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Exploring Freshwater Crab Diversity in Ban Tham Subdistrict, Phayao Province, Thailand Through Morphology and DNA Barcoding

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Abstract

Freshwater crabs are important biological resources in northern Thailand, yet their diversity remains poorly documented in many areas. This study investigated freshwater crab diversity in Ban Tham Subdistrict, Dok Khamtai District, Phayao Province, using morphological examination and mitochondrial cytochrome c oxidase subunit I (COI) DNA barcoding. A total of 69 specimens were assigned to four taxa. *Indochinamon bhumibol* and *Sayamia bangkokensis* were identified to species level, whereas two unidentified taxa were provisionally designated Potamidae sp. 1 and Potamidae sp. 2. Genetic diversity varied among taxa, with Potamidae sp. 2 showing the highest haplotype and nucleotide diversity, whereas *S. bangkokensis* showed no haplotype variation. Pairwise K2P distances revealed substantially greater divergence among taxa than within taxa. Phylogenetic analysis resolved the four taxa into distinct clades, while Automatic Barcode Gap Discovery (ABGD) recovered four molecular operational taxonomic units in the initial partition, corresponding to the four taxa. These results support the genetic distinctiveness of Potamidae sp. 1 and Potamidae sp. 2, although their species identities remain unresolved. Further taxonomic and molecular studies are required to clarify their identities. These findings provide baseline information for the conservation and sustainable management of freshwater crab resources in the study area.

Keywords: cytochrome c oxidase subunit I (COI); DNA barcoding; freshwater crabs; genetic diversity; species delimitation



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1. Introduction

Freshwater crabs of the order Decapoda are an important group of macroinvertebrates inhabiting inland freshwater ecosystems, particularly in tropical and subtropical regions. They account for approximately 20% of the total diversity of brachyuran crabs [1]. Primary freshwater crabs are a highly diverse group distributed throughout tropical and subtropical regions worldwide. Recent classifications recognize seven families: Potamidae, Trichodactylidae, Pseudothelphusidae, Gecarcinucidae, Potamonautidae, Deckeniidae, and

Epiloboceridae [2]. Their ecological importance is further highlighted by their role as bioindicators of water quality and their contribution to nutrient cycling within freshwater ecosystems [3]. More broadly, freshwater decapods may influence community biodiversity through trophic interactions and habitat modification [4]. Among freshwater crabs, burrowing activities have been reported to enhance sediment aeration and nutrient cycling, particularly in muddy substrates [5]. Importantly, many species also serve as valuable food resources for humans and contribute to local livelihoods and traditional fisheries in several regions.

Traditionally, freshwater crab species have been identified primarily based on morphological characteristics, including carapace shape and size, cheliped morphology, the structure of the anterolateral margin and epibranchial tooth, and the morphology of male gonopods [6,7]. Among these characters, the morphology of the male first gonopod (G1) is regarded as one of the most important diagnostic features in freshwater crab taxonomy because it typically exhibits species-specific traits that facilitate the discrimination of closely related species [6–8]. However, species identification based solely on morphology can be difficult because many freshwater crab species display high morphological similarity, phenotypic plasticity, ontogenetic variation, and sexual dimorphism [9]. In addition, morphological characters may vary among populations inhabiting different geographic regions, habitats, and hydrological environments [10] and developmental stages [11], potentially resulting in misidentification, taxonomic ambiguity, or underestimation of biodiversity. Consequently, molecular approaches such as DNA barcoding have become increasingly important for improving species identification and taxonomic resolution in freshwater crabs.

The application of DNA barcoding based on the mitochondrial cytochrome c oxidase subunit I (COI) gene has become a reliable approach for species identification and the detection of cryptic diversity across a wide range of taxonomic groups, including freshwater crustaceans [11,12]. This molecular approach provides improved accuracy in species delimitation, particularly among closely related taxa exhibiting limited morphological differentiation [13]. Previous studies have demonstrated the utility of COI-based DNA barcoding for crab identification in Thailand. For instance, Suthamrit and Thaewnon-Ngiw [8] identified six mountain crab species (Potamidae) from the Phetchabun Mountains, while Klinbunga et al. [14] identified the blue swimming crab *Portunus pelagicus* in Thai waters, demonstrating the usefulness of this approach for biodiversity assessment and taxonomic research. Moreover, this approach helps address the “taxonomic impediment”, which is associated with a shortage of taxonomic expertise and difficulties in species delimitation, potentially leading to inaccurate biodiversity assessments [15]. The availability of easily accessible molecular reference databases further improves the effectiveness of DNA barcoding for species identification and taxonomic validation [1].

Despite the ecological and socioeconomic importance of freshwater crabs, their diversity remains poorly documented in many parts of northern Thailand. In Ban Tham Subdistrict, Dok Khamtai District, Phayao Province, freshwater crabs are widely harvested and consumed by local communities as an important food resource. However, many of these crabs are identified primarily by local names, and information regarding their species diversity remains limited. As a result, the taxonomic identity and diversity of freshwater crabs in the area remain poorly understood. Therefore, this study aimed to investigate the species diversity of freshwater crabs in Ban Tham Subdistrict using morphological and mitochondrial COI-based DNA barcoding approaches. The resulting information provides a baseline for the sustainable utilization, conservation, and management of freshwater crab resources in the area, supporting the objectives of the Plant Genetic Conservation Project under the Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn.

2. Materials and Methods

2.1. Field Sampling

Information on freshwater crab distribution and habitat characteristics was gathered through informal interviews with local community members, and this local knowledge was used to inform the selection of sampling sites. Native freshwater crabs were subsequently collected from a range of natural water bodies in Ban Tham Subdistrict, Dok Khamtai District, Phayao Province, Thailand (Figure 1), from December 2021 to June 2022, by hand with the assistance of local residents. Live specimens were photographed immediately after collection, and key morphological traits were documented, including size, coloration, and diagnostic features such as the carapace, chelae, walking legs, abdomen, and the male first and second gonopods (G1 and G2). Specimens were identified to the lowest possible taxonomic level based on published morphological descriptions of Thai freshwater crabs using Naiyanetr [16] and Sripho and Chaiyarat [17].

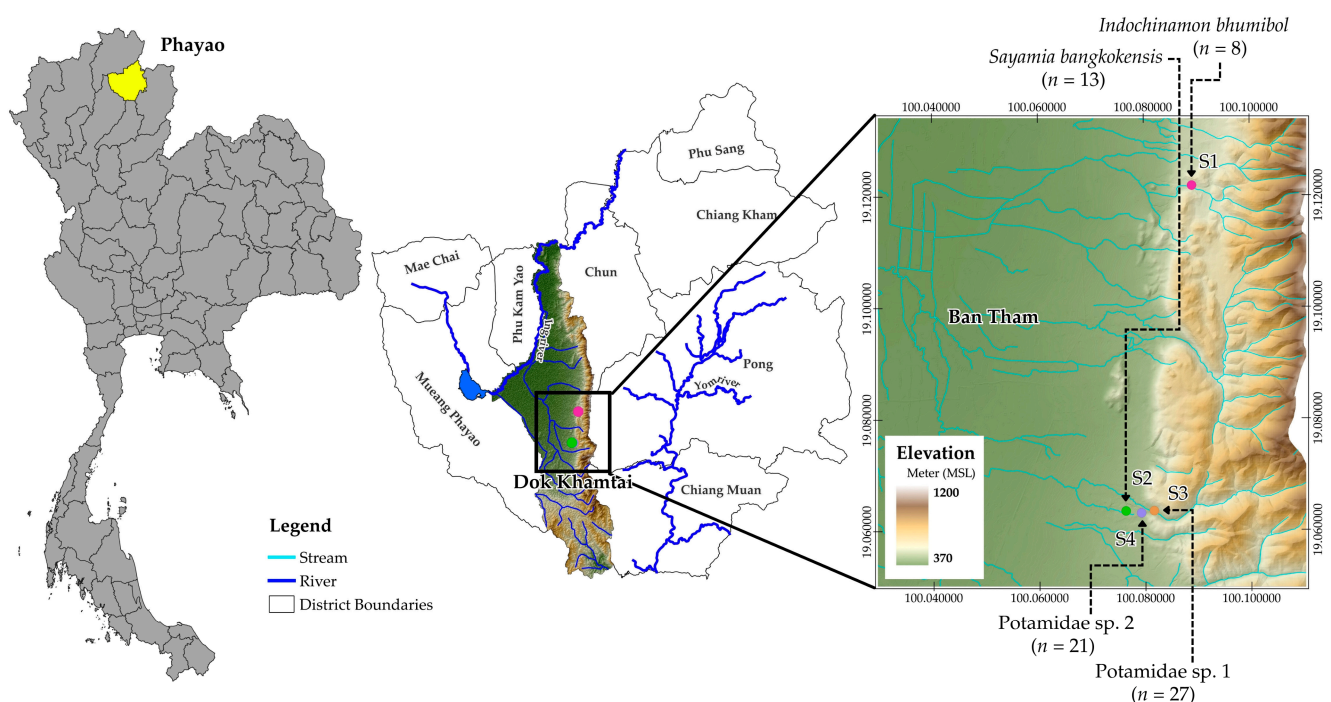


Figure 1. Location of the study area in Phayao Province, northern Thailand, showing Dok Khamtai District and Ban Tham Subdistrict, with four sampling sites (S1–S4) for four freshwater crab taxa: *Indochinamon bhumibol* (S1; $n = 8$; 19.146600° N, 100.200960° E), *Sayamia bangkokensis* (S2; $n = 13$; 19.063065° N, 100.077548° E), Potamidae sp. 1 (S3; $n = 27$; 19.063584° N, 100.081596° E), and Potamidae sp. 2 (S4; $n = 21$; 19.063111° N, 100.079132° E). The map was created using QGIS software version 3.40.

All specimens were preserved in absolute ethanol at room temperature prior to DNA extraction. Voucher specimens were deposited at the Division of Technology and Innovation for Fisheries, School of Agriculture and Natural Resources, University of Phayao, Thailand, with voucher numbers for all specimens provided in Supplementary Materials Table S1. The procedures were approved by the Institutional Animal Care and Use Committee, University of Phayao (approval no. 64 01 04 020).

2.2. DNA Barcoding for Species Identification

Genomic DNA was extracted from approximately 10–20 mg of muscle tissue from a walking leg of each crab specimen using GF-1 Tissue DNA Extraction Kit (Vivantis Technologies Sdn. Bhd., Subang Jaya, Selangor, Malaysia) following the manufacturer's protocol.

The quality and concentration of the extracted DNA were evaluated by electrophoresis on a 1.2% agarose gel and measured using NanoDrop One^C spectrophotometer (ThermoFisher Scientific Inc., Madison, WI, USA).

The mitochondrial *COI* gene was amplified by polymerase chain reaction (PCR) using the universal primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') [18]. The PCR was performed in a total reaction volume of 25 µL containing 2× *Taq* PCR MasterMix II (TIANGEN Biotech (Beijing) Co., Ltd., Changping, Beijing, China), 10 µM of each primer, and 20 ng of extracted DNA. The thermal cycling conditions consisted of 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s. The desired PCR products were subsequently purified using a TIANquick Midi Purification Kit (TIANGEN Biotech (Beijing) Co., Ltd., Changping, Beijing, China) according to the manufacturer's instructions.

Purified PCR products were subjected to bidirectional sequencing using the LCO1490 and HCO2198 primers by a commercial sequencing service (ATGC Co., Ltd., Pathumthani, Thailand) using the Illumina NovaSeq X Plus platform (Illumina, San Diego, CA, USA). The forward and reverse *COI* sequences obtained from each specimen were aligned using Clustal Omega version 1.2.4 [19] and manually trimmed to obtain a 709-bp consensus sequence for each specimen. The resulting sequences were examined for the presence of stop codons, insertions, and deletions using the ORF Finder website (<https://www.ncbi.nlm.nih.gov/orffinder/> (accessed on 1 August 2026)). Subsequently, sequence similarity searches were conducted by comparing the obtained *COI* sequences with reference sequences in the GenBank database using BLASTn [20] (<http://www.ncbi.nlm.nih.gov/blast> (accessed on 1 August 2026)) with default parameters and the Barcode of Life Data System (BOLD; <http://www.boldsystems.org> (accessed on 1 August 2026)) against the Animal Library (Public) using the Rapid Species Search mode. These analyses were performed to determine the most appropriate scientific names for the crab specimens. Sequence identities of >98%, 95–97%, and 85–94% were used as general criteria for species-, genus-, and family-level assignments, respectively (adapted from the criteria of Uchida et al. [21] and Panprommin et al. [22]).

Genetic diversity analyses for each taxon were performed using DnaSP version 6.00 [23], based on 709-bp *COI* sequences. The calculated indices included the number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (π), and the number of polymorphic (segregating) sites (S). One representative *COI* sequence was selected for each haplotype identified within each crab taxon and used to calculate pairwise genetic distances in MEGA version 7.0 [24] under the Kimura 2-parameter (K2P) model. The resulting distance matrix was exported and visualized as a heatmap in R version 4.5.0 [25] using the pheatmap package [26]. Haplotype networks were constructed separately for each taxon based on the haplotypes identified from the *COI* sequences. Statistical parsimony networks were generated using the Templeton, Crandall, and Sing (TCS) algorithm implemented in PopART v1.7 [27].

Phylogenetic relationships were assessed using representative *COI* haplotype sequences obtained in the present study together with reference sequences of related freshwater crab species retrieved from GenBank. All sequences were aligned using Clustal Omega version 1.2.4 [19] and trimmed to a uniform length of 709 bp. A maximum-likelihood (ML) phylogenetic tree was constructed using MEGA version 7.0 [24] with the Kimura 2-parameter (K2P) model and 1000 bootstrap replicates. *Rodriguezia adani* (Trichodactylidae; GenBank accession no. PQ064124) was selected as the outgroup because it belongs to a freshwater crab family distinct from the ingroup taxa. All positions containing gaps and missing data were eliminated from the analysis.

Species delimitation was performed using the Automatic Barcode Gap Discovery (ABGD) method [28], implemented in ABGDPy within iTaxoTools (<https://www.itaxotools.org/> (accessed on 1 August 2026)). Analyses were conducted on the COI dataset using the Kimura 2-parameter (K2P) distance model with a transition/transversion ratio of 2.0, a prior intraspecific divergence range (P) of 0.001–0.100, 20 recursive steps, and a relative gap width (X) of 1.5. Molecular Operational Taxonomic Units (MOTUs) were inferred from the initial partition, which produced stable delimitations across all tested prior divergence values.

3. Results

3.1. Morphological Identification and Habitat Characteristics

Morphological examination of 69 freshwater crab specimens distinguished four taxa (Figure 2). Two taxa were identified to the species level as *Indochinamon bhumibol* ($n = 8$) and *Sayamia bangkokensis* ($n = 13$) based on their morphological characteristics. The remaining two taxa, comprising 27 and 21 specimens, respectively, could not be identified to the species level. All freshwater crabs were collected from four locations within Ban Tham Subdistrict, where the four taxa showed differences in their observed habitats. *Indochinamon bhumibol* was primarily found in high-elevation mountainous areas, whereas *S. bangkokensis* occurred in lowland areas, particularly rice fields. Of the two unidentified taxa, the first was collected along the banks of relatively fast-flowing streams, whereas the other was found in slow-flowing streams in relatively flatter areas. Therefore, differences in sample sizes among taxa may have been influenced by variation in habitat accessibility. In addition, all four taxa were collected during both the dry and early wet seasons. *Indochinamon bhumibol* was more difficult to collect during the rainy season, whereas the other three taxa could be collected throughout the year.

3.2. Molecular Identification

All 69 freshwater crab specimens were successfully amplified for COI and sequenced, with high-quality sequences showing no stop codons, deletions, or insertions. COI-based identification confirmed the identification of all eight *I. bhumibol* specimens, with sequence similarities ranging from 98.16% to 99.15% in both the GenBank and BOLD databases (Supplementary Materials Table S1). Similarly, all 13 *S. bangkokensis* specimens showed sequence similarities of 99.85% in GenBank and 99.03% in BOLD. For the remaining two taxa, GenBank searches showed similarities to several species within the family Potamidae. The closest matches for the first taxon were *Potamiscus yiwuensis* (90.79–91.36%) and *P. montosus* (90.08–90.65%), whereas those for the second taxon were *P. yiwuensis* (91.26–92.10%) and *P. montosus* (91.11–91.96%). No matches were obtained for either taxon in the BOLD database. Therefore, these two taxa were designated as Potamidae sp. 1 and Potamidae sp. 2, respectively. All COI sequences generated in this study were deposited in GenBank under accession numbers PZ958406–PZ958474 (Supplementary Materials Table S1).

3.3. COI-Based Genetic Analyses

A total of 69 COI sequences representing four freshwater crab taxa were analyzed for genetic diversity. The number of haplotypes ranged from 1 to 15, with the highest value observed in Potamidae sp. 2 (15 haplotypes), followed by Potamidae sp. 1 (9 haplotypes), *I. bhumibol* (6 haplotypes), and *S. bangkokensis* (1 haplotype) (Table 1, Supplementary Materials Table S1). Haplotype diversity (H_d) was highest in Potamidae sp. 2 (0.962), followed by *I. bhumibol* (0.893) and Potamidae sp. 1 (0.860), whereas *S. bangkokensis* showed no haplotype variation ($H_d = 0$). A similar pattern was observed for nucleotide diversity

(π), with the highest value recorded in Potamidae sp. 2 (0.01005), followed by *I. bhumibol* (0.00589) and Potamidae sp. 1 (0.00525), while *S. bangkokensis* exhibited no nucleotide diversity ($\pi = 0$). The number of segregating sites (S) varied from 0 to 22, with the greatest value detected in Potamidae sp. 2 (Table 1).

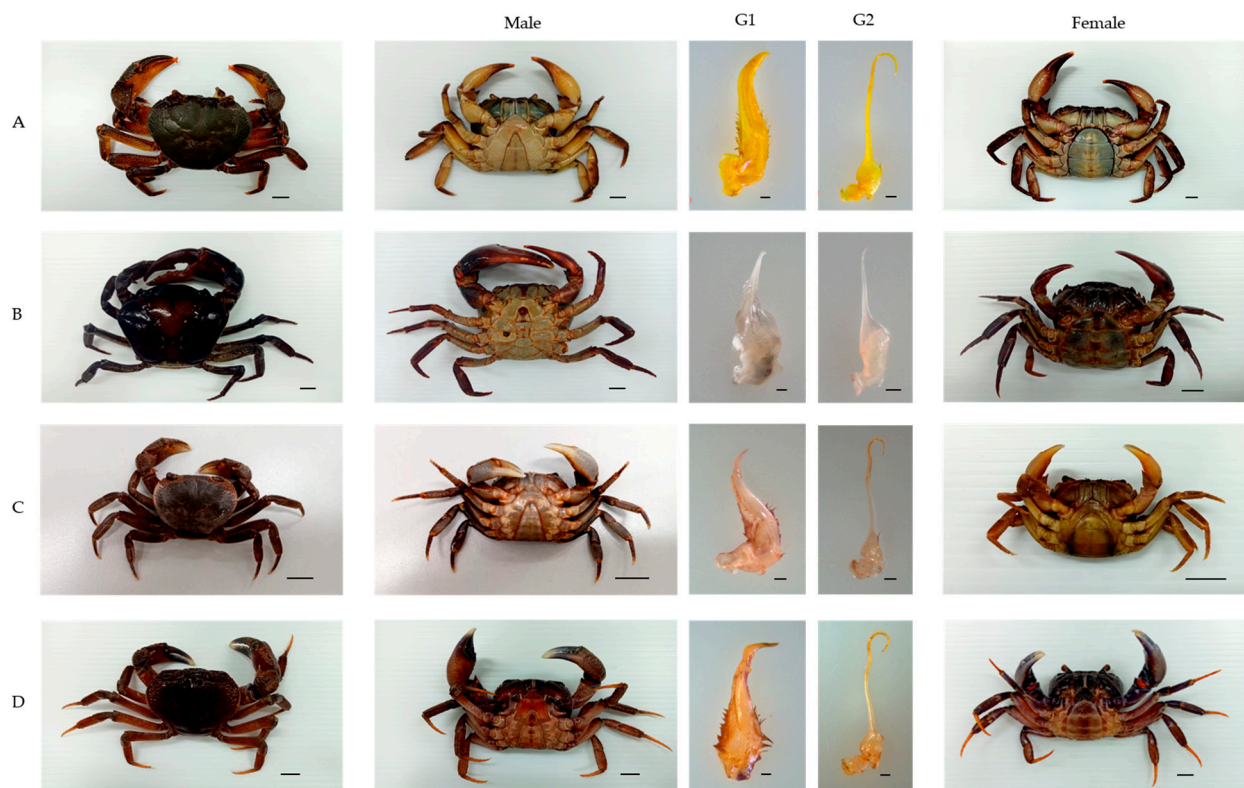


Figure 2. External morphology of four freshwater crab taxa collected in Ban Tham Subdistrict, Dok Khamtai District, Phayao Province, Thailand: (A) *Indochinamon bhumibol* (voucher nos. IB03, male; IB01, female), (B) *Sayamia bangkokensis* (voucher nos. SB04, male; SB02, female), (C) Potamidae sp. 1 (voucher nos. PS101, male; PS113, female), and (D) Potamidae sp. 2 (voucher nos. PS203, male; PS214, female). For each taxon, dorsal and ventral views of the male, the left first (G1) and second (G2) gonopods, and the female are shown. Scale bars = 1 cm for whole specimens and 1 mm for gonopods.

Table 1. Genetic diversity indices of four freshwater crab taxa based on mitochondrial *COI* sequences.

Taxa	Sampling Site	N	h	Hd	π	S
<i>Indochinamon bhumibol</i>	S1	8	6	0.893	0.00589	8
<i>Sayamia bangkokensis</i>	S2	13	1	0	0	0
Potamidae sp. 1	S3	27	9	0.860	0.00525	13
Potamidae sp. 2	S4	21	15	0.962	0.01005	22

Note: Sampling sites (S1–S4) correspond to those shown in Figure 1. N = number of sequences; h = number of haplotypes; Hd = haplotype diversity; π = nucleotide diversity; S = number of segregating (polymorphic) sites.

The TCS network of Potamidae sp. 1 comprised nine haplotypes (Figure 3A). Haplotype H4 was the most frequent and was directly connected to H2, H5, and H6. Haplotype H6 linked the H4 cluster to the H9 cluster, which included H1, H8, and H3, whereas H7 formed a peripheral lineage connected to H6 by two mutational steps. Overall, the network exhibited moderate haplotype variation, with the greatest divergence observed between H6 and H9, which were separated by five mutational steps. Potamidae sp. 2 comprised 15 haplotypes among 21 individuals (Figure 3B). Haplotypes H4 and H6 were the most frequent, while H3 and H8 were also shared by multiple individuals. The network displayed a more

complex pattern of relationships, with several lineages separated by one to five mutational steps. The TCS network of *I. bhumibol* comprised six haplotypes among eight individuals (Figure 3C). Haplotype H4 was the most common, whereas all remaining haplotypes were represented by single individuals. The network showed a simple linear structure, with haplotypes separated by one to four mutational steps and the greatest divergence occurring between H1 and H2.

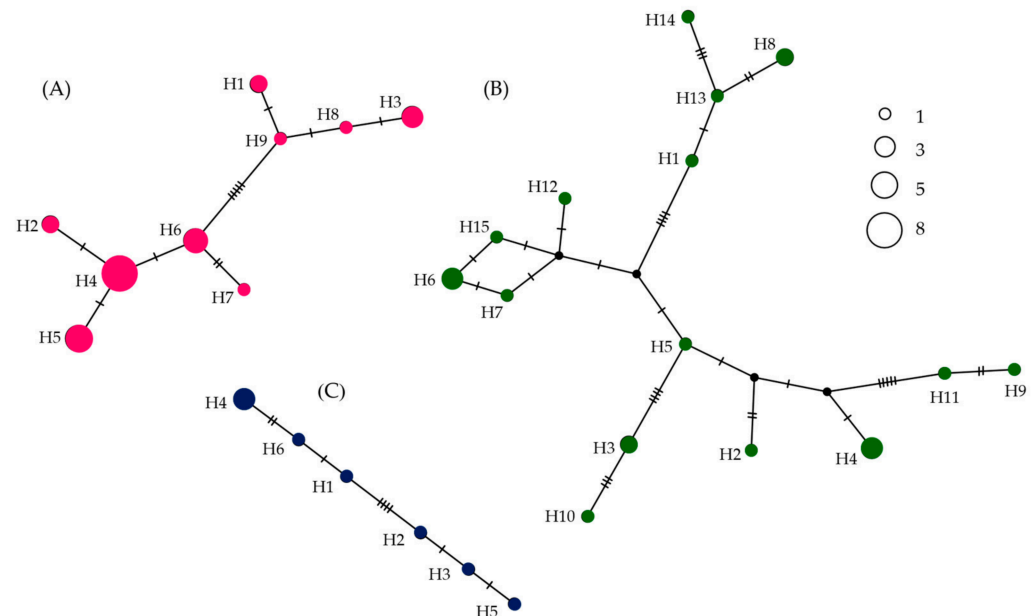


Figure 3. TCS haplotype networks based on *COI* sequences of (A) Potamidae sp. 1, (B) Potamidae sp. 2, and (C) *Indochinamon bhumibol*. Circle size is proportional to haplotype frequency. Hatch marks on connecting lines indicate mutational steps, and small unlabeled circles represent inferred intermediate haplotypes (median vectors).

Pairwise K2P genetic distances among representative *COI* haplotypes revealed clear genetic differentiation among the four freshwater crab taxa (Table 2, Figure 4). Within-taxon mean K2P distances were 0.0060 for *I. bhumibol* (IB), 0.0071 for Potamidae sp. 1 (PS1), and 0.0107 for Potamidae sp. 2 (PS2), with ranges of 0.0014–0.0114, 0.0014–0.0129, and 0.0014–0.0187, respectively (Table 2). *Sayamia bangkokensis* (SB) was represented by a single haplotype; therefore, within-taxon distance was not calculated. Intertaxon mean K2P distances ranged from 0.1073 to 0.2363. The lowest divergence was observed between PS1 and PS2, with a mean K2P distance of 0.1140 (0.1073–0.1214), whereas the highest was observed between PS1 and SB, with a mean of 0.2334 (0.2323–0.2363). Hierarchical clustering separated the haplotypes into four groups corresponding to SB, IB, PS1, and PS2 (Figure 4).

The ML phylogenetic tree clearly separated the four freshwater crab taxa obtained in the present study into four distinct clades (Figure 5). These taxa were further separated into two major clades. The first major clade comprised Potamidae sp. 1, Potamidae sp. 2, and *I. bhumibol* from the present study, together with reference sequences of *Potamiscus yizuwensis*, *Potamiscus montosus*, *Potamiscus yongshengensis*, *Potamiscus motuoensis*, *Indochinamon daweshanense*, *Bottapotamon lingchuanense*, *Aparapotamon huizeense*, and *Aparapotamon binchuanense* retrieved from GenBank. In addition, the reference sequences of *I. bhumibol* retrieved from GenBank clustered with the *I. bhumibol* sequences obtained in the present study. The second major clade comprised *S. bangkokensis* from the present study and *Somanthelphusa bawangensis*, together with *Esanthelphusa dugasti* and *Esanthelphusa* sp. Notably, *R. adani* was distinctly separated from the two major clades.

ABGD analysis of the 69 COI sequences revealed four MOTUs in the initial partition across all prior intraspecific divergence values ($0.0010 \leq p \leq 0.1000$). The recursive partition recovered four groups at $p \geq 0.0016$, whereas 12 groups were recovered at lower p values ($p \leq 0.0013$) (Figure 6A). The four MOTUs corresponded to the four taxa recognized in this study: *I. bhumibol*, *S. bangkokensis*, Potamidae sp. 1, and Potamidae sp. 2. Pairwise K2P genetic distances showed a clear barcode gap between 0.02 and 0.11. Intraspecific distances ranged from 0.00 to 0.02, whereas interspecific distances ranged from 0.11 to over 0.22 (Figure 6B).

Table 2. Pairwise K2P genetic distances based on 709-bp COI haplotype sequences within and among the four freshwater crab taxa. Values represent mean K2P distances, with minimum-maximum values given in parentheses. Diagonal values represent within-taxon distances, whereas lower-left values represent intertaxon distances.

Taxa	<i>Indochinamon bhumibol</i>	<i>Sayamia bangkokensis</i>	Potamidae sp. 1	Potamidae sp. 2
<i>Indochinamon bhumibol</i>	0.0060 (0.0014–0.0114)			
<i>Sayamia bangkokensis</i>	0.2340 (0.2313–0.2354)	–		
Potamidae sp. 1	0.1599 (0.1525–0.1652)	0.2334 (0.2323–0.2363)	0.0071 (0.0014–0.0129)	
Potamidae sp. 2	0.1554 (0.1469–0.1632)	0.2204 (0.2114–0.2270)	0.1140 (0.1073–0.1214)	0.0107 (0.0014–0.0187)

Note: –, not calculated because only one haplotype was detected.

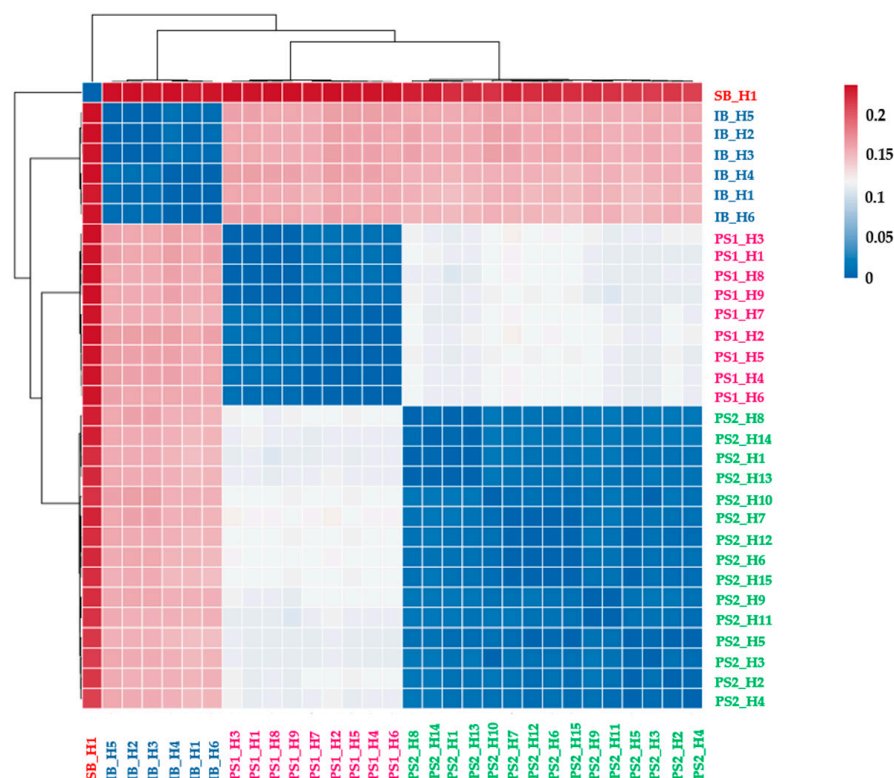


Figure 4. Heatmap of pairwise Kimura 2-parameter (K2P) genetic distances among representative COI haplotypes of four freshwater crab taxa from Ban Tham Subdistrict, Phayao Province, Thailand. Blue colors indicate low genetic distances and red colors indicate high genetic distances. Dendrograms represent hierarchical clustering of haplotypes into four groups corresponding to *Sayamia bangkokensis* (SB), *Indochinamon bhumibol* (IB), Potamidae sp. 1 (PS1), and Potamidae sp. 2 (PS2).

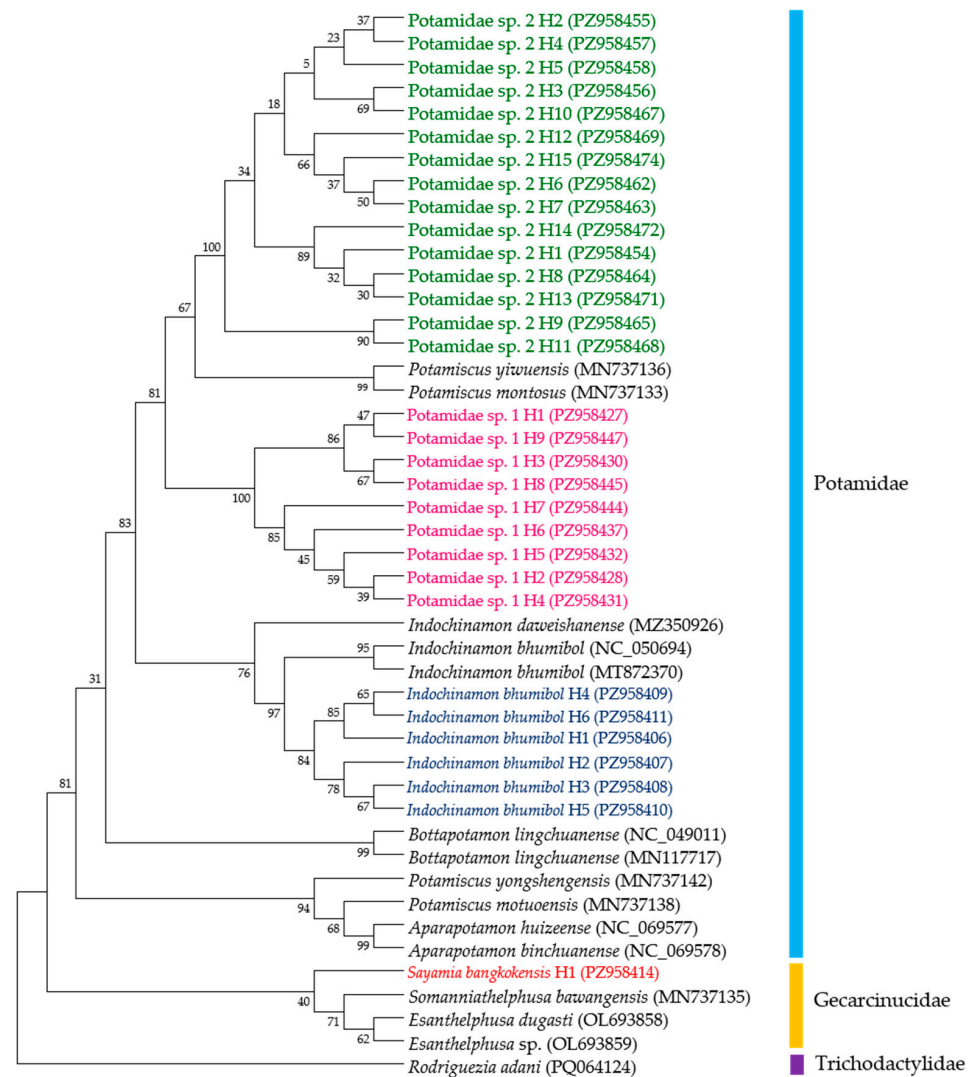


Figure 5. Maximum-likelihood (ML) phylogenetic tree based on 709-bp COI haplotype sequences of four freshwater crab taxa from Ban Tham Subdistrict, Dok Khamtai District, Phayao Province, Thailand, together with reference sequences retrieved from GenBank. The tree was constructed using the Kimura 2-parameter model with 1000 bootstrap replicates. Bootstrap values are shown at the nodes. *Rodriguezia adani* (Trichodactylidae) was used as the outgroup. Branch lengths are shown for illustrative purposes and are not proportional.

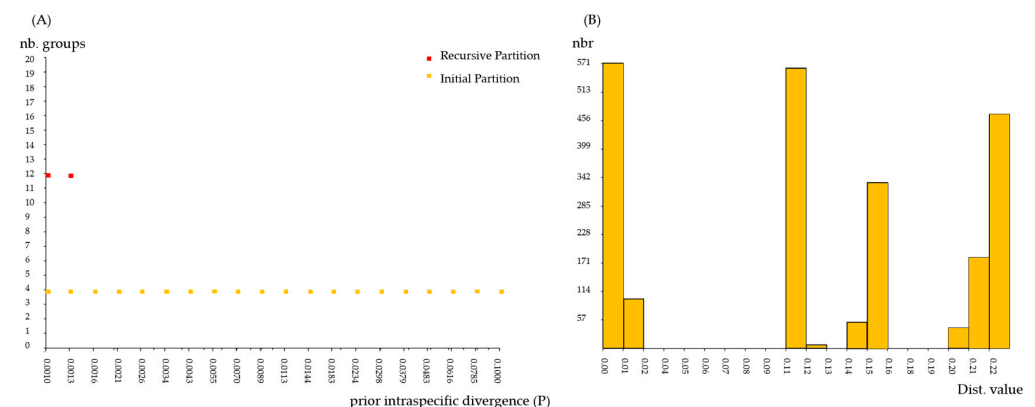


Figure 6. Automatic Barcode Gap Discovery (ABGD) analysis of 69 COI sequences. **(A)** Number of molecular operational taxonomic units (MOTUs) recovered across different prior intraspecific divergence values (p). **(B)** Distribution of pairwise K2P genetic distances showing the barcode gap between intra- and interspecific distances.

4. Discussion

The morphological characteristics observed in the present study were consistent with previous descriptions of *I. bhumibol* and *S. bangkokensis*. *Indochinamon bhumibol* was characterized by a carapace broader than long, with a dark brown carapace, chelipeds, and walking legs and orange coloration at the tips of the walking legs. Its G1 was broad, with a short terminal segment directed outward and curved downward at the tip, whereas the distal segment of G2 was more than half the length of the basal segment, consistent with the description of Sripho and Chaiyarat [17]. Similarly, *S. bangkokensis* (originally described as *Somanniathelphusa bangkokensis*) exhibited a strongly convex and smooth dorsal surface of the carapace and a T-shaped male abdomen, with concave lateral margins of the sixth abdominal segment. In addition, its G1 had an unexpanded basal portion and a slender distal portion that extended straight or curved slightly outward, as described by Naiyanetr [16]. However, the remaining two taxa could not be identified to the species level based on external morphological characteristics, highlighting the need for molecular identification using COI sequences. Notably, the four taxa were collected from distinct habitats within Ban Tham Subdistrict: *I. bhumibol* from mountainous areas, *S. bangkokensis* from lowland rice fields, and the two unidentified taxa from streams differing in flow conditions and surrounding topography.

Comparison of the COI sequences with reference databases showed that only two of the four taxa could be identified to the species level, namely *I. bhumibol* and *S. bangkokensis*, with sequence similarities exceeding 98%. These sequence similarities are consistent with those reported for species-level identification by Mohrbeck et al. [29]. However, the remaining two taxa could only be assigned to the family level. Potamidae sp. 1 and Potamidae sp. 2 showed relatively low COI sequence similarities to reference sequences in GenBank, ranging from 87.01% to 91.36% and from 86.50% to 92.10%, respectively. Although these values were insufficient for reliable species-level identification, their closest matches consistently belonged to the family Potamidae. Similarly, Kang et al. [30] assigned specimens with COI sequence similarities below 98% to the family level. This may reflect a limitation of DNA barcoding when closely related or conspecific reference sequences are absent from public databases, as molecular identification depends on the availability and taxonomic coverage of reference sequences [31]. Thus, neither external morphology nor COI DNA barcoding could provide reliable species-level identification of Potamidae sp. 1 and Potamidae sp. 2.

Genetic diversity varied among the four freshwater crab taxa. Potamidae sp. 2 showed the highest genetic diversity, with high haplotype diversity ($H_d = 0.962$), the highest nucleotide diversity ($\pi = 0.01005$), and 15 haplotypes among 21 individuals. Its TCS network also showed a complex pattern with multiple haplotypes separated by several mutational steps. According to Grant and Bowen [32], this combination of high haplotype and nucleotide diversity corresponds to the fourth category and may reflect a large, stable population with a long evolutionary history. The complex haplotype network observed in Potamidae sp. 2 further supports the presence of substantial mitochondrial genetic variation within this taxon. *Indochinamon bhumibol* and Potamidae sp. 1 also exhibited high haplotype diversity ($H_d = 0.893$ and 0.860 , respectively), but lower nucleotide diversity ($\pi = 0.00589$ and 0.00525 , respectively). This pattern is consistent with the second category described by Grant and Bowen [32], in which high haplotype diversity is accompanied by relatively low nucleotide diversity. Such a pattern may reflect population expansion following a period of low effective population size, during which newly accumulated mutations were retained as population size increased. Similar patterns of high haplotype diversity and relatively low nucleotide diversity have been reported in other freshwater crabs, including *Potamon elbursi* [33] and *Potamonautes flavusjo* [34]. In addition, Suthamrit and Thaewnon-Ngiw [8]

reported 10 haplotypes among 14 *I. bhumibol* specimens collected from Loei Province, Thailand, compared with six haplotypes among eight specimens from Phayao Province in the present study. Although the specimens were collected from geographically distinct populations, the relatively high number of haplotypes in both studies suggests considerable haplotype diversity in *I. bhumibol*. In contrast, all 13 *S. bangkokensis* specimens shared a single haplotype with no nucleotide variation, indicating low genetic diversity. This pattern corresponds to the first category of Grant and Bowen [32] and may reflect a recent population bottleneck or founder event. Overall, the contrasting diversity patterns among the four taxa suggest differences in their demographic histories, and further population-level studies are needed to confirm these interpretations.

The clear separation between within- and intertaxon K2P distances supports the genetic differentiation of the four freshwater crab taxa. Low intraspecific *COI* divergence has also been reported in Asian Potamidae. For example, the maximum intraspecific K2P distances of *Geothelphusa lanyu* and *G. lutao* from Taiwan were 0.49% and 0.65%, respectively [35]. Notably, even the lowest divergence between Potamidae sp. 1 and Potamidae sp. 2 was substantially greater than the variation observed within either taxon. The intertaxon K2P divergence between these two taxa (10.73–12.14%) was comparable to interspecific *COI* divergence reported in other Asian Potamidae. For example, interspecific genetic distances among 12 species of *Nanhaipotamon* from China ranged from 2.39% to 15.52% [36]. Likewise, the interspecific K2P distance between *Candidiopotamon rathbuni* and *C. penglai* from Taiwan ranged from 10.72% to 13.65% [37]. Ratnasingham and Hebert [31] also noted that *COI* barcode records showing more than 2% sequence divergence from existing records assigned to the same species represent substantial sequence divergence. Thus, the high genetic divergence observed between Potamidae sp. 1 and Potamidae sp. 2 provides further support for their recognition as distinct genetic lineages.

Hierarchical clustering of the K2P distances further supported the genetic differentiation among the four taxa, with haplotypes clustering according to their respective taxa. This pattern was consistent with the ML phylogenetic analysis, in which the four taxa formed distinct clades. At the family level, Potamidae sp. 1, Potamidae sp. 2, and *I. bhumibol* were grouped within the Potamidae clade together with reference sequences of several potamid species retrieved from GenBank, supporting the assignment of the two unidentified taxa to this family. Moreover, the placement of *I. bhumibol* within Potamidae agrees with previous mitochondrial phylogenetic analyses [38], while its clustering with conspecific GenBank reference sequences further supports its molecular identification. In contrast, *S. bangkokensis* was grouped with *S. bawangensis* and other gecarcinucid reference taxa within the Gecarcinucidae clade, supporting its placement in a separate family from the three potamid taxa. Notably, *R. adani* (Trichodactylidae), used as the outgroup, was distinctly separated from the Potamidae and Gecarcinucidae lineages. This pattern is consistent with the broader phylogenetic framework reported by Tsang et al. [39], in which Trichodactylidae was genetically distinct from the Old World freshwater crab lineages, including Potamidae and Gecarcinucidae. Overall, the phylogenetic results provided additional molecular support for the taxonomic assignments of the four freshwater crab taxa.

The ABGD analysis further supported the genetic separation of the four freshwater crab taxa, with the initial partition recovering four MOTUs corresponding to these taxa. However, the recursive partition recovered 12 groups at low *p* values ($p \leq 0.0013$), suggesting potential over-splitting [28]. The clear barcode gap between intra- and intertaxon distances indicates marked *COI* divergence among these groups. ABGD is a simple and rapid method that uses the barcode gap between intra- and interspecific divergence to partition sequence data into candidate species [28]. Similar *COI*-based species delimitation has successfully distinguished genetically differentiated lineages in the potamid freshwater

crabs [40]. Importantly, the separation of Potamidae sp. 1 and Potamidae sp. 2 into distinct MOTUs in the initial partition, together with their distinct phylogenetic clades, provides further evidence that they represent distinct genetic lineages.

From a conservation perspective, *I. bhumibol* is of particular concern because it is classified as endangered on the International Union for Conservation of Nature (IUCN) Red List (<https://www.iucnredlist.org/> (accessed on 1 August 2026)), with habitat destruction and pollution as threats to the species [17]. The unresolved identities of Potamidae sp. 1 and Potamidae sp. 2 warrant further investigation using additional molecular markers and complete mitogenomes. This is particularly important for Potamidae sp. 1, for which the local community has established a conservation area along an approximately 4-km section of a relatively fast-flowing stream. The area is overseen by the Ban Tham Subdistrict Municipality, with the mayor and municipal staff encouraging local residents to reduce harvesting and to collect only larger individuals, allowing smaller crabs to grow before capture. Accurate species identification is therefore essential for the conservation and sustainable use of freshwater crab resources and supports the objectives of the Plant Genetic Conservation Project under the Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn.

5. Conclusions

This study provides baseline information on the diversity and genetic variation of freshwater crabs in Ban Tham Subdistrict, northern Thailand. Morphological examination identified *I. bhumibol* and *S. bangkokensis* to the species level, whereas COI-based analyses supported four genetically distinct taxa. Potamidae sp. 1 and Potamidae sp. 2 remained taxonomically unresolved. Genetic diversity, K2P distances, phylogenetic analysis, and ABGD consistently supported the genetic differentiation of the four taxa, particularly the separation of the two unidentified potamid crabs. Further integrative taxonomic studies combining detailed morphological comparisons with closely related Thai potamids, particularly G1, carapace, and cheliped characters, additional molecular markers, and complete mitogenomes are needed to clarify the taxonomic identities of Potamidae sp. 1 and Potamidae sp. 2. Given the conservation concern for *I. bhumibol* and the community-based conservation of Potamidae sp. 1, accurate species identification is important for developing appropriate conservation and sustainable management strategies for freshwater crab resources in the region.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/d18100623/s1>, Table S1: molecular identification, GenBank accession numbers, COI haplotypes, sequence lengths, voucher numbers, and sex of four taxa specimens collected from sampling sites S1–S4, based on comparisons with the GenBank and BOLD databases.

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Data Availability Statement: Voucher specimens are deposited at the Division of Technology and Innovation for Fisheries, School of Agriculture and Natural Resources, University of Phayao, Thailand. The COI sequences generated in this study are available in GenBank under accession numbers PZ958406–PZ958474.

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