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GENETIC DIVERSITY OF SCORPIONS IN THIEN DUONG AND TIEN SON CAVES BASE ON MITOCHONDRIAL COI GENE ANALYSIS

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ABSTRACT

The mitochondrial cytochrome c oxidase subunit I (COI) genes of scorpions *Vietbocap* from the Thien Duong and Tien Son caves were sequenced and compared to assess the genetic diversity of populations. Phylogenetic trees and evolutionary maps were constructed to reveal the genetic affinities among the populations. A total of 32 polymorphic sites were identified in the 628-bp sequence of the mtDNA COI gene following sequence correction. The polymorphic loci were more than 10, the haplotype diversity was > 0.5, and the nucleotide diversity (Pi) was > 0.005. A total of five haplotypes were detected based on nucleotide variation among sequences. Scorpions in both Thien Duong and Tien Son caves were divided into three groups with significant genetic differences. The group in Tien Son cave exhibited clear distinctions when compared to the two groups in Thien Duong cave, with genetic distances of 4.33% and 4.64%, respectively. The two groups in Thien Duong cave were less differentiated, with a genetic distance of 0.68%. These results provide important genetic information for taxonomic study and offer a scientific basis for the existence of more than one species of scorpion within the populations at Thien Duong and Tien Son caves.

Keywords: COI gene, evolutionary analysis, genetic diversity, scorpion, *Vietbocap*

INTRODUCTION

Scorpions have been very recently surveyed in karst cave systems in Vietnam, and several specimens of a new pseudochactid scorpion were collected in the Tien Son cave, which is part of the Phong Nha - Ke Bang system in Quang Tri province. These specimens were described as a new genus (*Vietbocap*) and new species (*Vietbocap canhi*), which represents a true troglobitic element (Lourenco and Pham, 2010). Subsequent surveys in the cave systems of Phong Nha - Ke Bang have been carried out, and three more species of pseudochactid scorpions were collected in the Thien Duong

cave: *Vietbocap thienduongensis* (Lourenco and Pham, 2012); *Vietbocap aurantiacus* (Lourenco *et al*., 2018); *Vietbocap quinquemilia* (Lourenco *et al.,* 2018). The genus *Vietbocap* now includes 4 species distributed in Tien Son and Thien Duong caves. However, Prendini *et al.* (2021) reported only one species of the genus *Vietbocap* distributed in the Tien Son and Thien Duong caves. Thus, the taxonomic issues surrounding the genus *Vietbocap* remain unsolved, particularly in terms of the number of species distributed in these caves.

The mitochondrial cytochrome c oxidase subunit I (COI or COX1) gene is a widely utilized genetic marker located within the mitochondrial DNA (mtDNA) of eukaryotic organisms (Hebert *et al*., 2003). It resides in the mitochondrial genome, distinct from nuclear DNA, and encodes the cytochrome c oxidase subunit I, an essential enzyme in the mitochondrial electron transport chain (Funk and Omland, 2003). In most animals, the COI gene spans approximately 1,500 base pairs (bp), with a ~650 bp region frequently employed for DNA barcoding due to its balance of variability and universality (Folmer *et al*., 1994). This region contains highly conserved sequences, enabling the design of universal primers, as well as variable regions that facilitate differentiation among species or populations (Hebert *et al*., 2003). The COI gene is a standard marker for DNA barcoding, a method for species identification using short, standardized gene sequences (Ratnasingham and Hebert, 2007). Its extensive adoption is attributed to its high interspecies variability coupled with low intraspecies variation (Hebert *et al.,* 2004). As mtDNA is typically maternally inherited and lacks recombination, COI is particularly valuable for tracing maternal lineages and elucidating evolutionary relationships (Avise, 2000). COI sequences are instrumental in resolving phylogenetic relationships, identifying cryptic species, and refining taxonomic classifications (Moritz and Cicero, 2004). Furthermore, the gene is widely applied to investigate genetic diversity, population structure, and gene flow within species (Avise, 2004).

In this study, we used COI sequence data from barcode regions to assess the diversity of populations of *Vietbocap* at the Tien Son and Thien Duong caves. The study provides important genetic information for studying taxonomy and serves as a scientific basis for the existence of more than one species here.

MATERIALS AND METHODS

Sampling

Scorpion individuals were collected across the Tien Son (17°34'46.7"N 106°16'42.5"E) and Thien Duong (17°31'10.0"N 106°13'23.7"E) caves using stainless steel forceps and preserved in 96% alcohol before being brought to the laboratory for analysis.

DNA extraction

Total DNA was extracted from leg tissue using a GeneJET Genomic DNA Purification Kit and diluted to 50 ng/µL. The genomic DNA solution was stored in a refrigerator at −20°C until it was used in the analysis.

Primer design, gene amplification, and sequencing

Based on the published full mtDNA sequence of scorpions (NCBI GenBank: NC_010765, AY803353, MF975702, KR190462, NC_012817, OP716846, OP727991, NC_072214, AJ716204,

DQ340065, KR024030, EU523756), universal primers for the amplification of the scorpion COI gene were designed with the help of the DNAStar software (v.5.0). The amplification location is shown in Figure 1. The primer sequences were as follows F (BcL1490): 5'-ATTCDACDAATCATAAG GATATTGG-3', and R (BcH2198): 5′-TAAACTTCAGGATGYCCAAAGAACC A-3′. The primers were synthesized by the PhuSa Genomics Co. (Can Tho, Vietnam). The PCR amplification mix consisted of 1 µL of DNA template (50 ng of DNA), 0.75 µL of each of the upstream and downstream primers (10 pmol/µL), 12.5 µL of 2× PCR Mastermix, and 10 µL of ultra-pure water. The PCR amplification parameters were set as follows: 94°C for pre-denaturation for 5 minutes, followed by 35 cycles of 94°C denaturation for 60 seconds, 50°C annealing for 60 seconds, and 72°C extension for 60 seconds. The PCR products were subjected to 0.8% agarose gel electrophoresis. The size of PCR products was observed by the Gel Documentation System, Germany. The products were subsequently sent to the Macrogen Co. (Seoul, Korea) for direct sequencing using the forward primer BcL1490. Multidimensional analyses of the data were performed to ensure the comprehensiveness and accuracy of the results.

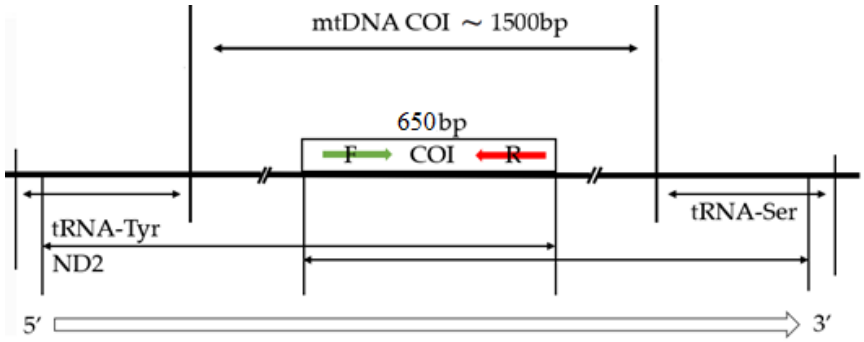

Figure 1. Map of design of primers for PCR of COI gene region on the mitochondrial genome.

Statistical analysis

The COI gene sequences were clipped and corrected using DNA Baser Assembler 5.21.0 (www.DnaBaser.com). The parameters polymorphic sites, parsimony informative sites, haplotype number, haplotype diversity, and nucleotide diversity were plotted using Arlequin V3.5.2.2 (Excoffier *et al*., 2010). Genetic distances and the maximum-likelihood tree were calculated through 1000 resamplings of bootstrap using the MEGA 11 software under the Kimura 2- parameter evolutionary model (Tamura *et al*., 2021). Haplotype network relational maps were constructed using "Popart1.7" software (Bandelt *et al*., 1999).

RESULTS

DNA extraction, amplification and sequencing

DNA was successfully extracted from a total of 21 scorpion individuals. Although we did not electrophoretically test total DNA, the successful PCR results showed that genomic DNA was well extracted. PCR products observed a specific band when

electrophoresed on 0.8% agarose gel (Figure 2). The PCR products confirmed with the DNA ladder, which was consistent with the expected target fragment size (from 600-700 bp). The band in well 7 exhibited a slightly lower size compared to those in other wells, likely attributable to an elevated concentration of PCR product. The bands were clear and well-defined, indicating that the samples were not degraded and showed good specificity. Moreover, no additional bright bands were observed in the sample lanes, suggesting that the samples were free from contamination. The PCR products were sequenced to obtain high-quality fluorescence histograms and clear peaks (data not show here). After eliminating ambiguous nucleotide positions at both ends, a 628-bp fragment was obtained for all 21 individuals.

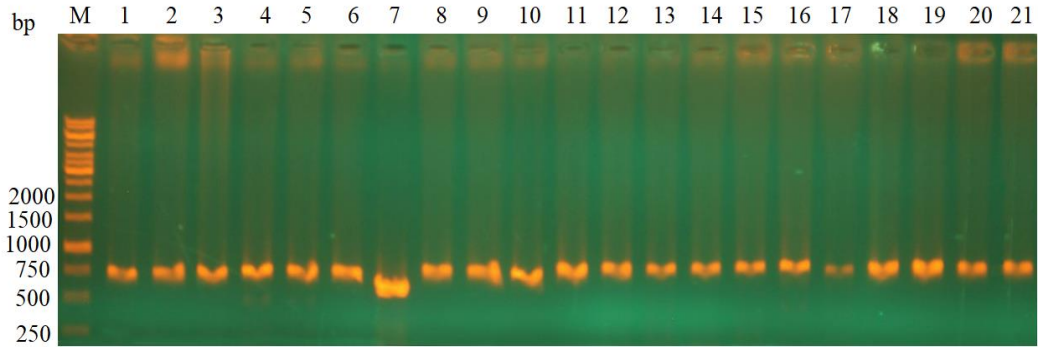

Figure 2. Gel electrophoresis on a 0.8% agarose gel of PCR products for the amplification of the partial COI gene. Lane M: DNA ladder (250–10,000 bp), lanes 1-21: PCR sample products (in order from voucher number small to large, Sc001 to Sc028). Electrophoresis was carried out at 150 V for 28 min. Gels were stained with RedGel-safe stain and then DNA was visualized by an ultra-violet (UV) transilluminator using BioDocAnalyze (Biometra, Germany).

Sequence variation

The COI dataset, comprising 21 sequences from scorpion individuals representing *Vietbocap* populations at Tien Son and Thien Duong caves, was included in the analysis. The final alignment consisted of 628 sites, with 596 conserved sites and 32 variable sites, 30 of which were parsimony-informative. The transition-transversion bias (R) was estimated at 2.14. Nucleotide frequencies were A = 31.23%, T = 24.47%, C = 24.27%, and G = 20.03%. The final length of the analyzed sequences (628 bp) revealed the existence of 32 polymorphic sites, which accounted for approximately 5.1% of the total number of sequence sites (32/628). A total of five haplotypes of the COI gene sequences were identified among the 21 scorpion specimens analyzed. The haplotype diversity was low, with Hd = 0.748, and the variance and standard deviation of haplotype diversity were 0.00268 and 0.052, respectively. Nucleotide diversity was greater than 0.005 (Pi = 0.02119). Variable positions, including 46, 64, 142, 151, 154, 172, 175, 226, 244, 268, 271, 343, 358, 379, 382, 394, 448, 457, 464, 466, 472, 487, 520, 526, 547, 586, 589, 595, 601, and 619 (Figure 3).

	Individual	Group	Location	46	64	111	142	151	154	172	175	226	244	268	271	343	358	379	382
Hap1	Sc007,008,012,014,017,023,024,025	1	Thien Duong	C	A	T	T	A	T	G	C	T	G	A	A	A	C	G	G
Hap2	Sc009	1	Thien Duong	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap3	Sc011,016,026,027,028	2	Thien Duong	T	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
Hap4	Sc015	2	Thien Duong	T	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
Hap5	Sc001,002,004,020,021,022	3	Tien Son	T	G	.	C	G	C	A	T	C	A	G	C	G	T	A	A

				394	448	457	464	466	472	485	487	520	526	547	586	589	595	601	619
Hap1	Sc007,008,012,014,017,023,024,025	1	Thien Duong	T	A	C	A	C	A	G	G	A	C	G	T	A	A	G	G
Hap2	Sc009	1	Thien Duong	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap3	Sc011,016,026,027,028	2	Thien Duong	.	.	.	.	.	G	.	.	.	T	.	.	.	.	.	.
Hap4	Sc015	2	Thien Duong	.	.	.	.	.	G	A	.	.	T	.	.	.	.	.	.
Hap5	Sc001,002,004,020,021,022	3	Tien Son	C	G	T	G	T	G	.	A	G	.	A	C	G	T	A	A

Figure 3. Distribution of variable loci based on haplotypes of the mtDNA COI gene. The dot symbol indicates base-identical sequences.

Molecular phylogeny

The phylogenetic analysis was conducted using all substitutions in COI. The maximum-parsimony (MP) tree of 21 scorpion individuals split into three groups with high bootstrap support values of 97, 97, and 100, respectively (Fig. 4). Group 1 included the majority of individuals collected near the Thien Duong cave entrance. Group 2 included individuals collected deeper inside the Thien Duong cave (where there was no light). Group 3 included individuals collected at Tien Son cave. Here, we considered these groups to be distinct populations, and a subsequent analysis was performed to determine the genetic differences and affinities between them in order to provide important data for the taxonomic diversity study.

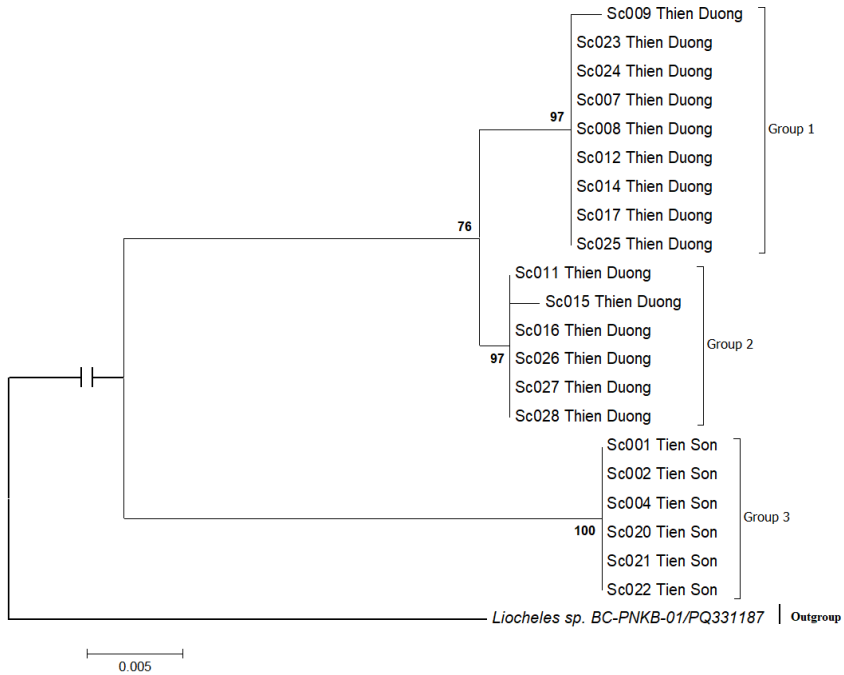

Figure 4. Phylogeny of scorpions in Thien Duong and Tien Son Caves.

Genetic distances

The uncorrected *p*-distances based on the COI gene sequences of the studied individuals are presented in Table 1. Genetic uncorrected *p*-distances between individuals ranged from 0% (between Sc007 and Sc008) to 4.78% (e.g., between Sc009 and Sc001). The variation in Group 3 (Tien Son) was zero, while this one in Groups 1 and 2 (Thien Duong) was 0.0053% and 0.0035%, respectively (values not shown in the table). The uncorrected genetic *p*-distances between Group 1 and Group 2 were determined to be 0.68%, while that between Group 2 and Group 3 was 4.33%, and between Group 1 and Group 3 was 4.64%. Two groups from Thien Duong cave were clearly distinct from this in Tien Son, but they differ less from each other (*p*-distance = 0.68%). The intragroup uncorrected p-distances were 0.0% for Group 3 from Tien Son Cave and 0.1% for Groups 1 and 2 from Thien Duong Cave.

Genetic variation among populations

Analysis of Molecular Variance (AMOVA) revealed significant genetic differentiation among the three scorpion populations, as indicated by Fst values ($P < 0.001$). The results demonstrated that 99.01% of the total genetic variation was attributed to differences among populations, with only 0.99% occurring within populations. The Fst statistic was statistically significant ($P < 0.05$), confirming substantial population structure (Table 2). Pairwise Fst values were calculated to assess genetic distinctiveness between populations, with all comparisons showing significant differentiation ($P < 0.05$). Notably, the genetic divergence between the scorpion population from Tien Son Cave and those from Thien Duong Cave was particularly pronounced, as evidenced by higher pairwise Fst values, visually represented by darker blue shading in the heatmap (Figure 5).

Table 1. Uncorrected *p*-distances (percentages) between individuals and groups based on mitochondrial COI sequences.

No	Specimen ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	Sc007 Thien_Duong (Group 1)																				
2	Sc008 Thien_Duong (Group 1)	0.00																			
3	Sc009 Thien_Duong (Group 1)	0.16	0.16																		
4	Sc012 Thien_Duong (Group 1)	0.00	0.00	0.16																	
5	Sc014 Thien_Duong (Group 1)	0.00	0.00	0.16	0.00																
6	Sc017 Thien_Duong (Group 1)	0.00	0.00	0.16	0.00	0.00															
7	Sc025 Thien_Duong (Group 1)	0.00	0.00	0.16	0.00	0.00	0.00														
8	Sc024 Thien_Duong (Group 1)	0.00	0.00	0.16	0.00	0.00	0.00	0.00													
9	Sc023 Thien_Duong (Group 1)	0.00	0.00	0.16	0.00	0.00	0.00	0.00	0.00												
10	Sc011 Thien_Duong (Group 2)	0.64	0.64	0.80	0.64	0.64	0.64	0.64	0.64	0.64											

11	Sc015 Thien_Duong (Group 2)	0.80	0.80	0.96	0.80	0.80	0.80	0.80	0.80	0.80	0.16											
12	Sc016 Thien_Duong (Group 2)	0.64	0.64	0.80	0.64	0.64	0.64	0.64	0.64	0.64	0.00	0.16										
13	Sc026 Thien_Duong (Group 2)	0.64	0.64	0.80	0.64	0.64	0.64	0.64	0.64	0.64	0.00	0.16	0.00									
14	Sc027 Thien_Duong (Group 2)	0.64	0.64	0.80	0.64	0.64	0.64	0.64	0.64	0.64	0.00	0.16	0.00	0.00								
15	Sc028 Thien_Duong (Group 2)	0.64	0.64	0.80	0.64	0.64	0.64	0.64	0.64	0.64	0.00	0.16	0.00	0.00	0.00							
16	Sc001 Tien_Son (Group 3)	4.62	4.62	4.78	4.62	4.62	4.62	4.62	4.62	4.62	4.30	4.46	4.30	4.30	4.30	4.30						
17	Sc002 Tien_Son (Group 3)	4.62	4.62	4.78	4.62	4.62	4.62	4.62	4.62	4.62	4.30	4.46	4.30	4.30	4.30	4.30	0.00					
18	Sc004 Tien_Son (Group 3)	4.62	4.62	4.78	4.62	4.62	4.62	4.62	4.62	4.62	4.30	4.46	4.30	4.30	4.30	4.30	0.00	0.00				
19	Sc020 Tien_Son (Group 3)	4.62	4.62	4.78	4.62	4.62	4.62	4.62	4.62	4.62	4.30	4.46	4.30	4.30	4.30	4.30	0.00	0.00	0.00			
20	Sc021 Tien_Son (Group 3)	4.62	4.62	4.78	4.62	4.62	4.62	4.62	4.62	4.62	4.30	4.46	4.30	4.30	4.30	4.30	0.00	0.00	0.00	0.00		
21	Sc022 Tien_Son (Group 3)	4.62	4.62	4.78	4.62	4.62	4.62	4.62	4.62	4.62	4.30	4.46	4.30	4.30	4.30	4.30	0.00	0.00	0.00	0.00	0.00	

Table 2. AMOVA of the three groups (populations).

Source of variation	Sum of squares	Variance components	Percentage variation	P-value
Among populations	131.325	9.56186	99.00928	< 0.0001
Within populations	1.722	0.09568	0.99072	
Total	133.048	9.65754		

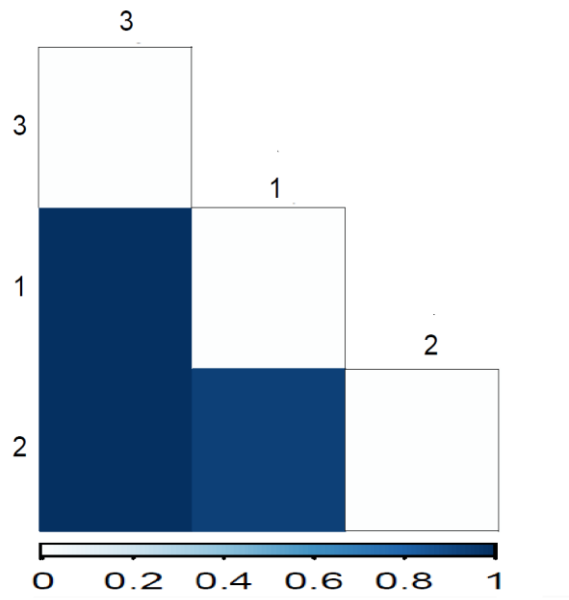

Figure 5. The graphic pairwise Fst. The left column lists the populations (numbers 1, 2, and 3 correspond to Group 1, Group 2, and Group 3). The empty square represents a population being compared to itself. The colors indicate the strength of the Fst value for each pairwise comparison. Darker blue shades indicate stronger genetic distinctiveness between populations, while the Fts values near zero suggest that two populations are not genetically distinct.

Haplotype distribution

The results of the study show that the haplotypes of scorpions from Thien Duong and Tien Son caves had a centralized star-like distribution (Figure 6). In total, five haplotypes were detected based on nucleotide variation among the sequences. The highest number of haplotypes was found in the Thien Duong populations with four haplotypes, while the Tien Son population contained only 1 haplotype. A median-joining network diagram was constructed based on COI gene haplotypes, showing that the haplotype in Tien Son (red) is completely separate from the haplotype in Thien Duong (bright green).

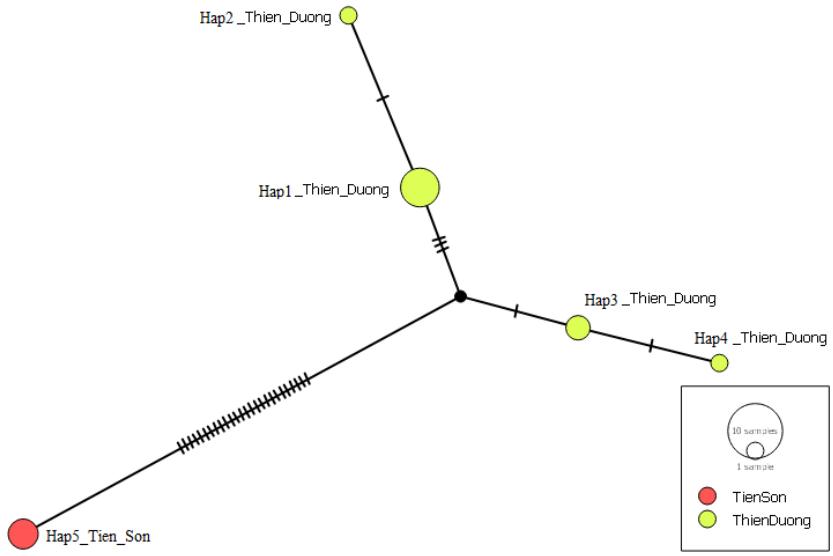

Figure 6. A median-joining network diagram was constructed based on COI gene haplotypes. Each circle represents a unique haplotype, the colour indicating the corresponding group, and the size of the circle being proportional to the number of isolates it contains. The lines (shaded markers) on the branches represent the location of mutations, with one line for each mutation.

DISCUSSION

The analysis of the genetic diversity of scorpions in Thien Duong and Tien Son caves is an important step in the conservation and management of this biological resource. In the present study, we analyzed the genetic diversity of populations of *Vietbocap* from two different geographical locations, using mitochondrial COI gene sequences. By sequencing and analyzing the mitochondrial COI gene sequence, it was found that there are three scorpion groups in Thien Duong and Tien Son caves, with significant genetic differences among them. In a previous comparison, Prendini *et al.* (2021) also utilizing the mitochondrial COI gene, did not perform AMOVA analyses. AMOVA is a statistical method used to analyze genetic distribution in different populations as well as genetic differentiation between different groups or populations based on genetic variation data. In this study, we provided evidence for the potential existence of three scorpion populations of the *Vietbocap* in the Thien Duong and Tien Son caves. However, the lack of morphological data provides no evidence to determine the taxonomic rank of the populations. Further morphological studies are needed to confirm and determine whether these populations are distinct species or subspecies.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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