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REVIEW

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Paragonimus bangkokensis and *P. harinasutai*: Reappraisal of Phylogenetic Status

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Abstract

Paragonimus bangkokensis and *P. harinasutai* were first found in the same crab host in central Thailand, in 1967 and 1968, respectively. In addition to the repeated finding of both species in Thailand, they have also been found in China and Lao PDR. In Vietnam, only *P. bangkokensis*, but not *P. harinasutai*, was found recently in the northern provinces. These two species can be easily distinguished from each other by the morphological features of their metacercarial stage. Adult worms can also be easily distinguished from each other by the arrangement of the cuticular spines. However, recent phylogenetic analysis has revealed that the DNA sequences of the nuclear ribosomal second internal transcribed spacer region (ITS2) and the partial mitochondrial cytochrome oxidase subunit 1 (CO1) genes of *P. bangkokensis* and *P. harinasutai* are very similar. In addition, several *Paragonimus* spp–*P. paishuihoensis*, *P. menglensis,* and *P. xiangshanensis* in China–were found to be genetically similar to *P. bangkokensis*. The Thai isolate of *P. paishuihoensis* was also shown to be genetically similar to *P. bangkokensis*/*P. harinasutai*. Moreover, a possible monophyletic relationship of *P. ohirai* and its synonyms, *P. sadoensis* and *P. iloktuenensis*, with *P. bangkokensis* and *P. harinasutai*, has been reported repeatedly. In this review, the conflict and the congruence of morphological and molecular identifications of *P. bangkokensis* and *P. harinasutai*, and their phylogenetic status among the *Paragonimus* spp, are discussed.

Keywords: *Paragonimus*, *P. bangkokensis, P. harinasutai*, species complex

Introduction

The genus *Paragonimus* comprises more than 50 nominal species [1], the majority (about 30 species) being found and named in China [2]. In an array of *Paragonimus* spp, six species–*P. heterotremus*, *P. westermani*, *P. siamensis*, *P. bangkokensis*, *P. harinasutai*, and *P. macrorchis*–were found in Thailand [3,4]. Recently, *P. pseudoheterotremus* was added as a new species found in Thailand [5], although molecular data suggest this would be a sister species to *P. heterotremus* [6]. Among the 6 or 7 *Paragonimus* spp in Thailand, *P. siamensis* [7], *P. bangkokensis* [8] and *P. harinasutai* [9] were, as seen by their names, first found in Thailand. *P. siamensis* was soon found in Ceylon (Sri Lanka) [10] and the Philippines [11]. Later, *P. siamensis* was placed in the *P. westermani* species complex by molecular phylogenetic

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Fig 1 Geographical distribution of *P. bangkokensis, P. harinasutai, P. paishuihoensis* and *P. ohirai* complex.

analysis [12-14]. In contrast, *P. bangkokensis* and *P. harinasutai* were considered local species in central Thailand, because they were hardly found anywhere else for a long time after their first discovery. Recently, however, new endemic areas of *P. bangkokensis* and *P. harinasutai* have been found in and around Thailand. For example, *P. bangkokensis* [15,16] and *P. harinasutai* [17-19] were found separately in China. In Lao PDR, both species were concurrently found in the same crab hosts [20]. Recently, *P. bangkokensis* was found in Vietnam [21]. Molecular phylogenetic analyses of those geographically different isolates revealed that, although *P. bangkokensis* and *P. harinasutai* were easily distinguishable by morphology, they were very similar to each other in ITS2 and CO1 DNA sequences, and were considered to form a species complex [21], The aim of this review is to compile recent molecular information on the mutual relationship of *P. bangkokensis* and *P. harinasutai* and to elucidate their phylogenetic status among the *Paragonimus* spp.

Morphology of *P. bangkokensis* and *P. harinasutai*

In the *Paragonimus*-endemic areas like central Thailand, freshwater crabs often harbor several morphologically different types of *Paragonimus* metacercariae. For example, metacercariae of *P. bangkokensis* and *P. harinasutai* were found together with metacercariae of *P. westermani* and/or *P. heterotremus* [8,9]. In a recent survey, metacercariae of these 4 *Paragonimus* spp were found in the same crab, *Larnaudia beusekomae,* collected in Nakhon Nayok Province, central Thailand [22,23]. In Vientiane Province, Lao PDR, *P. harinasutai*, *P. bangkokensis,* and *P. heterotremus* were found concurrently in the same crab hosts with *P. harinasutai* predominating (84.6%) [20]. In northern Vietnam, *P. heterotremus* [24] was

Table 1 Morphological characteristics of ***Paragonimus*** species/species complex.

	P. westermani	*P. heterotremus*	*P. bangkokensis/P. harinasutai*		*P. skrjabini/P. miyazaki*	
			P. bangkokensis	*P. harinasutai*	*P. skrjabini*	*P. proliferus*
Metacercariae						
Outer cyst	uncommon	uncommon	thin	thin	uncommon	
Inner cyst wall	thick	thick	thin	thin	thick	thin
Size in µm (average)	400	212	450	600	425	1,000
Shape	round, no space	round, no space	C, U shape with space	C, U shape with space	round, no space	C, U shape with space
Excysted form (µm)	707 x 333	318 x 173	960 x 327	1,200 x 400	800 x 500	2,500 x 720
Adults						
Shape	oval	oval	elongate	elongate	elongate	spindle
Cuticular spine	single	single	grouped	single	single	grouped
Oral/ventral sucker	ventral ≈ oral	oral >>> ventral	ventral > oral	ventral > oral	ventral > oral	ventral > oral
Branching of ovary	5 lobes	complex	complex	complex	complex	complex
testes	5-6 lobes	complex	simple	simple	simple	Simple

Table 2 Indels in ITS2 sequences of ***P. bangkokensis*** and ***P. harinasutai*** of various geographical isolates.

Species	Locality	n	AT deletion						ATC insertion					
			240	241	242	243	244	245	270	271	272	273	274	275
P. ohirai	Japan	1	T	A	T	G	T	G	C	–	–	–	T	A
P. harinasutai	Thailand	3	•	•	•	•	•	•	•	–	–	–	•	•
	China	1	•	•	•	•	•	•	•	A	T	C	•	•
	Lao PDR	4	•	–	–	•	•	•	•	A	T	C	•	•
P. bangkokensis	Thailand	1	•	•	•	•	•	•	•	–	–	–	•	•
	Lao PDR	5	•	•	•	•	•	•	•	A	T	C	•	•
	Vietnam (Quangninh)	4	•	•	•	•	•	•	•	A	T	C	•	•
	Vietnam (Sinho)	11	•	•	•	•	•	•	•	–	–	–	•	•

n = number of samples examined for each locality the dominant species in freshwater crabs, with cohabitation by *P. bangkokensis* [21], *P. vietnamensis* [24,25], and/or *P. proliferus* [26] to varying degrees.

In all these recent discoveries, each *Paragonimus* species was primarily differentiated by the size and appearance of the metacercariae, and then by the morphological and morphometric appearance of the adult worms obtained by experimental infections of dogs and/or cats. Several key issues regarding the morphological features of *P. bangkokensis* and *P. harinasutai*, compared with *P. westermani*, are summarized in Table 1.

As described by Miyazaki and Vajrasthira [8,9], *P. bangkokensis* and *P. harinasutai* can be differentiated from other *Paragonimus* species, and also can be differentiated from each other, by their morphology. Encysted *P. bangkokensis* metacercariae are about 400-450 µm in diameter, and a metacercaria presents as a "C" or "U" shape, with an obvious space between the body and the cyst wall. In contrast, *P. westermani* metacercariae are somewhat smaller (350-400 µm) than *P. bangkokensis*, round and symmetrical in appearance, with a filled thick-walled inner cyst, without any space. Metacercariae of *P. harinasutai* are much larger, about 550-600 µm. As one of the key features of adult worms, *P. bangkokensis* had grouped cuticular spines, whereas *P. harinasutai* had singly arranged cuticular spines. Adult worms of these two species obtained by experimental infection shared some morphological features, but were distinguishable from each other by the shape of the cuticular spines and some other morphological and morphometric features [7,8,20,21].

Geographical distribution in and outside of Thailand

P. bangkokensis and *P. harinasutai* were originally found in the same intermediate host crabs, *Potamon smithianus*, collected in Nakhon Nayok Province, central Thailand [7,8]. Since then, *P. bangkokensis* was repeatedly found in central Thailand; in Saraburi [27,28], and in Nakhon Nayok [22,23]. *P. bangkokensis* was also found in Chanthaburi, eastern Thailand [27], and in Surat Thani, southern Thailand [29,30]. Thus, *P. bangkokensis* is widely distributed in Thailand.

Outside Thailand, *P. bangkokensis* was found in Hainan Island, China [15,16]. Recently, Odermatt *et al* [20] found cohabitation of *P. bangkokensis* with *P. harinasutai* and *P. heterotremus* in the same crab, *Potamon lipkei*, in Vientiane Province, Lao PDR. *P. bangkokensis* was found in Laichau Province in the northwest and Quangninh Province in the northeast of Vietnam [21].

After the first discovery in Nakhon Nayok [8], *P. harinasutai* was found repeatedly in Saraburi [28,31] and Nakhon Nayok [22,23], central Thailand. However, unlike *P. bangkokensis*, *P. harinasutai* was not recorded in places other than central Thailand. Outside Thailand, *P. harinasutai* was found in Ninghai, Zhejang Province, China [17-19]. As mentioned as above, Odermatt *et al* [20] reported the cohabitation of *P. harinasutai* with *P. bangkokensis* and *P. heterotremus* with a huge predominance of *P. harinasutai* in the same crab collected in Vientiane Province, Lao PDR. *P. harinasutai* metacercariae were also found in *Indochinamon ou* (Crustacea: Potamidae) collected in Luang Prabang Province, Lao PDR [32].

Molecular identification and phylogenetic analysis for *Paragonimus* spp

At least 6 *Paragonimus* spp are present in Thailand. Intapan *et al* [28] reported that 5 *Paragonimus* species in Thailand could be differentiated using RAPD (random amplified polymorphic DNA) markers. Although RAPD is useful for the identification of species, this method has limitations when assessing phylogenetic relationships [33]. Blair *et al* [1,34] pointed out that the DNA sequences of ITS2 would be a good inter-species marker, whereas that of the CO1 gene would be an intra-species marker for the molecular phylogenetic analysis of the genus *Paragonimus*. Since then, these two sequence data for the *Paragonimus* spp have been growing in the DNA database, and the phylogenetic relationship of *Paragonimus* spp has been re-evaluated on a molecular basis [1,14,34]. In this way, some *Paragonimus* spp were placed as synonyms of others, and some were classified as sister species, to be placed in the same species complex [12-14,16,35,36].

Compared with the extensive study of *P. westermani* [12,13] and *P. skrjabini* [36] for their probable construction of a species complex [14], little information has been available regarding the molecular phylogenetic status of *P. bangkokensis* and *P. harinasutai* until recently. The first breakthrough was made by Sugiyama and colleagues [29,30] who reported that the ITS2 sequence of *P. bangkokensis* from Surat Thani Province, southern Thailand, was highly similar (only one base difference) to *P. harinasutai*. Meanwhile, Qian *et al* [19] determined one each of the ITS2 and CO1 sequences of *P. harinasutai* from Ninghai, Zhejang Province, China, and showed that the ITS2 and CO1 nucleotide identities were 95.6 and 89.5%, respectively, similar to the Thai isolate of *P. harinasutai*. Such incongruence of the greater intra-species genetic variation of *P. harinasutai* than the inter-species variation of *P. bangkokensis* and *P. harinasutai* strongly suggests that *P. bangkokensis* and *P. harinasutai* would form a species complex. This possibility has been substantiated recently by Doanh *et al* [21], who reported that, using Lao isolates and Vietnamese isolates in comparison of all previously known sequences in the DNA database, *P. bangkokensis* and *P. harinasutai* were inseparable from each other and formed a single cluster.

In spite of the overall homogeneity of all available CO1 and ITS2 sequences of *P. bangkokensis* and *P. harinasutai*, Doanh *et al* [21] found that some geographical isolates had distinctive variations in their ITS2 sequences. As shown in Fig 2, AT deletion at the 241 base position was seen only in Laotian isolates of *P. harinasutai*. An ATC insertion appeared at the 271 base position in Laotian isolates of *P. bangkokensis* and *P. harinasutai,* and *P. bangkokensis* from Quang Ninh, but not of Laichau isolates from Vietnam. This ATC insertion was also seen at the same position in the ITS2 sequence of the *P. harinasutai* Chinese isolate (AB354603: Qian *et al*, 2006), but not in Thai isolates of *P. bangkokensis* or *P. harinasutai* or any other *Paragonimus* spp examined. Since those indels were consistently seen in all specimens of particular geographical isolates, this would be a good marker for tracking the geo-diversity of this species complex.

In terms of the phylogenetic relationship of *P. bangkokensis* and *P. harinasutai* to other *Paragonimus* spp, Cui *et al* [16] reported that ITS2 and CO1 sequences of *P. paishuihoensis*, *P. menglaensis*, and *P. xiangshanensis,* obtained in Yunnan Province were similar to those of *P. bangkokensis* obtained in Hainan Island. Based on a morphological study [37], *P. menglaensis* was shown to be an invalid species, due to mixing-up *P. microrchis* and *P. proliferus* metacercariae. Since the ITS2 and CO1 sequences of *P. proliferus* were identical to those of *P. hokuoensis* [26,38], and since *P. hokuoensis* is placed in the *P. skrjabini* complex [36], *P. proliferus*/*P. hokuoensis* should be placed into the *P. skrjabini* complex. Conversely, the molecular similarities of invalid *P. menglaensis* with *P. bangkokensis* [16] was supposedly due to contaminated *P. microrchis*, whose metacercariae were misidentified as *P. menglaensis*. Molecular identification of *P. microrchis* is urgently needed to clarify this point. Recently, Rangshiruji *et al* [39] reported that, based on ITS2 and CO1 sequences, *P. paishuihoensis* found in Chanthaburi Province, Thailand, was nested within a single clade with *P. bangkokensis* and *P. harinasutai*.

In addition to the phylogenetic similarities of several *Paragonimus* spp from China, mentioned above, similarities of the ITS2 and CO1 sequences of *P. ohirai* (including its synonyms: *P. sadoensis* and *P. iloktsuenensis* [35]) to those of *P. bangkokensis* and *P. harinasutai* were noted in several studies [24,26,34,40]. Until recently the ITS2 and CO1 sequence data of *P. bangkokensis*, *P. harinasutai,* and *P. ohirai* in the DNA database have been limited, so that statistical analysis of their phylogenetic relationships was not feasible. Due to the exhaustive work of Doanh *et al* [21], all *P. bangkokensis* and *P. harinasutai* samples from Thailand, Lao PDR, and Vietnam were found to differ from each other by only 0-7 bp for ITS2 and 0-37 bp for CO1, and were clustered in a single clade with bootstrap values of 56 and 38%, respectively. The Chinese isolate of *P. harinasutai* was clustered with *P. ohirai* in the ITS2 tree, whereas the Chinese isolates of *P. harinasutai* and *P. ohirai* were clustered separately as a sister clade of *P. bangkokensis*/*P. harinasutai* in CO1. Because of the obscurity in both ITS2 and CO1 phylogenetic trees and relatively low bootstrap support values for each node, possible genetic groupings of *P. bangkokensis*, *P. harinasutai,* and *P. ohirai,* were examined further by statistical analysis (see details in Ref 21). The possible monophyly of *P. ohirai* with *P. bangkokensis/P. harinasutai* was supported in the CO1 dataset. This possibility could not be tested for ITS2 because only one set of sequence data was available for *P. ohirai* during the study period. Further sampling of *P. ohirai* and related species is necessary to strengthen our understanding of the possible monophyly of *P. ohirai* to *P. bangkokensis*/*P. harinasutai*.

Bio-geographical distribution of *P. bangkokensis/ P. harinasutai* complex

As shown by Doanh *et al* [21] and as reviewed in this study, *P. bangkokensis* and *P. harinasutai* isolated in the Indochina peninsula are genetically close to, and mixing with, each other to form a single clade in both ITS2 and CO1 phylogenetic trees. In addition, several *Paragonimus* spp in China, for example, *P. paishuihoensis*, *P. xiangshanensis,* and probably *P. microrchis*, would be included in this clade. Moreover, although the evidence is not strong enough due to too few samples for phylogenetic analysis, *P. ohirai* is likely to form a species complex with *P. bangkokensis*/*P. harinasutai*. When the geographic distribution of all these *P. bangkokensis/P. harinasutai* and related species are plotted on a map (Fig 1), this probable species complex occupies a diverse area almost comparable to that of *P. westermani*. While the *P. westermani* complex is distributed in India, Sri Lanka, Malaysia, and the Philippines [12-14], the *P. bangkokensis*/ *P. harinasutai* complex or related species have not yet been found in those countries.

Concluding remarks

The taxonomic relationship of *Paragonimus* spp has been changing drastically along with the accumulation of genetic data and the development of molecular phylogenetic analysis. Already, some species, *eg*, *P. iloktuenensis* and *P. sadoensis,* have been proven synonyms of *P. ohirai* [35]. Recently, *P. hokuoensis* was proven genetically identical to *P. proliferus* [26,38]. *P. miyazakii* is considered a sub-species or sister species of *P. skrjabini* [36], and *P. siamensis* is included in the *P. westermani* clade [13,14]. The concept of "species complex" is now widely accepted, at least for the *P. skrjabini* [36] and *P. westermani* complexes [13,14]. Here, we propose the *P. bangkokensis/P. harinasutai* complex as a 3rd species complex in the genus *Paragonimus*. We have tentatively used the term "*P. bangkokensis*/ *P. harinasutai* complex" because, if *P. ohirai* were included in this species complex, it should be named *P. ohirai* complex because *P. ohirai* was registered far earlier [41] than *P. bangkokensis*/ *P. harinasutai*. Since a possible monophyletic grouping of *P. bangkokensis*, *P. harinasutai*, and *P. ohirai* was repeatedly recognized by using larger taxon samplings [14,24,26], it is highly likely that this species complex would be categorized as *P. ohirai* complex. The presence of *P. ohirai* in China was recently proven by molecular genetic methods [42], suggesting wider distribution of this species than ever thought.

For the morphological discrimination of *P. bangkokensis* and *P. harinasutai* adult worms, the arrangements of the cuticular spines have been considered the most simple and reliable marker; the former have grouped spines, while the latter have singly arranged spines (Table 1). Nevertheless, molecular phylogenetic analysis has shown that these two species should be considered a species complex. A similar discrepancy was already recognized in the *P. westermani* complex, in that the adult *P. westermani* worm has singly arranged spines, whereas the adult *P. siamensis* worm, which is allocated to the same *P. westermani* complex by molecular genetics [12-14], has grouped spines. Such a discrepancy is also seen in the *P. skrjabini* complex, in that *P. skrjabini* has singly arranged spines, while *P. proliferus* has grouped spines [26]. These results strongly suggest the necessity for a re-evaluation of the morphological parameters and/or the introduction of statistical analysis of multi-parameters for the morphology/morphometry of the *Paragonimus* spp.

In terms of the *P. bangkokensis*/*P. harinasutai* complex, several *Paragonimus* spp isolated in China, *eg*, *P. paishuihoensis* and *P. xiangshanensis,* are supposed to be included in this complex [16]. This possibility was strengthened further by the DNA sequence similarities of the Thai isolate of *P. paishuihoensis* to *P. bangkokensis*/*P. harinasutai* [39]. Since those rare *Paragonimus* spp were discriminated from *P. bangkokensis*/*P. harinasutai* or any other *Paragonimus* spp, and identified as new species based on morphology, their possible inclusion in one species complex indicates morphological variations in this complex.

So far as we could gather, despite the overall sequence similarities, this *P. bangkokensis*/*P. harinasutai* complex is likely to consist of distinct geographical isolates, because each geographical isolate was extremely homogeneous and distinguishable from each other by minor DNA sequence differences [21]. In particular, the ATC insertion/AT deletions in ITS2 were found conserved in the Laotian isolate of *P. bangkokensis* and *P. harinasutai* and some parts of the Vietnamese isolates of *P. bangkokensis* [21]. Such a unique marker beyond the species would be useful to pursue the biogeographical diversification of this species complex. For this purpose, we need to accumulate more DNA sequence data from various geographical locations, especially from Thailand, where the original *P. bangkokensis* and *P. harinasutai* were found. The most serious concern is that these and other *Paragonimus* spp would become extinct before the completion of our study, due to drastic environmental changes.

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