Journal of Biotechnology **18**(4): 653-662, 2020

MOLECULAR EVOLUTIONARY RELATIONSHIPS OF VIETNAMESE AND GLOBAL PULMONARY *PARAGONIMUS* SPECIES IN THE FAMILY PARAGONIMIDAE AND SUBORDER XIPHIDIATA (PLATYHELMINTHES: TREMATODA)

Nguyen Thi Khue[1,2], Pham Thi Khanh Linh[1], Do Thi Roan[1], Doan Thi Thanh Huong[1,2], Pham Ngoc Doanh[3], Le Thanh Hoa[1,2,✉]

[1]*Institute of Biotechnology, Vietnam Academy of Science and Technology*
[2]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology*
[3]*Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology*

✉To whom correspondence should be addressed. E-mail: imibtvn@gmail.com

Received: 09.8.2020
Accepted: 30.9.2020

SUMMARY

Paragonimiasis, caused by *Paragonimus* species belonging to the family Paragonimidae of the suborder Xiphidiata (Platyhelminthes: Trematoda), often occurs in poor, upland, ethnic minorities, in Vietnam and the world. Asian *Paragonimus* species are distributed from Japan, South Korea, along with North and Southeast China, North-West and Central Vietnam, the Philippines, Thailand, Bangladesh, India, and Sri Lanka. There are various genetic variants, strains, and genotypes forming different complexes and evolutionary lineages. The 18S, 28S rDNA sequences and the intergenic transcribed spacer regions (ITS-1, ITS-2) of nuclear ribosomal transcription units are commonly used as molecular markers in genetic studies and phylogenetic analyses. We obtained a portion of 28S rDNA (domains D1–D3) of *Paragonimus* spp. including *P. heterotremus* (from Vietnam), *P. ohirai* (Japan), *P. iloktsuenensis* (Japan), and *P. westermani* (India and Vietnam) and conducted phylogenetic analysis for molecular evolutionary studies. The results showed that the family Paragonimidae formed the biggest cluster in a phylogenetic tree, which comprises of 46 sequences of 11 species belonging to 11 subgroups, among which the *P. westermani* complex of strains originating from China, Korea, Japan, India, Philippines, Malaysia, and Vietnam is present. *P. westermani* complex is arranged in a position of "*sister*" (sister group) with the subgroup *P. siamensis*. The *P. heterotremus* and *P. ohirai* complexes, and the *P. miyazakii*, *P. harinasutai*, *P. mexicanus*, *P. kellicotti*, and *P. macrorchis* complexes are clustered in a common population. *P. westermani* of Vietnam is in close proximity to the East Asian strains, as of which has been previously reported. *P. ohirai* and *P. iloktsuenensis* are considered "*sibling*" species, sharing the same clade. Phylogenetic analysis using the 28S rDNA sequences directly presented species position and their molecular evolutionary relationships in the families Paragonimidae, Troglotrematidae, Nanophyetidae, and Collyriclidae. Evolutionary analysis has also clarified a number of complex delineation problems and made a clear nomenclature for *Paragonimus* sp. of Vietnam, in particular, which has scientific grounds merited to recognize as that it is really the *P. westermani* species.

Keywords: 28S rDNA/rRNA, ribosomal transcription unit, *Paragonimus* spp., *Paragonimus westermani*, phylogenetic, complex, evolution, Vietnam

INTRODUCTION

The genus *Paragonimus* of the Paragonimidae family, suborder Xiphidata (Platyhelminthes: Trematoda), contains, epidemiologically, numerous species of veterinary and medical importance due to the

paragonimiasis disease, which often occurs in poor, upland, ethnic minorities in the remote mountainous areas, in Vietnam and the world. Infection rate is often very high in primary and secondary school children, as they have a habit of eating uncooked/undercooked infected crabs (Le *et al.*, 2006; Blair *et al.*, 2014; Yoshida *et al.*, 2019). Paragonimiasis is one of the most common neglected, re-emerging diseases in many countries in Asia, Africa, and America. About 23 million people are infected and 292 millions are at risk of infection (Blair, 2014; Blair *et al.*, 2016). Over 50 species are recognized as valid species, of which, about 10 species cause serious diseases in humans and are transmitted from animals, including *P. westermani*, *P. heterotremus*, *P. ohirai*, *P. miyazakii*, *P. skrjabini*, *P. iloktsuenensis*, *P. kellicoti,* and *P. mexicanus*. They are divided into the complexes of *P. westermani*, *P. heterotremus*, *P. ohirai*, *P. skrjabini* etc…, of which each complex has many different genotypes and strains (Blair, 2008; 2014; Blair *et al.*, 2016). Paragonimiasis is also divided into groups according to geographical distribution: Asian group, African group, and American group (Procop, 2009; Blair, 2014; Blair *et al.*, 2016; Cumberlidge *et al.*, 2018; Le *et al.*, 2019). Asian *Paragonimus* species have many different and complicated strains, distributed from Japan, Korea, along with North and Southeast China, North-west and Central Vietnam, the Philippines, Thailand, Bangladesh, India and Sri Lanka (Le *et al.*, 2006; Blair, 2014; Sanpool *et al.*, 2013; 2015; Blair *et al.*, 2016; Doanh *et al.*, 2013; Yoshida *et al.*, 2019).

The ribosomal transcription unit (rTU) or simply rDNA positioned in the nuclear genome, they are of 7–10 kb for each in parasites, which contains three ribosomal coding genes (18S, 5.8S, and 28S rRNA genes) separated by two intergenic regions (internal transcribed spacer 1 and 2, that ITS-1 is between 18S and 5.8S; and ITS- 2 between 5.8S and 28S rDNA, respectively). The rTUs are up to hundreds of units connected in arrays by nucleotide sequences containing many repetitive structures, called IGS (non-transcribed intergenic spacer). Each unit forms a characteristic structural frame: IGS-18S-ITS1-5.8S-ITS2-28S-IGS, and arranged in series. In the human genome, rTU is located in the secondary constriction, or NOR (nucleolar organizing region), in chromosomes 13, 14, 15, 21, and 22 (McStay, 2016). The ribosomal coding genes including 18S, 28S rRNA genes or/and the intergenic regions (ITS-1, ITS-2) are commonly used in the taxonomic analyses, taxonomic relationships, and species originality studies (Weider *et al.*, 2005; Blair, 2006; Blair, 2014; 2016). The rTU molecular markers are also used in species identification and paternality identification for independent species, either for "exotic species" or "hybrid or introgressive hybridization" (Blair, 2014; Blair *et al.*, 2016).

Up to now, there is not enough data of the entire ribosomal transcription unit (rDNA/ rTU) for species in the family Paragonimidae and suborder Xiphidiata, especially for *Paragonimus* and some recently reported species in Vietnam (Doanh *et al.*, 2009). For other members in Troglotremata/Xiphidiata as well, there are limited complete rDNA data, although several distinct ribosomal genes and regions (18S, 28S or ITS regions) have been captured and used in diagnostic and epidemiological studies (Doanh *et al.*, 2013). All of these sequences (18S, 28S, ITS-1, ITS-2) were used as molecular markers in the analysis of phylogeny, classification, and molecular evolutionary relationships between species (Weider *et al.*, 2005; Blair, 2006; Pérez-Ponce de León, Hernández-Mena, 2019).

In this paper, we present the acquisition of partial 28S rDNA (domains D1–D3) of *Paragonimus* spp. including: *P. heterotremus* (Vietnam), *P. ohirai* (Japan), *P. iloktsuenensis* (Japan) and *P. westermani* (India and Vietnam) and some other species, including *P. westermani* (sample discovered in Vietnam); and used to analyze phylogenetic relationships between species to assess their molecular evolution of pulmonary flukes in the family Paragonimidae and suborder Xiphidiata.

MATERIALS AND METHODS

Parasite samples of species in the family Paragonimidae

The samples in this study were the adult pulmonary flukes and metacercariae which were identified by morphology and verified by molecular analysis. Samples were freshly frozen forms, either 70% ethanol preserved or genomic DNA, store at –20°C. Nine strains of 5 species of *Paragonimus* spp. in this study (Table 1), they are: 1) *Paragonimus heterotremus* (Vietnam) including 3 strains: strain LC, designated as Phete-LC-VN; strain D2YB, designated as Phete-D2YB-VN; strain D3YB, symbol Phete-D3YB-VN; 2) *Paragonimus westermani* including 3 strains: strain QT2 (Vietnam), designated as Pwest-QT2-VN; strain Meghalaya(2n) (India), designated as Pwest-Meghalaya(2n)-IN; strain Bogil(3n) (Korea), designated as Pwest-Bogil(3n)-KR; 3) *Paragonimus ohirai* (Japan) including 2 strains: strain Nagoya, designated as Pohir-Nagoya-JP; and strain Kochi, designated as Pohir-Kochi-JP; 4) *Paragonimus miyazakii* (Japan), strain OkuST1, designated as Pmiya-OkuST1-JP; 5) *Paragonimus iloktsuenensis* (Japan), strain Amami, designated as Pilok-Amami-JP.

Table 1. List and information of 56 strains/species providing the 28S rDNA sequences (D1–D3) used to construct a phylogenetic tree for analyzing the species relationship, determining the taxonomic position and molecular evolution of species in the suborder Xiphidiata (Trematoda: Platyhelminthes).

No	Family/Species	Abbreviati-on	Sequence designation	Country of isolation	Genbank accession No
	Collyriclidae(1)/1				
1	*Collyriclum faba*	Cfaba	Cfaba-Orlicke-CZ	Czech	JQ231122
	Paragonimidae (11)/46				
2	*Paragonimus harinasutai*	Phari	Phari-Nakorn-TH	Thailand	HM172616
3	*Paragonimus heterotremus*	Phete	Phete-(egg)-IN	India	DQ836249
4		Phete	Phete-(egg5)-IN	India	HM172615
5		Phete	Phete-D2YB-VN	Vietnam	This study
6		Phete	Phete-D3YB-VN	Vietnam	This study
7		Phete	Phete-sp2uz2017-VN	Vietnam	MK828944
8		Phete	Phete-L2017-VN	Vietnam	MK817556
9		Phete	Phete-LC-VN	Vietnam	This study
10		Phete	Phete-Manipur-IN-	India	KF781294
11		Phete	Phete-PheteroChi-CN	China	HM172617
12	*Paragonimus iloktsuenensis*	Pilok	Pilok-CN	Japan	AY116875
13		Pilok	Pilok-Amami-JP	Japan	This study
14	*Paragonimus kellicotti*	Pkell	Pkell-Missouri-US	United States	HQ900670
15	*Paragonimus macrorchis*	Pmacr	Pmacr-Chanta-TH	Thailand	HM172618
16	*Paragonimus mexicanus*	Pmexi	Pmexi-Concordia-EC	Ecuador	HM172619
17	*Paragonimus miyazakii*	Pmiya	Pmiya-Kochi-JP	Japan	HM172620
18		Pmiya	Pmiya-OkuST1-JP	Japan	This study
19	*Paragonimus ohirai*	Pohir	Pohir-Kinosaki-JP	Japan	HM172621
20		Pohir	Pohir-Nagoya-JP	Japan	This study
21		Pohir	Pohir-Kochi-JP	Japan	This study

22	*Paragonimus pseudoheterotremus*	Ppseuhet	Ppseuhet-(Llarn)-TH	Thailand	HM004189
23	*Paragonimus siamensis*	Psiam	Psiam-m1Assam-IN	India	JQ322628
24		Psiam	Psiam-m2Assam-IN	India	JQ322629
25		Psiam	Psiam-m3Assam-IN	India	JQ322630
26		Psiam	Psiam-PspSLan12-LK	Sri Lanka	HM172624
27	*Paragonimus westermani*	Pwest	Pwest-JP	Japan	AY116874
28		Pwest	Pwest-Manipur-IN	India	KF781290
29		Pwest	Pwest-Meghalaya(2n)-IN	India	This study
30		Pwest	Pwest-Meghalaya(2n-IN	India	DQ836244
31		Pwest	Pwest-mp18Pradesh-IN	India	JN656181
32		Pwest	Pwest-mp23Pradesh-IN	India	JN656180
33		Pwest	Pwest-mp27Pradesh-IN	India	JN656179
34		Pwest	Pwest-mt1Assam-IN	India	JN656176
35		Pwest	Pwest-mt3Assam-IN	India	JN656175
36		Pwest	Pwest-mt4Assam-IN	India	JN656174
37		Pwest	Pwest-mt10Assam-IN	India	JN656177
38		Pwest	Pwest-mtn1Assam-IN	India	JN656178
39		Pwest	Pwest-mtn4Assam-IN	India	JN656173
40		Pwest	Pwest-Pradesh-IN	India	DQ836247
41		Pwest	Pwest-PwJpnMie3-JP	Japan	HM172626
42		Pwest	Pwest-PwKor1(3n)-SR	South Korea	HM172627
43		Pwest	Pwest-PwLiguhe-CN	China	HM172628
44		Pwest	Pwest-PwSorso4-PH	Philippines	HM172629
45		Pwest	Pwest-PwUruLan1-MY	Malaysia	HM172630
46		Pwest	Pwest-PwXigu4n-CN	China	HM172631
47		Pwest	Pwest-QT2-VN	Vietnam	This study
	Troglotrematidae (8)/8				
48	*Nanophyetus japonensis*	Njapo	Njapo-NJ142-JP	Japan	LT796170
49		Njapo	Njapo-NJ161-JP	Japan	LT796169
50	*Nanophyetus salminicola*	Nsalm	Nsalm-Oregon-US	United States	AY116873
51		Nsalm	Nsalm-OK42-US	United States	MG806919
52		Nsalm	Nsalm-Karp55-RU	United States	MG806919
53	*Nanophyetus schikhobalowi*	Nschi	Nschi-03Karp1442-RU	Russia	LN871820
54	*Nephrotrema truncatum*	Ntrun	Ntrun-(adult)	N/A	AF151936
55	*Skrjabinophyetus neomidis*	Sneom	Sneom-UA	Ukraine	AF184252
	Schistosomatidae (1)/1				
56	*Schistosoma haematobium*	Shaem	Shaem-N10-ML*	Mali	AY157263

Note: Species/strain: Those in parentheses () are the numbers of species in a family; after the slash (/) is the number of strains providing nucleotide sequences for phylogenetic analysis in this study. *Outgroup sequence (from *Schistosoma haematobium*). N/A: not available.

Total genomic DNA extraction

Total genomic DNA was extracted from a ~10mg section of an adult worm using the GeneJET™ Genomic DNA Purification Kit (Thermo Scientific Inc., MA, USA) as instructed, eluted in 100 µL, and stored at −20°C until use. The concentration of DNA was estimated using a GBC UV/visible 911A spectrophotometer (GBC Scientific Equipment Pty. Ltd., Australia). A working concentration of 50 ng/µL was prepared and 2 µL of this used as a template for PCR in a 50 µL reaction volume.

Primers used for acquisition of rDNA unit

Each rDNA unit (5' 18S-ITS1-5.8S-ITS2-28S-IGS 3') has a length of about 7–8 to 9–10 kb, depending on each trematode species. There are some samples for which only the entire coding region, not the IGS region were obtained. The majority of the rTU universal primers used were previously reported and some were added for use in this study (Le *et al.*, 2017; 2020; Le Thanh Hoa *et al.*, 2019). PCR is applied with the combination of alternative primers to obtain different long and short DNA fragments.

PCR amplification and sequencing

PCR reactions of 50 µL were prepared using 25 µL of DreamTaq PCR Master Mix (2x) (Thermo Fisher Scientific Inc., MA, USA) and 2 µL of DNA template (50 ng/µL), 2 µL of each primer (10 pmol/µL), 2 µL DMSO (dimethyl sulphoxide) and 17 µL of water, performed in an MJ PTC-100 Thermal Cycler. Initiation was at 94°C for 5 min, followed by 35 cycles consisting of denaturation for 30 sec at 94°C, annealing at 52°C for 30 sec, extension at 72°C for 6 min and a final extension at 72°C for 10 min. The PCR products (10 µL of each) were examined on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light (Wealtec, Sparks, NV, USA). The amplicons were purified by GeneJET PCR Purification Kit; or the right band of the expected size was eluted by GeneJET Gel Extraction Kit (Thermo Fisher Scientific Inc.) if multiple bands present. The PCR products were sent to a service company for direct sequencing or primer-walking in both directions until the complete sequence for the whole fragment was obtained.

Sequence and data processing, identifying rDNA characteristics of species

The entire rDNA sequence for each species/isolate was obtained after editing chromatograms (Chromas 2.6.6; http://technelysium.com.au/wp/chromas/) and 18S, 5.8S, and 28S rRNA genes were determined by using the previously published reference sequences and those available in GenBank. The majority of the PCR products were directly sequenced; the overlapped sequences were compared and connected to obtain the entire rDNA coding sequence. For some species, only a fragment of 28S rDNA region (D1–D3 domains) of about 1,200–1,250 bp was obtained and used for comparative analysis and constructing phylogenetic tree.

Phylogenetic analyses

Fifty-six 28S rDNA sequences of ~1100 bp, comprising of 9 sequences from 5 *Paragonimus* species in this study including *P. westermani* of Vietnam (strain QT2, designated as Pwest-QT2-VN) and 46 sequences from species in the families of Collyriclidae, Paragonimidae, Nanophyetidae (Troglotrematidae) of the suborder Xiphidiata, and a sequence of *S. haematobium* as an outgroup, were aligned and used to perform phylogenetic analyses (Table 1).

The final 28S rDNA alignment was composed of 56 species/isolates and MEGA X was used to perform Neighbor-Joining (NJ), Maximum Composite Likelihood replacement model phylogenetic reconstruction with 1000 bootstrap resamplings. MEGA X identified the general time-reversible GTR + G + I model (γ rate heterogeneity and a proportion of invariant sites) as the most appropriate model for phylogenetic reconstruction based on the lowest Bayesian information criterion score (Kumar *et al.*, 2018).

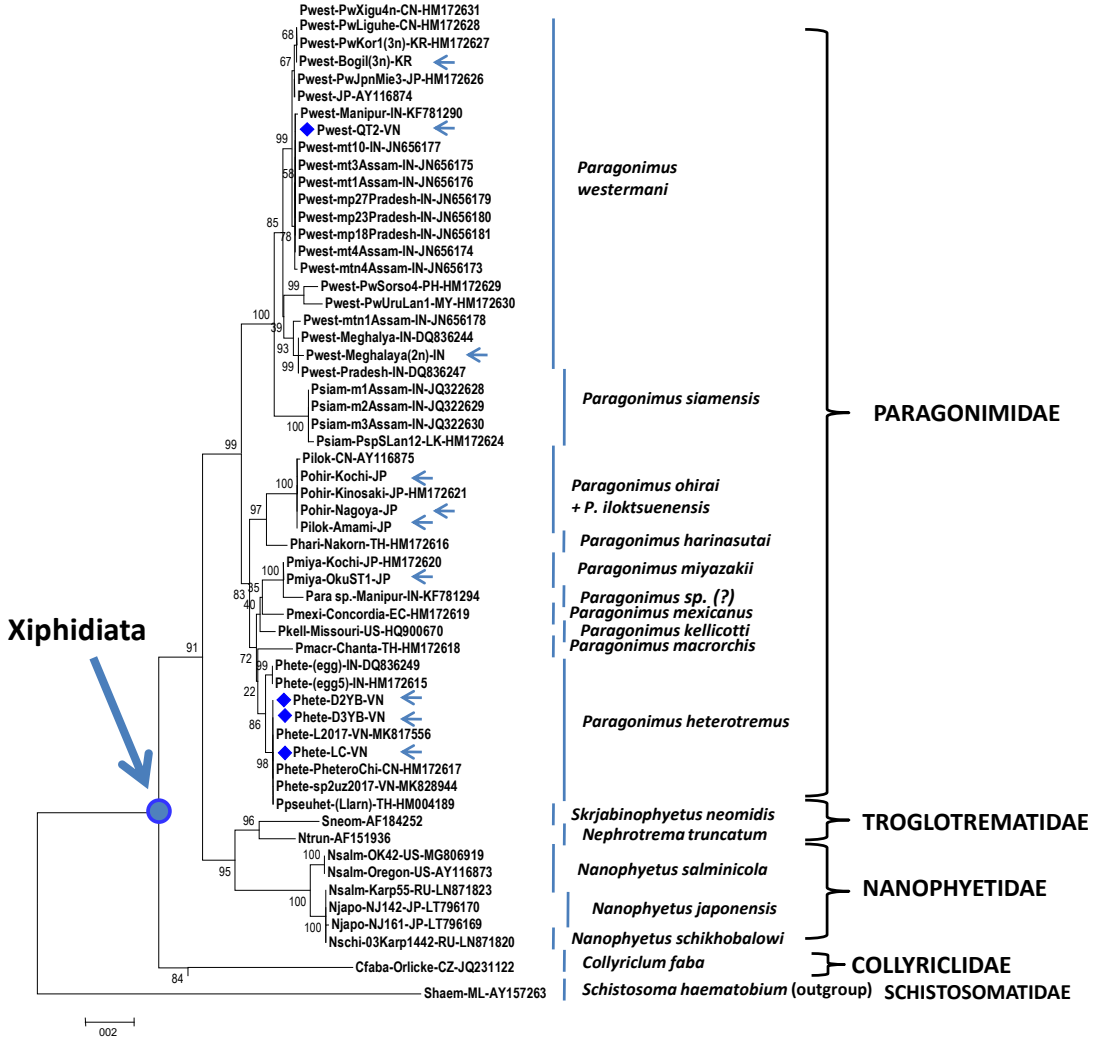

Figure 1. Phylogenetic tree determining species and taxonomic relationships of species in the family Paragonimidae based on nucleotide sequence analysis of 28S rDNA (~1,100 bp of D1–D3 region). Nine strains of 5 species in this study (indicated by arrows) are subjects of this study: *P. heterotremus* species including Phete-LC-VN; Phete-D2YB-VN and Phete-D3YB-VN strains; *P. westermani* species including Pwest-QT2-VN and Pwest-Meghalaya(2n)-IN; *P. ohirai* species including Pohir-Nagoya-JP and Pohir-Kochi-JP; *P. miyazakii* species including Pmiya-OkuST1-JP; and *P. iloktsuenensis* including Pilok-Amami-JP. Recently discovered *P. westermani* of Vietnam (Pwest-QT2-VN) and the Vietnamese *P. heterotremus* isolates are marked with a diamond-shaped icon. The 28S sequences were analyzed by MEGA X program, using the method of Neighbor-Joining (NJ) with a bootstrap of 1000 replicates (Kumar *et al.*, 2018). The bootstrap was recorded at each branch group. Note: species names and Genbank accession numbers are fully recorded (see Table 1). In each sequence on a branch, there are an abbreviation of the species name, followed by strain designation (if any) and the country of isolation, and the Genbank accession numbers given at the end. The outgroup is a 28S rDNA sequence of *Schistosoma haematobium* (Schistosomatidae) to be used.

RESULTS AND DISCUSSION

Taxonomic position and species relationship of *Paragonimus* species of Vietnam and the world, including strains of 5 species: *P. heterotremus* (Phete-LC-VN; Phete-D2YB-VN; and Phete-D3YB-VN); *P. westermani* (Pwest-QT2-VN and Pwest-Meghalaya(2n)-IN); *P.*

ohirai (Pohir-Nagoya-JP and Pohir-Kochi-JP); *P. miyazakii* (Pmiya-OkuST1-JP); and *P. iloktsuenensis* (Pilok-Amami-JP) have been identified in the phylogenetic tree well-positioned in the family Paragonimidae and suborder Xiphidiata (Fig. 1).

The tree in Fig. 1, besides the outgroup, clearly distinguished are 4 included families, **Paragonimidae**, **Troglotrematidae, Nanophyetidae,** and **Collyriclidae**, with clustered species for each family.

1. The largest one is the group of species of the family **Paragonimidae**, consisting of 46 sequences of 11 species in 11 subgroups, one of which is the *P. westermani* complex which includes strains originating from different countries, ie., China, Korea, Japan, India, Philippines, Malaysia, and Vietnam. The *P. westermani* complex arranged in the position "*sister*" group with the subgroup *P. siamensis*, including strains from India and Sri Lanka. The *P. heterotremus* complex and the *P. ohirai* complex and the *P. miyazakii*, *P. harinasutai*, *P. mexicanus*, *P. kellicotti*, and *P. macrorchis* complexes were arranged together. The *P. westermani* species (strain QT2) of Vietnam is close to the East Asian strains of *P. westermani*, as previously noted when analyzed by other investigators (Doanh *et al.*, 2009). *P. ohirai* and *P. iloktsuenensis* are considered "*sibling*" species (Blair *et al.*, 2016), they share the same branch in taxonomic position in the phylogenetic tree.

2. The second group of species belongs to the family **Troglotrematidae**, including 2 species of *Skrjabinophyetus neomidis* and *Nephrotrema truncatum*. Troglotrematidae is a family of controversial grouping and their nomenclature is not yet to be elucidated (Blair, 2008).

3. The third group, recently named **Nanophyetidae,** seems to have all species of the genus *Nanophyetus* including strains *N. japonensis*, *N. salminicola* and *N. schikhobalowi*.

4. The fourth group is the **Collyriclidae** family, has only one species available for comparison , *Collyriclum faba* with 28S rDNA sequence in GenBank (JQ231122, Czech). Some of the sequences of *Collyriclum* spp. registered in Genbank are 18S rDNA sequences, not 28S rDNA.

The reference group selected for the tree as outgroup is *Schistosoma haematobium* (family: Schistosomatidae), completely separate, which supports the more precise classification of the Paragonimidae family on the phylogenetic tree of the suborder Xiphidiata (Fig. 1).

SOME DISCUSSION

Paragonimus spp. draw out attention with high diversity in morphology and genetics, forming relatively controversial complexes, so far to some extent, the exact taxonomic criteria have not been determined (Blair *et al*., 2016). In the family tree in Fig. 1, the family Paragonimidae is a collection of many separate groups formed from strains/species with wide and far geographical distribution. The 28S rDNA analysis has ensured a relatively accurate and reliable arrangement when handled by the method of "Neighbor-Joining" (NJ) or "Maximum Likelihood" (ML)) in the MEGA X program with a bootstrap of 1000 resamplings (Kumar *et al*., 2018).

Species placement in the phylogenetic tree formed from analysis of 55 28S rDNA sequences of 17 species of 4 families in the suborder Xiphidiata in this study, excluding *S. haematobium* (outgroup), basically agreed with some studies of morphology classification and recent molecular analysis. Paragonimidae, previously imported in the family Troglotrematidae (Tkach *et al.*, 2000; Olson *et al.*, 2003), has recently been proposed to convert into an independent family of *Paragonimus* spp. in the taxonomic clarification; and likewise, the family Nanophyetidae comprising all the *Nanophyetus* spp. Such rearrangement of species and families have made a proposal of the change for suborder Troglotremata to suborder Xiphidiata (Blair *et al*., 2008; 2016; Ponce de

León, Hernández-Mena, 2019). In this study, the family Paragonimidae is divided into different complexes including *P. westermani* complex, *P. heterotremus* and *P. ohirai* that they were recognized and identified in the same groups as indicated in the phylogenetic tree (Fig. 1). However, the suborder Xiphidiata replacing the suborder Troglotremata might make some jumping positions for species since the suborder Xiphidiata is too large to cover morphologically and genetically distinct species (Ponce de León, Hernández-Mena, 2019). Categorization and taxonomic classification for taxa and subfamilies related to pulmonary flukes still have many issues to consider, but at least, the use of the nuclear ribosomal markers (18S and 28S rDNA) contributes to an increasingly clear identification of species and families, as is the case with species of the family Paragonimidae and other families in the suborder Xiphidiata.

Thus, the 28S rDNA marker extracted from the ribosomal transcription unit (rTU) data has been effectively utilized in molecular classification, in species relationship determination, and molecular evolution. It also provides scientific evidence for the nomenclature and reclassification of species/genera and families Paragonimidae, Troglotrematidae, Nanophyetidae, and Collyriclidae in suborder Xiphidiata (Tkach *et al*., 2000; Olson *et al*., 2003; Ponce de León, Hernández-Mena, 2019). Especially, this study once again established a clear taxonomic position of *P. westermani* species of Vietnam (collected metacercaria samples from crabs in Quang Tri) together with the valid *P. westermani* species in the taxonomic classification system.

CONCLUSION

Phylogenetic tree and taxonomic position of 9 *Paragonimus* strains of 5 species, *P. heterotremus*, *P. westermani*, *P. ohirai*, *P. miyazakii* and *P. iloktsuenensis*, through analysis of 28S ribosomal markers and phylogeny along with 46 sequences of 17 species of the family Paragonimidae, Troglotrematidae, Nanophyetidae and Collyriclidae in the suborder Xiphidiata were identified. Phylogenetic analysis using the 28S rDNA sequences showed clearly molecular evolutionary relationships of species in the family Paragonimidae, Troglotrematidae, Nanophyetidae, and Collyriclidae and clarified a number of complex identification and nomenclature problems of *P. westermani* of Vietnam to recognize as that it is really *P. westermani* species.

Acknowledgments: *Thanks are expressed for funding from the VAST Program to support scientific research activities for senior research fellows in 2020, code NVCC08-04/20-20 for Prof. Dr. Le Thanh Hoa to implement this project.*

REFERENCES

Blair D (2006) Ribosomal DNA variation in parasitic flatworms. In: Maule A, editor. Parasitic Flatworms: Molecular Biology, Biochemistry, Immunology and Control. CAB International pp. 96–123.

Blair D (2008) Family Paragonimidae Dollfus, 1939. In: Bray RA, Gibson DI, Jones A, eds. Keys to the Trematoda. Vol. 3. London: CAB International and Natural History Museum.

Blair D (2014) Paragonimasis. In: Toledo R, Fried B (editors). Digenetic Trematodes. New York: Springer Science, pp. 115–152.

Blair D, Nawa Y, Mitreva M, Doanh PN (2016) Gene diversity and genetic variation in lung flukes (genus *Paragonimus*). *Trans R Soc Trop Med Hyg* 110(1): 6–12.

Cumberlidge N, Rollinson D, Vercruysse J, Tchuem Tchuenté LA, Webster B, Clark PF (2018) *Paragonimus* and paragonimiasis in West and Central Africa: unresolved questions. *Parasitology* 145(13): 1748–1757.

Doanh PN, Horii Y, Nawa Y (2013) *Paragonimus* and paragonimiasis in Vietnam: an update. *Korean J Parasitol* 51(6): 621–627.

Doanh PN, Shinohara A, Horii Y, Habe S, Nawa Y (2009) Discovery of *Paragonimus westermani* in Vietnam and its molecular phylogenetic status in *P. westermani* complex. *Parasitol Res* 104(5): 1149–1155.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol Biol Evol* 35: 1547–1549

Le TH, De NV, Blair D, McManus DP, Kino H, Agatsuma T (2006) *Paragonimusheterotremus* Chen and Hsia, 1964, in Vietnam: a molecular identification andrelationships of isolates from different hosts and geographical origins. *Acta Trop* 98(1): 25–33.

Le TH, Nguyen KT, Nguyen NT, Doan HT, Dung DT, Blair D (2017) The ribosomal transcription units of *Haplorchis pumilio* and *H. taichui* and the use of 28S sequences for phylogenetic identification of common heterophyids in Vietnam. *Parasites & Vectors* 10: 17.

Le TH, Nguyen KT, Nguyen NTB, Doan HTT, Agatsuma T, Blair D (2019) The complete mitochondrial genome of *Paragonimus ohirai* (Paragonimidae: Trematoda: Platyhelminthes) and its comparison with *P. westermani* congeners and other trematodes. *PeerJ* 7: e7031.

Le TH, Pham KLT, Doan HTT, Le TKX, Nguyen KT, Lawton SP (2020) Description and phylogenetic analyses of ribosomal transcription units from species of Fasciolidae (Platyhelminthes: Digenea). *J Helminthol* 94: e136.

Le Thanh Hoa, Nguyen Thi Bich Nga, Doan Thi Thanh Huong, Le Thi Thi Kim Xuyen, Nguyen Thi Khue (2019) Structure and characterization of the ribosomal transcription units of small liver flukes, *Opisthorchis viverrini*, *O. felineus* and *Clonorchis sinensis*. *Journal of Biotechnology* 17(3): 561–567 (in Vietnamese with English abstract).

McStay B (2016) Nucleolar organizer regions*:* genomic ′dark matter′ requiring illumination. *Genes & Development* 30(14): 1598–1610.

Pérez-Ponce de León G, Hernández-Mena DI (2019) Testing the higherlevel phylogenetic classification of Digenea (Platyhelminthes, Trematoda) based on nuclear rDNA sequences before entering the age of the ‘next-generation’ Tree of Life. *Journal of Helminthology* 93: 260–276.

Procop GW (2009) North American paragonimiasis (caused by *Paragonimus kellicotti*) in the context of global paragonimiasis. *Clin Microbiol Rev* 22(3): 415–446.

Sanpool O, Intapan PM, Thanchomnang T, Janwan P, Laymanivong S, Sugiyama H, Maleewong W (2015) Morphological and molecular identification of a lung fluke, *Paragonimus macrorchis* (Trematoda, Paragonimidae), found in central Lao PDR and its molecular phylogenetic status in the genus *Paragonimus*. *Parasitol Int* 64(6): 513–518.

Sanpool O, Intapan PM, Thanchomnang T, Janwan P, Nawa Y, Blair D, Maleewong W (2013) Molecular variation in the *Paragonimus heterotremus* complex in Thailand and Myanmar. *Korean J Parasitol* 51(6): 677–681.

Tkach V, Pawlowski J, Mariaux J (2000). Phylogenetic analysis of the suborder Plagiorchiata (Platyhelminthes, Digenea) based on partial lsrDNA sequences. *Int J Parasitol* 30(1): 83–93.

Weider LJ, Elser JJ, Crease TJ, Mateos M, Cotner JB, Markow TA (2005). The functional significance of ribosomal rDNA variation: Impacts on the evolutionary ecology of organisms. *Annu Rev Ecol Evol Syst* 36: 219–242.

Yoshida A, Doanh PN, Maruyama H (2019). *Paragonimus* and paragonimiasis in Asia: An update. *Acta Trop* 199: 105074.

QUAN HỆ TIẾN HÓA PHÂN TỬ SÁN LÁ PHỔI *PARAGONIMUS* VIỆT NAM VÀ THẾ GIỚI TRONG HỌ PARAGONIMIDAE VÀ PHÂN BỘ XIPHIDIATA (PLATYHELMINTHES: TREMATODA)

Nguyễn Thị Khuê[1,2], Phạm Thị Khánh Linh[1], Đỗ Thị Roan[1], Đoàn Thị Thanh Hương[1,2], Phạm Ngọc Doanh[3], Lê Thanh Hòa[1,2]

[1]Viện Công nghệ sinh học, Viện Hàn lâm Khoa học và Công nghệ Việt Nam
[2]Học viện Khoa học và Công nghệ, Viện Hàn lâm Khoa học và Công nghệ Việt Nam
[3]Viện Sinh thái và Tài nguyên sinh vật, Viện Hàn lâm Khoa học và Công nghệ Việt Nam

TÓM TẮT

Bệnh sán lá phổi (paragonimiasis) do *Paragonimus*, họ Paragonimidae, thuộc phân bộ Xiphidiata

(Platyhelminthes: Trematoda) gây ra, thường xảy ra trong cộng đồng nghèo, vùng cao, dân tộc thiểu số, ở Việt Nam và thế giới. Các loài *Paragonimus* của châu Á phân bố từ Nhật Bản, Hàn Quốc, dọc theo Bắc và Đông-Nam Trung Quốc, Tây-Bắc và miền Trung Việt Nam, Philippin, Thái Lan, Bangladesh, Ấn Độ và Sri Lanka. Sán lá phổi châu Á có nhiều biến chủng di truyền, phức tạp và tạo nên các phức hệ và dòng tiến hóa khác nhau. Các chuỗi gen 18S, 28S và vùng giao gen (ITS-1, ITS-2) trong đơn vị sao chép ribosome được sử dụng làm chỉ thị phân tử trong nghiên cứu di truyền và phân tích phả hệ. Chúng tôi thu nhận một phần chuỗi gen 28S rRNA (vùng D1–D3) của các loài *Paragonimus* spp. gồm: *P. heterotremus* (Việt Nam), *P. ohirai* (Nhật Bản), *P. iloktsuenensis* (Nhật Bản) và *P. westermani* (Ấn Độ và Việt Nam) và xác lập cây phả hệ phân tích tiến hóa phân tử. Kết quả cho thấy, trên cây phả hệ tập hợp lớn nhất là các loài thuộc họ Paragonimidae, bao gồm 46 chuỗi của 11 loài thuộc 11 phân nhóm, trong đó phức hệ *P. westermani* gồm các chủng có nguồn gốc Trung Quốc, Hàn Quốc, Nhật Bản, Ấn Độ, Philippin, Malaysia và Việt Nam. Phức hệ *P. westermani* sắp xếp ở vị trí "*chị em*" (sister group) với phân nhóm *P. siamensis*. Phức hệ *P. heterotremus* và phức hệ *P. ohirai* và các loài *P. miyazakii, P. harinasutai, P. mexicanus, P. kellicotti, P. macrorchis* nằm trong một tập hợp chung. *P. westermani* của Việt Nam ở vị trí gần với các chủng Đông Á, như trước đây đã được ghi nhận. Hai loài *P. ohirai* và *P. iloktsuenensis* được coi là "*đồng hình*" (sibling species), chia sẻ cùng nhánh phả hệ. Phân tích phả hệ sử dụng chỉ thị 28S rDNA đã cho thấy mối quan hệ về loài và tiến hóa phân tử trong họ Paragonimidae, Troglotrematidae, Nanophyetidae và Collyriclidae**.** Nghiên cứu cũng đã làm sáng tỏ một số vấn đề phân định phức hệ và danh pháp của *P. westermani* của Việt Nam và có cơ sở để công nhận đó thực sự chính là loài *P. westermani*.

Từ khóa: 28S rDNA/rRNA, đơn vị sao chép ribosome, *Paragonimus*, *Paragonimus westermani*, phả hệ, phức hệ, tiến hóa, Việt Nam