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Microsatellite Loci Reveal Heterozygosis and Population Structure in the Critically Endangered Southern River Terrapin (*Batagur affinis* ssp.) of Peninsular Malaysia †

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Abstract: These freshwater turtles are found across Indochina, mostly in large rivers. There is a lack of genetic research concentrating on Malaysia's southern river terrapin (*Batagur affinis*) population. We used minimally intrusive methods to collect blood samples from a total of 80 individuals in four different sites in peninsular Malaysia. The genetic difference within and between locations was examined using five microsatellite loci. Our findings indicated that each locus was polymorphic. High numbers of heterozygotes were observed when the percentage of alleles in each locus was compared. Pairwise F_{ST} and Nei matrixes revealed considerable genetic differences across individuals from distinct geographical locations. Our population structure analysis shows a significant proportion of assigned individuals are linked to certain collection locations.

Keywords: turtles; Indochina; genetic; blood sample; polymorphic; population structure

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1. Introduction

Batagur affinis is a freshwater turtle species that once frequented large rivers in Vietnam, Cambodia, Thailand, peninsular Malaysia, Singapore, and Sumatra [1]. Currently, the IUCN Red List 2000 classifies it as critically endangered [2]. *B. affinis* [3] was divided into two subspecies based on minor morphological differences, colouration, nesting ecology, and three mitochondrial (mtDNA) and three nuclear DNA markers: *B. affinis affinis*, the western nominate population, and *B. affinis edwardmolli*, the eastern population [1,4]. The appoint subspecies *B. affinis affinis* is found solely on the Malaysian Peninsula's western coast; it is undoubtedly extinct in Sumatra [5]. On the other hand, the eastern coast of peninsular Malaysia subspecies *B. a. edwardmolli*, which previously extended from Singapore to Indochina, is now believed to be extinct in Singapore, Thailand, and Vietnam [1]. Overall, *B. a. edwardmolli* survives in populations along the Malaysian peninsula's east coast and a relict population exists in Cambodia [1], making the Malaysian population the only genetic pool persisting in all of Indochina.

A microsatellite is one of the most frequently used markers. A microsatellite is a DNA sequence consisting of two to six tandem repetitions. Due to its codominant nature, polymorphism, ability to be inherited according to Mendelian's rule, and ability to identify variations between closely related species, the microsatellite is an excellent tool for investigating population patterns and pedigree studies [6]. As a result, microsatellites are one of the most frequently used genetic markers by scientists in a wide variety of biological studies.

We investigated the genetics of the Southern river terrapin population. Then, we examined the genetic diversity, genetic structure, genetic divergence, and distributional range of several groups in peninsular Malaysia. Our purpose was to demonstrate that Southern river terrapin populations' genetic diversity and structure are related to the collection location and restricted migrations, with little effect at broader scales due to the Southern river terrapin's lack of broad migratory movements. Following that, we analysed and assessed the genetic diversity of Southern river terrapin populations. Finally, we hypothesised a genetic population structure characterised by a large proportion of individuals allocated to certain places. We demonstrate that human disturbance affects the Southern river terrapin's genetic diversity by presenting our findings in various quantitative methods.

2. Materials and Methods

2.1. Populations Description

This study comprised 80 *Batagur affinis* ssp. individuals from four population regions crossing the East and West Malaysia peninsula (Figure 1): Pasir Gajah, Kemaman (KE), Terengganu (4.2524° N, 103.2957° E); Bukit Pinang, Kepala Batas (BP), Kedah (4.2221° N, 100.4370° E); Bota Kanan, Bota (BK), Perak (4.3489° N, 100.8802° E); and Bukit Paloh, Kuala Berang (KB), Terengganu (5.0939° N, 102.7821° E). A total of 20 individuals from the *B. affinis* ssp. population were sampled at each location in 2020. Blood was drawn using two venepuncture techniques: the subcarapacial venous plexus (SVP) and the jugular vein.

2.2. DNA Extraction and Microsatellite Analysis

Nucleic acids were isolated from each EDTA whole blood sample volume of 200 µL. Following cell lysis and protein denaturation, extractions were carried out utilising an automated system, the ReliaPrep™ Blood gDNA Miniprep System (Promega, Madison, WI, USA), with Binding Column technology following the manufacturer's protocol. The quality and concentration of extracted DNA were estimated using the Thermo Scientific™ NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) model ND −2000 and visualised in 1% agarose with molecular markers. The DNA was stored at −20 °C until further use.

Five pairs of microsatellite loci established by [7] were used for microsatellite amplification. Go Taq Flexi PCR (Promega, Madison, WI, USA) comprises 1.6 µL of $MgCl_2$, 0.2 µL of Taq polymerase, 0.4 µL of dNTPs, 4 µL of buffer, 11 µL of ddH_2O, 2 µL of DNA, and 0.4 µL of forward and reverse microsatellite primers. Thermal cycling parameters for PCRs using microsatellite markers of *B. trivittata* included an initial denaturation at 95 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 1 min, primer annealing at Ta °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. The full amplification procedure retained a constant temperature of 10 °C. To check for contamination during the experiment, all amplifications were carried out using negative controls. The microsatellites' PCR products were tested on a 2% high-resolution agarose gel. To create the gel, 4 g of MetaPhor™ Agarose powder (Lonza, Rockland, ME, USA) were combined with 60 mL of 1× TBE buffer (Promega, Madison, WI, USA). A 50 bp DNA ladder (Promega, Madison, WI, USA) was also employed as a standard DNA size marker. Numerous DNA polymorphisms were revealed in the PCR results with numerous fluorescence bands. Microsatellite screening was then executed on the samples to determine the anticipated size of PCR products. At this step, colourless MyTaq Red Mix and suitable primers with appropriate fluorescent colour markers (FAM) were used for PCR amplification. In addition, for agarose gel electrophoresis, loading dye was added to the PCR products.

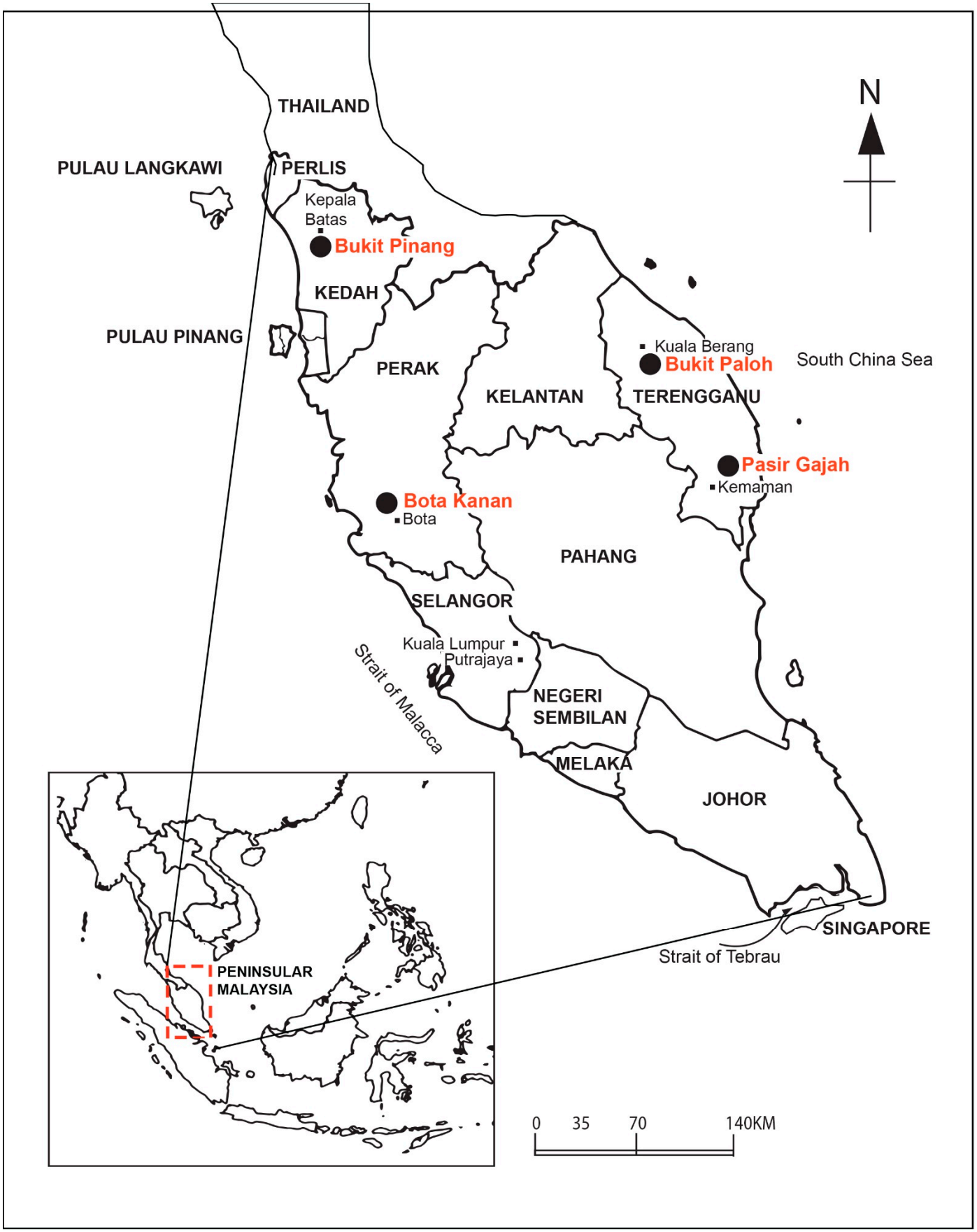

Figure 1. Sampling locations of *Batagur affinis* in four areas of peninsular Malaysia.

All PCR products were wrapped in aluminium foil and sent through the Applied Biosystems Genetic Analyzer to analyse fragments. Using GeneMapper version 5.0 [8], fragment sizes were interpreted in accordance with the 500-ROX DNA size standard. The fragment analysis findings were then used in statistical analyses of microsatellites.

2.3. Statistical Analysis

The genotypic data were interpreted into the appropriate forms for microsatellite analysis using the CONVERT 1.31 programme [9]. The GENEPOP and ARLEQUIN formats were among them. The allelic frequencies of the microsatellite loci observed in the *Batagur affinis* ssp. populations were also calculated using the CONVERT programme.

2.3.1. Genetic Diversity

The programme MicroChecker 2.2.3 [10] was used to check for genotyping errors, notably those caused by null alleles and allele dropouts, which were discovered throughout the study. According to the results of this study, the allelic richness (R_s), genetic diversity (H_s), observed heterozygosity (H_o), and expected heterozygosity (H_e) were estimated using FSTAT version 2.9.3.2 [11].

2.3.2. Population Structure

Furthermore, ARLEQUIN version 3.0 [12] was used to evaluate the significance of spatial variation in genetic diversity of *B. affinis* ssp. populations applied in AMOVA. Analyses of molecular variance (AMOVA) [13] were then performed to test the genetic relationships between the different groups. Spatial analysis of molecular variance (SAMOVA) was carried out with a microsatellite marker using SAMOVA 2.0 [14] to recognise groups of populations that are phylogeographically homogeneous and maximally differentiated from each other, taking into account the geographic distances. This analysis permits the identification of the maximally differentiated groups that parallel predefined genetic barriers by optimising the proportion of total genetic differences due to dissimilarities between groups [15]. Two measures must be considered to select the optimal number of groups (K). First, F_{CT} values would reach a maximum or a plateau. Second, the structures with one or more single-population groups should be left out, as this indicates that the group structure is disappearing [16]. We implemented analyses for K = 2 to 3 to identify the most likely number of groups, with the four populations genotyped with microsatellites.

The other fixation indices were also measured using the ARLEQUIN software to examine the genetic differentiation among *B. affinis* ssp. populations [17]. Finally, assignment tests in GenAlEx 6.5 [18] were conducted by estimating the probability of individuals from each population and pairwise net matrix.

3. Results and Discussion

We identified 133 alleles at five nuclear microsatellite loci. For all locations, all loci were polymorphic (range: 21–37 alleles per locus). Marine vertebrates are thought to have more significant allele variations at their microsatellite primers than freshwater animals, which is mainly compatible with their larger population evolutionary size [19]. Batr25, Batr30, and Batr36 were identified as null alleles or linkage disequilibrium. Additionally, null alleles resulted in an excess of homozygotes in the groups examined [20,21].

Compared to [22], *B. Baska* research in Bangladesh does present any null allele. This, in turn, affects the F_{IS} inquiry. According to [23], null alleles at microsatellite loci are often observed. As a result, using more heterozygous loci allows a more precise resolution of *B. affinis* ssp. population structure. Finally, overexploitation of *B. affinis* ssp. across Malaysia's rivers and habitat changes such as sand mining has reduced the size of this species' proper breeding population [24].

Additionally, for all populations, the observed heterozygosity (H_o) was less than the expected heterozygosity (H_e) (Table 1). Except for Batr10 and Batr25, the number of heterozygotes was higher than the number of homozygotes at practically all loci (Figure 2).

On the other hand, various judgements revealed an ample difference between homozygote/heterozygote associations. Kuala Berang (KB) had the highest allelic richness (R_s) (13.6) and genetic diversity (H_s) (0.88), whereas Kemaman (KE) had the lowest. Bukit Pinang (BP) had the lowest R_s (9) and H_s (0.81), whereas Bota Kanan (BK) had the highest. Therefore, *B. affinis edwardmolli* has a higher level of genetic diversity than *B. affinis affinis*.

Table 1. Descriptive statistics for each population including a range of alleles, expected heterozygosity (H_e), and observed heterozygosity (H_0), allele richness (R_s), gene diversity (H_s), sample size (N).

Locality	Statistical						
	H_o	H_e	Allele Range	p Value	R_s	H_s	N
KE	0.66	0.85	7–17	0.02	11.4	0.86	20
KB	0.49	0.87	9–16	0	13.6	0.88	20
BP	0.51	0.8	8–11	0	9	0.81	20
BK	0.37	0.81	7–13	0.01	10	0.81	20

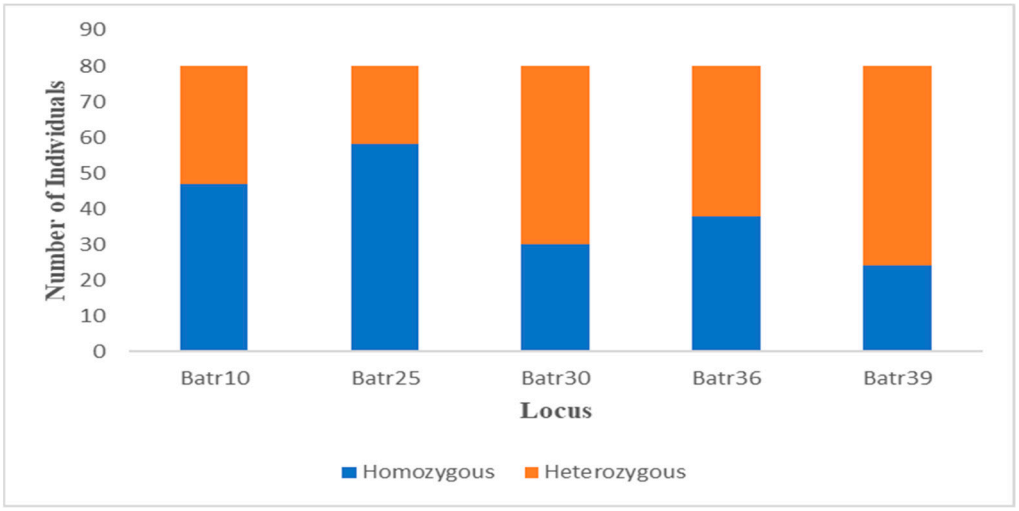

Figure 2. Frequencies of homozygous/heterozygous individual alleles represented for the five loci.

Thus, total R_s indicated that the examined populations used cross-amplified primers at a greater rate than the *B. baska* population [22]. The sample size may affect the allelic richness and H_s. The authors in [25] established the beneficial impacts of sampling populations of 25–30 individuals. They did, however, emphasise the importance of collecting 5–100 samples per collection in order to avoid uncommon non-informative alleles.

The F_{ST} and Nei comparisons (Table 2) were both significant, with the greatest genetic difference between BP and BK. (F_{ST} = 0.122; Nei = 1.011) and the lowest genetic divergence between KB and BK. (F_{ST} = 0.064; Nei = 0.574). Nei's pairwise estimations of genetic distances [26], the pairwise F_{ST} values, which indicate a high degree of interaction across the populations investigated [27,28], are similar to those found in *Emys orbicularis* populations, with F_{ST} values ranging from 0.02 to 0.30. [29] Assignment tests confirmed this, revealing a high proportion of properly assigned individuals, indicating significant genetic differences across groups.

Table 2. Pairwise genetic distance based on the F_{ST} matrix (below diagonal) and Nei Matrix (upper diagonal), a measure of divergence among the *B. affinis* ssp. populations. * $p < 0.05$.

Region	Kemaman	Kuala Berang	Bukit Pinang	Bota Kanan
Kemaman	-	0.962	0.894	0.823
Kuala Berang	0.080 *	-	0.749	0.574
Bukit Pinang	0.096 *	0.083 *	-	1.011
Bota Kanan	0.088 *	0.064 *	0.122 *	-

To determine population genetic structure, a hierarchical AMOVA was used. Between the identified populations, 8% of the variance was observed. Individuals explained 55.32% of the population variance (Table 3). Similarly, differences in AMOVA of 7% were seen in the *Emys orbicularis* population [29]. This situation may be impacted by the high rate of gene flow across populations [28]. Fixation indices revealed a high degree of genetic structuring across populations ($F_{ST} = 0.07$; $p < 0.05$) and a moderate degree of inbreeding within them ($F_{IS} = 0.39$ and $F_{IT} = 0.44$; $p > 0.05$). In light of the SAMOVA findings, the geographical distribution of populations revealed three groups. These three categories were as follows: group I (KB and BK), group II (KE), and group III (BP). SAMOVA distribution values were consistent with those obtained for AMOVA, with individuals within populations bringing the greatest value (55.03%), followed by disparities across populations (5.34%), Table 3. The Bayesian cluster technique may determine the population structure [30]. The results of the three clusters of *B. affinis* ssp. populations with individuals collected in the east and west of peninsular Malaysia in the SAMOVA were unexpected.

Table 3. Summary of AMOVA and SAMOVA statistics for alternative groupings of populations.

Source of Variation	*df*	Sum of Squares	Variance Components	Percentage of Variance	Fixation Indices
AMOVA.					
Among populations within groups	3	30.869	0.18346	8.00	$F_{IS} = 0.39871$
Among individuals within populations	76	224.3	0.84128	36.68	$F_{ST} = 0.07999$
Among individuals within individuals	80	101.5	1.26875	55.32	$F_{IT} = 0.44680$
SAMOVA, 3 groups					
Among groups	2	22.994	0.07244	3.14	$F_{IS} = 0.39871$
Among populations within groups	1	7.875	0.12309	5.34	$F_{SC} = 0.05512$
Among individuals within populations	76	224.3	0.84128	36.49	$F_{CT} = 0.03142$
Among individuals within individuals	80	101.5	1.26875	55.03	$F_{IT} = 0.44970$

On the other hand, we observed large excesses of heterozygotes in populations of *B. affinis* ssp. in Malaysia, as determined by a single statistical comparison. High levels of heterozygosis have been documented insufficiently in mammals and reptiles such as baboons [31], domestic sheep [32], vampire bats [33], and snapping turtles [34].

Excess heterozygosity has been attributed to various factors [33,35]: (1) It could be the result of small reproductive populations with only a few capable breeders. (2) Outbreeding may occur due to the most heterozygous individuals being subjected to selection factors. (3) it could be the outcome of asexual reproduction, or (4) It could result from random mating behaviour in dense populations. We terminated hypothesis three due to the species' natural biology. Due to the species' existing conservation status, small reproductive populations of *B. affinis* ssp. are not conceivable [36]. Therefore, the most logical explanation for the high heterozygosity would be dense populations of the Southern river terrapins,

with little assortative mating behaviour under the influence of natural selection pressures. However, [37] noted the positive association between inbreeding and heterozygosity. It should be investigated further by using other polymorphic markers, which exhibit a higher percentage of linkage disequilibrium.

4. Conclusions

Overall, genetic diversity in *B. affinis* ssp. was higher than reported by [22] for other *Batagur* species. Furthermore, levels of genetic divergence and population differentiation among our sampling sites may change over time, primarily due to habitat changes caused by human activities. Nevertheless, our results showed consistency with expected genetic diversity and population differentiation for a species affected by human activities. A novelty is the first study on two subspecies of *B. affinis*. Four populations spanning the east, and west coasts of peninsular Malaysia were disclosed using microsatellites. The study showed that *B. affinis edwardmolli* had more genetic diversity than *B. affinis affinis*, making this research beneficial beyond Malaysia to the Indochina region. This will serve as a key source for future genetic association and functional analysis to enhance breeding programmes for long-term sustainability.

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Data Availability Statement: Not applicable.

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