibson 2000).

The high polymorphism and heterozygosity of TkD1Int5 detected in our study could potentially also be used as a genetic marker to elucidate the genetic variation, population genetics, as well as hybridization of these echinostomes in future studies. The intron of TK has previously been successfully characterized and used to detect genetic variation, genetic differentiation, and heterozygosity of medically important parasitic trematodes *Paragonimus* spp. and *Fasciola* spp. (Jarilla et al. 2013; Saijuntha et al. 2016, 2018). Moreover, it has also been successfully used to detect DNA recombination of *F. hepatica* and *F. gigantica* in the hybrid fasciolid, *Fasciola* species (Saijuntha et al. 2018), as well as between diploid (2n) and triploid (3n) *P. westermani* in Japan (Saijuntha et al. 2016).

Even though DNA recombination between *E. miyagawai* and *E. revolutum* was not observed in our study, there is evidence that these echinostomes have been utilizing sympatric host species, e.g., domestic ducks (Nagataki et al. 2015). Additionally, a duck KK-DI was found in this study to be coinfected by *E. miyagawai* and *E. revolutum*. Therefore, hybridization between these echinostomes may have occurred in the past, maybe ongoing, and can occur in the future. This is feasible as the possible hybridization of *Echinostoma lieli* with other African species examined by isoenzyme analysis has been reported (Voltz et al. 1988). Further comprehensive investigations of the echinostomes by using this intron sequence to reveal heterozygosity and hybridization should shed light on their systematics and population structure. High levels of polymorphism in intron sequences have been previously detected in a number of parasite species, for instance, in *Opisthorchis viverrini*, *Paragonimus* spp., and *Fasciola* spp. (Pitaksakulrat et al. 2018; Saijuntha et al. 2016, 2018). Our

Table 2 Molecular diversity indices and neutrality tests

	E. miyagawai	*E. revolutum*	*A. malayanum*
Number of samples examined	58	5	8
Nucleotide sequence length (bp)	867–876	863–868	843
Number of polymorphic sites	94	20	0
Number of transitions	76	12	0
Number of transversions	21	8	0
Number of indels	21	7	0
Number of indel patterns	16	3	0
Number of haplotypes	74	7	1
Number of unique haplotypes	68	6	0
Haplotype (gene) diversity ± SD	0.9922 ± 0.0046	0.9987 ± 0.0625	0.0000 ± 0.0000
Nucleotide diversity ± SD	0.0105 ± 0.0054	0.0087 ± 0.0051	0.0000 ± 0.0000
Tajima's D	− 2.17330**	− 1.27706	N/A
Fu and Li's D	− 5.07275*	− 1.29356	N/A
Fu and Li's F	− 4.65090*	− 1.43464	N/A

*$P < 0.05$, **$P < 0.01$, N/A = not analyzed

study has shown that TkD1Int5 has extremely high polymorphism, which can now be used as an alternative high polymorphic genetic marker to examine genetic variations in the 37 collar-spined echinostomes, as well as in other medically important echinostomes, globally.

A previous study using mitochondrial DNA sequences (Nagataki et al. 2015) demonstrated that there was no population sub-structuring of *E. miyagawai* and *E. revolutum* related to different geographical localities in Thailand, which our study confirms using TkD1Int5; however, genetic clusters related to the continental scale were observed by the mitochondrial DNA sequence analyses (Nagataki et al. 2015; Anucherngchai et al. 2019). Unfortunately, we cannot compare intron sequences with those other geographical localities because the specific isolates have not been examined in our study.

Importantly, the results from our research have shown that the TkD1Int5 region can be used as a genetic marker to provide comprehensive analyses of genetic variation, genetic differentiation, population genetics, genetic structure, heterozogosity, and potential hybridization of the 37 collar-spined echinostomes that currently are distributed worldwide.

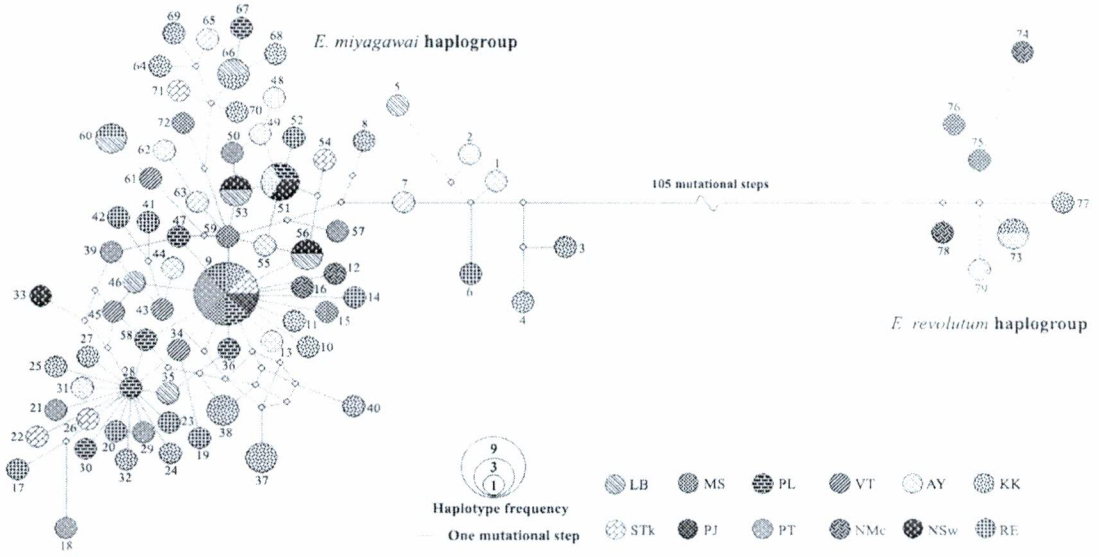

Fig. 2 Haplotype network constructed by TkD1Int5 sequences excluding indel sites. Haplotype network classified into *Echinostoma miyagawai* and *E. revolutum* haplogroups. Each circle represents a haplotype identified with a different number. The area of the circle represents the number of samples found in each haplotype. The length of each branch demonstrates the number of mutational steps (ms). Different printed patterns in the haplotype networks correspond to their geographical localities separated into 12 different localities (more details in Table 1)

Moreover, it can also be further used as a genetic marker for molecular differential diagnosis of these morphospecies of echinostomes, as previously mitochondrial genes have been used effectively as genetic markers to develop multiplex PCR to differentiate medically important echinostomes in Thailand (Tantrawatpan and Saijuntha 2020).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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