

Morphological characteristics and mitochondrial COI gene of the green water dragon in Phu Quoc island, Vietnam

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Abstract:

The green water dragons of Phu Quoc island, Vietnam have been given a VU rating (vulnerable) by the Vietnam red data book and the International Union for conservation of nature and natural resources. A description of the morphological characteristics and sequenced mitochondrial cytochrome oxidase subunit 1 COI gene was completed following a survey of thirty individuals from September 2018 to May 2019. Their body weights ranged from 115 to 850 g and their snout-vent length ranged from 120 to 280 mm. They were identified to have the highest similarity to the species *Physignathus cocincinus* by the barcode of life data system. Their COI gene sequence was compared to GenBank (KM272197.1) and the following alternations were found: C5483T, A5486C, C5537A, A5564G, A5591G, T5633C, G5684A, A5771G, A5849C, C5927T, T5942C, C5993T, G6002A, and A6020G. The neighbourjoining tree of their COI sequence divergence indicated a distance (compared to the same published species) from 2.3 to 2.8%. Thus, it might be possible that the green water dragons of Phu Quoc island are evolving into a specific subspecies. However, more green water dragon specimens from Phu Quoc should be sequenced to ensure a conclusion and further research into farming should be initiated to protect this vulnerable species.

Keywords: COI, gene sequence, morphology, Phu Quoc, *Physignathus cocincinus*.

Classification number: 3.4

Introduction

Phu Quoc island, Vietnam, boasts idyllic beaches and evergreen forests. The northern part of the island is relatively untouched due to a National park, which was assessed to have high biodiversity and conservation value. In this park, green water dragons need conservation due to their entry as a vulnerable species in the Vietnam red data book [1] and International Union for Conservation of nature and natural resources [2]. Green water dragons were firstly described by Cuvier (1829) [3]. They belong to the species *Physignathus cocincinus*, genus *Physignathus*, subfamily Amphibolurinae, family Agamidae, suborder Iguania, order Squamata, class Reptilia, phylum Chordata. Their distribution areas are in China, Thailand, Laos, Cambodia, and Vietnam [2]. In Vietnam, they have a wide geographic range that reaches

the mountainous areas of the North, Central, Central Highlands, Eastern South, and Phu Quoc island [4]. They are large in size, have good meat, and an attractive appearance; so much so that they are often hunted for food, medicine, pets, and export [5]. Currently, the green water dragons of central Vietnam have been studied to clarify their morphological characteristics, ecology, behaviour, and diets [6] for breeding and expanding successfully [4]. However, these studies are limited in both their genetic information and sampling of wild populations in different geographies.

The genetic information contained in deoxyribonucleic acid (DNA) sequences of the genome play a decisive role in forming the biological characteristics of each species. The mitochondrial genome evolves about 17 times faster than the nucleus genome, so studies on genetic information

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often originate from the mitochondrial genome [7]. In the mitochondrial genome rests, the mitochondrial cytochrome c oxidase gene subunit 1 (COX-1, COXI or COI) with a size of 1547 bp for *Physignathus cocincinus* (GenBank #KM272197.1). The COI gene is responsible for translating the protein of cytochrome oxidase, which is a component of the respiratory chain that catalyses the reduction of oxygen to water in cells [8, 9]. Alternation of the mitochondrial COI gene would impact cellular respiration, a cell's energy release, and body function. Therefore, the mitochondrial COI gene is often used for taxonomic and phylogenetic analyses [10]. This study aims to provide scientific data on the morphological characteristics and DNA sequence of the mitochondrial COI gene of the green water dragons distributed in Phu Quoc island, Vietnam, for further research on their conservation and breeding.

Materials and methods

Sample collection

The sampling was carried out from September 2018 to May 2019. The green water dragons were collected in National park of Phu Quoc (10°19'30"N, 103°57'0"E), Phu Quoc city, Kien Giang province, Vietnam. There were thirty individuals collected over three collection times (~10 lizards/time) and all samples were licensed by the Forest protection department of Kien Giang province. After coordinating with the staff of the Forest protection the streams (Fig. 1) were surveyed and, when discovering a lizard, a noose was used to catch it and then a code was assigned to manage each individual.

After collecting the samples, photographs of the individuals were recorded, their morphologies measured, and 10% of the lizards were selected randomly to sample tail tissue for DNA isolation [11]. Then, the lizards were handed over to the National park of Phu Quoc. Morphological measurements were conducted according to Schulte II, et al. (2004) [12] including body weight, body length, snout-vent length, tail length, armpit-groin length, head length, head width, head height, front limb length, hindlimb length, snout-eye length, and eye diameter.

Mitochondrial COI gene sequencing

DNA extraction: the method of DNA isolation was conducted according to Werman, et al. (1996) [13]. Briefly, 50-100 mg of sample was crushed in 300 µl TE, 50 µl SDS 10%, 5 µl proteinase K, and warmed to 65°C for 20 min. Then, 400 µl CTAB 10% in NaCl 0.7 M was added and warmed to 65°C for 20 min. Continuously, 200 µl of chloroform:isoamyl (24:1) was added and centrifuged at 13,000 rpm for 5 min to collect 300 µl supernatant. Next, the supernatant was dissolved in 300 µl Ethanol 95% and centrifuged at 13,000 rpm for 5 min. Then, the supernatant was removed again and washed twice with 500 µl ethanol 70%. Finally, the isolated DNA was dissolved in 100 µl TE for PCR tests or stored at -20°C until analysis. The DNA quality was estimated using the absorbance values at 260 nm by a UV-Vis1800 spectrometer of Shimadzu, Japan.

DNA sequencing: primers were used to amplify mitochondrial DNA designed with the Primer-BLAST program [14], which is based on the

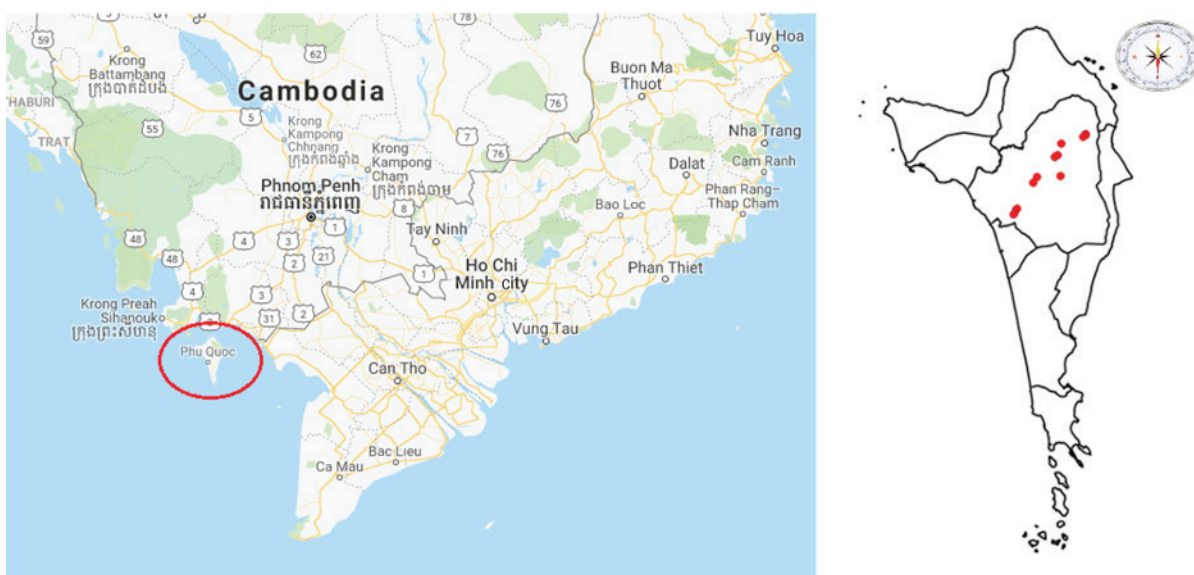


Fig. 1. The collection locations (red dots) for the 30 green water dragons in Phu Quoc island.

DNA sequence of the mitochondrial COI gene of *Physignathus cocincinus* in GenBank (KM272197.1) from base 5169 to 6715. The forward primer was 5'CTAGGTGCCCCAGACATAGC3', and the reverse was 5'GCTCGGGTGTCAATGTCAAG3' with the amplified product of 650 base pairs (bp). The polymerase chain reaction (PCR) was performed in a volume of 50 μ l with components including 25 μ l Master mix 2X, 2 μ l $MgCl_2$ 25 mM, 1 μ l Taq polymerase 5 u/ μ l, 2 μ l genomic DNA, 2.5 μ l forward primer 10 pM, 2.5 μ l reverse primer 10 pM, and nuclease-free water up to 50 μ l. The amplification was performed in a thermocycler programmed at 95°C for 5 min, 35 cycles of 95°C for 30 secs, 58°C for 30 secs, 72°C for 45 secs, and a final extension at 72°C for 5 min. The PCR was done in a GeneAmp® PCR System 9700 of Life Technologies, USA, with 96 wells. The PCR products were confirmed by 1.2% agarose gel electrophoresis. The DNA fragment sequence was determined using Sanger, et al.'s method (1975) [15] and manually edited based on their traces by BioEdit 7.2.5.3 [16].

Data and DNA analysis

The descriptive statistical parameters of the morphological measurements were calculated in Microsoft Excel 2010. Linear regression analyses of the morphological measurements were run on Minitab 18.1. Translations of the mitochondrial COI gene into amino acids was done by BioEdit 7.0.5.3 [16]. The barcode of life data identification system for COI, which accepts sequences at <http://www.barcodinglife.org>, was used for taxonomy assignment and returns a species-level identification when one was possible [17]. The COI sequence of the samples was checked with GenBank (KM272197.1) for identifying alternations by the basic local alignment search tool for highly similar sequences at <https://blast.ncbi.nlm.nih.gov> [18].

Results and discussion

Morphological characteristics

An external photograph of the green water dragons in Phu Quoc island is given in Fig. 2. Their morphological measurements are shown in Table 1.



Fig. 2. Green water dragons from (A) Phu Quoc island and (B) Thua Thien - Hue province [6].

Table 1. The morphological measurements of the green water dragons in Phu Quoc island and Thua Thien - Hue province.

Traits	Phu Quoc island (n=30)				Thua Thien - Hue [6]
	Min.	Max.	Mean	Standard deviation	
Body weight, g	115	850	326	162	10.4-333
Snout-vent length, mm	120	280	188	38.9	42.4-260
Tail length, mm	300	540	422	76.7	109-550
Head length, mm	31.0	68.0	43.6	9.68	13.1-73.5
Head width, mm	21.0	48.0	30.7	6.32	9.50-38.1
Head height, mm	21.0	46.5	30.2	5.94	7.90-36.8
Front limb length, mm	55.0	120	81.7	17.0	21.6-114
Hindlimb length, mm	95.0	200	145	26.1	16.4-191
Snout-eye length, mm	15.0	31.0	21.2	4.51	5.60-25.3
Armpit-groin length, mm	75.0	160	107	22.0	16.1-111
Eye diameter, mm	7.80	18.0	11.4	2.08	1.70-9.20

Table 2. Linear relationships (n=30) between morphological measurements and snout-vent length of the green water dragons in Phu Quoc Island.

Relationships	Intercept	Slope	R ²	RSD	p
Snout-vent length vs. body weight	116±6.74	0.220±0.019	0.83	16.2	0.001
Snout-vent vs. head length	23.3±11.9	37.7±2.67	0.88	13.9	0.001
Snout-vent length vs. head width	9.36±12.2	58.0±3.88	0.89	13.2	0.001
Snout-vent length vs. head height	0.434±12.3	62.0±4.01	0.90	12.8	0.001
Snout-vent length vs. front limb length	12.8±12.8	21.4±1.54	0.87	14.1	0.001
Snout-vent length vs. hindlimb length	-9.67±16.8	13.6±1.14	0.84	16.0	0.001

Figure 2A shows external observations of the green water dragons from Phu Quoc island. We found that their snout was longer than their eye and that the area of the eye is concave. The eyes were clear, covered with many small scales, and the edges were slightly rough. The body was covered with small scales, evenly spaced, which were smaller than abdominal scales and limbs. Abdominal scales and under limbs were hexagonal. Tail scale size was similar to the abdominal scales and had burrs. The neck area was covered with oval-shaped scales that were small and all facing forward. On each cheek, there were three or four pointed protrusions. The upper body had a row of hard spikes from the neck to the tail. The tail length was nearly 2/3 of the body length. These characteristics were similar to the green water dragons distributed in Thua Thien - Hue province (Fig. 2B) [6].

Table 1 shows that the green water dragons from Phu Quoc have a larger sized body than those observed by Hoang (2018) [6] in Thua Thien - Hue province. For example, the snout-vent length ranged from 120 to 280 mm (vs. 42.4-260 mm) and the body weight ranged from 115 to 850 g (vs. 10.4-333). However, these figures were the same as the measurements carried out by Chung, et al. (2007) [19] with an average weight of 355 g (max 650 g). The snout-vent length is a stable morphological index often used to assess species [20]. The relationship analysis of the morphological measurements to the snout-vent length is presented in Table 2.

Table 2 shows that the snout-vent length has a close linear relationship to the body weight, head length, head width, head height, front limb length, and hindlimb length with a coefficient of determination R² from 0.83 to 0.90 (p<0.05). The base-10 logarithm (log) of the snout-vent length had a closer relationship to log (body weight) with R²=0.85 (Fig. 3). Similarly, Hoang (2018) [6] has identified a relationship of the snout-vent length to other morphological measurements of green water dragons in Thua Thien - Hue, Vietnam with R² ranging from 0.64 to 0.94. In other reptiles, these relationships were also close, e.g., *Oligosoma microlepis* with R²=0.75 [21] and *Euphlyctis cyanophlyctis* with R²=0.84 [22].

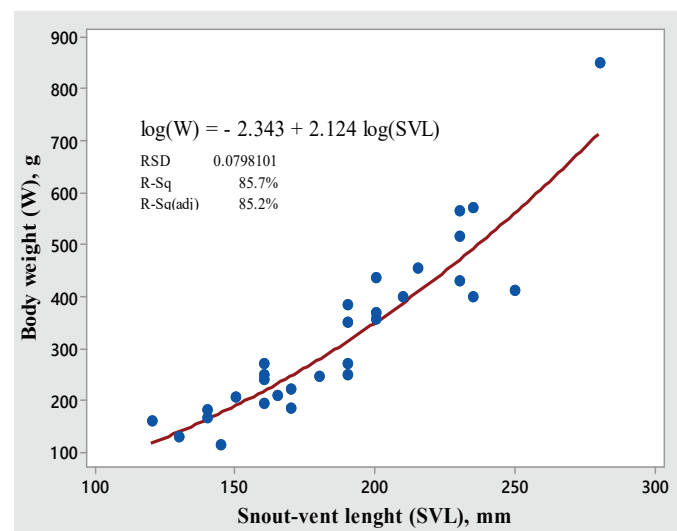


Fig. 3. Relationship between body weight and snout-vent length of green water dragons in Phu Quoc island, Vietnam.

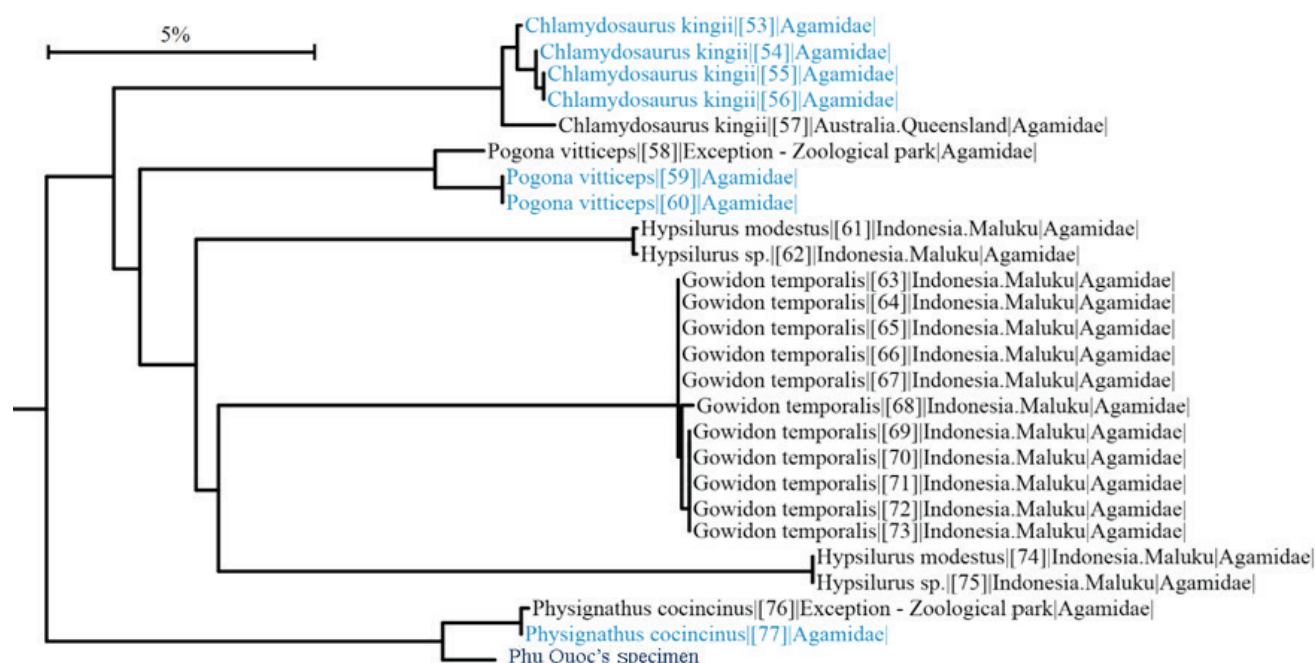


Fig. 4. The neighbor-joining tree for *Physignathus cocincinus* in Phu Quoc island, Vietnam.

Mitochondrial COI gene

The total isolated DNA was confirmed to be high purity. All samples had an optical density within 1.73-2.01 with high cleanliness that was guaranteed for sequencing. The PCR product analysis on 1.2% agarose gel found the obtained specific DNA fragments had a size of about 650 bp. Sequence reading was done in two dimensions. The PCR products were successfully sequenced and the fluorescence peaks were very clear, which corresponded to each specific alignment of DNA. After eliminating the primer, the DNA sequence of the COI gene was 604 bp for all specimens. This shows that the results of sequencing were highly reliable. The DNA sequence of the COI gene of three individuals of green water dragon in Phu Quoc island were 100% similar. There was no detectable variation of the COI gene in the population of green water dragons in Phu Quoc island, Vietnam. The COI sequences were not identified as a specific species level match in the barcode of life data system, but the nearest match was with *Physignathus cocincinus* at a 97.7% probability of placement.

The neighbor-joining tree is given in Fig. 4 and one can see the distance compared to the same published species is around 2.3 to 2.8%. Consequently, this might

confirm that the green water dragons in Phu Quoc island are evolving into a specific subspecies. Some observations of sequence divergence for intraspecific comparisons of Reptilia (Testudines and Squamata) have been less than 3% [23] or even lower in intraspecific sequence divergences for birds [24], mammals [25], and fishes [26].

The comparison with GenBank (KM272197.1) by the Basic Local Alignment Search Tool for highly similar sequences found 14/604 new alternations in COI sequences of the green water dragons from Phu Quoc Island (Fig. 5). The changes were at localities of C5483T, A5486C, C5537A, A5564G, A5591G, T5633C, G5684A, A5771G, A5849C, C5927T, T5942C, C5993T, G6002A, and A6020G. The protein sequences translated from the COI gene of the green water dragons in Phu Quoc island would alter three amino acids in the cytochrome oxidase I enzyme structure compared to the GenBank. The changes were from three alanine molecules transformed into two glycines and one threonine. Alanine and glycine are nonpolar molecules while threonine is polarized, so it is possible that there is an impact on the function of cytochrome c oxidase I enzyme, which is involved in the electron transport of cellular respiration by reducing oxygen into water [9].

UNVERIFIED: *Physignathus cocincinus* mitochondrion sequence
Sequence ID: [KM272197.1](#) Length: 16451 Number of Matches: 1

Range 1: 5476 to 6079 [GenBank](#) [Graphics](#) [Next Match](#) [Prev Match](#)

Score	Expect	Identities	Gaps	Strand
1038 bits(562)	0.0	590/604(98%)	0/604(0%)	Plus/Plus
Query 1	TCTGACTTCTCCACCATCATACCTCCTACTAATATCAACCACCTGATTCAACTCTGGAG	60		
Sbjct 5476	TCTGACTCCTACCACCATCATACCTCCTACTAATATCAACCACCTGATTCAACTCTGGAG	5535		
Query 61	TAGGAACAGGATGAACCATTTATCCTCCGATCAGGAAACCTTGACACGCAGGGCCAT	120		
Sbjct 5536	TCGGAACAGGATGAACCATTTATCCTCCATCAGGAAACCTTGACACGCAGGACCAT	5595		
Query 121	CTATAGACCTAGCCATCTTCTCCCTACATCTAGCAGGCGCATCCTCCATTCTAGGCGCAA	180		
Sbjct 5596	CTATAGACCTAGCCATCTTCTCCCTACATCTAGCAGGTGCATCCTCCATTCTAGGCGCAA	5655		
Query 181	TTAACTTCATCACAACCTGCATCAACATATCCCCACCACACATAAAGCCATTCAACTGAC	240		
Sbjct 5656	TTAACTTCATCACAACCTGCATCAACATATCCCCACCACACATAAAGCCATTCAACTGAC	5715		
Query 241	CCTTATTCGTTTGATCAGTATTCTTAACAGCCACCTACTCTACTATCATTACCGGTAC	300		
Sbjct 5716	CCTTATTCGTTTGATCAGTATTCTTAACAGCCACCTACTCTACTATCATTACCGGTAC	5775		
Query 301	TAGCCGCAGCAATCACAATATTACTAACAGACCGAAATCTCAACACAACATTCTTCGACC	360		
Sbjct 5776	TAGCCGCAGCAATCACAATATTACTAACAGACCGAAATCTCAACACAACATTCTTCGACC	5835		
Query 361	CTAATGGTGGAGGCGATCCAGTCTATTCCAACATCTATTCTGATTTTTCGGACACCCAG	420		
Sbjct 5836	CTAATGGTGGAGGAGATCCAGTCTATTCCAACATCTATTCTGATTTTTCGGACACCCAG	5895		
Query 421	AAGTTTACATCCTCATTCTACCAGGATTCGGTATTATCTCCACATCGTCACACACCACG	480		
Sbjct 5896	AAGTTTACATCCTCATTCTACCAGGATTCGGCATTTATCTCCACATTGTCACACACCACG	5955		
Query 481	CCAGTAAAAAAGAACCAATTTGGCTACGTCAGCATAGTTTGGGCCATACTAGCAATCACTA	540		
Sbjct 5956	CCAGTAAAAAAGAACCAATTTGGCTACGTCAGCATAGTCTGGGCCATGCTAGCAATCACTA	6015		
Query 541	TTCTGGGATTTCATTGTATGAGCACATCACATATTTACAGTAGGACTTGACATTGACACCC	600		
Sbjct 6016	TTCTAGGATTTCATTGTATGAGCACATCACATATTTACAGTAGGACTTGACATTGACACCC	6075		
Query 601	GAGC	604		
Sbjct 6076	GAGC	6079		

Fig. 5. The COI sequences of *Physignathus cocincinus* in Phu Quoc island and GenBank.

Conclusions

The present study shows that the green water dragons from Phu Quoc island, Vietnam are lizards with body weights up to 850 g and snout-vent lengths up to 280 mm. The relationships of the body weight to the head length, head width, head height, front limb length, and hindlimb length had coefficients of determination (R^2) from 0.83 to 0.90 ($p < 0.05$). The logarithm of the snout-vent length to base 10 had a closer relationship to log (body weight) with $R^2 = 0.85$. Their COI gene sequence had the highest similarity to the species *Physignathus cocincinus* with a

divergence ranging from 2.3 to 2.8%. Thus, it might be confirmed that the green water dragons from Phu Quoc island are evolving into a specific subspecies, however, there needs to be more sequencing of different specimens to ensure a significant conclusion. More research on farming should be done to contribute to protecting this vulnerable species.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

REFERENCES

- [1] Ministry of Science and Technology (2007), *Vietnam Red Data Book, Part I. Animals*, Natural Science and Technology Publisher, Hanoi, Vietnam.
- [2] B. Stuart, M. Sumontha, M. Cota, N. Panitvong, T.Q. Nguyen, T. Chan-Ard, T. Neang, D.Q. Rao, J. Yang (2019), “*Physignathus cocincinus*, Chinese water dragon”, *The IUCN Red List of Threatened Species*, <http://dx.doi.org/10.2305/IUCN.UK.2019>.
- [3] P.M.L.B. Cuvier (1829), *Le Règne Animal*, Distribue D’Après Son Organisation, Pour Servir de Base, A L’histoire Naturelle des Animaux et D’introduction a L’anatomie Comparée II(8), <https://gallica.bnf.fr/ark:/12148/bpt6k65211382/f17.item.texteImage>.
- [4] Ngo Dac Chung, Tran Huu Khang, Tran Xuan Thanh (2012), *Water Dragon Farming*, Agricultural Publisher, 41pp (in Vietnamese).
- [5] N.Q. Truong, N.N. Hai, P.T. Cuong, N.V. Hoang, N.D. Chung, M. Van Schingen, T. Ziegler (2018), “First population assessment of the Asian water dragon (*Physignathus cocincinus* Cuvier, 1829) in Thua Thien - Hue province, Vietnam”, *Nature Conservation*, **26**, pp.1-14.
- [6] N.V. Hoang (2018), *Study on the Population Status of the Asian Water Dragon (Physignathus cocincinus Cuvier, 1829) with Implications for Conservation in Thua Thien - Hue*, Doctoral biology thesis, Hue College of Education, Hue University, pp.39-43.
- [7] N. Neckelmann, K. Li, R.P. Wade, R. Shuster, D.C. Wallace (1987), “cDNA sequence of a human skeletal muscle ADP/ATP translocator: lack of a leader peptide, divergence from a fibroblast translocator cDNA, and coevolution with mitochondrial DNA genes”, *Proceedings of the National Academy of Sciences of the United States of America*, **84**(21), pp.7580-7584.
- [8] T. Yonetani (1960), “Studies on cytochrome oxidase. II. Steady state properties”, *The Journal of Biological Chemistry*, **235**, pp.3138-3143.
- [9] F. Fontanesi, I.C. Soto, D. Horn, A. Barrientos (2006), “Assembly of mitochondrial cytochrome c-oxidase, a complicated and highly regulated cellular process”, *American Journal of Physiology-Cell Physiology*, **291**, pp.1129-1147.
- [10] Y.P. Kartavtseva, J.S. Lee (2006), “Analysis of nucleotide diversity at the cytochrome b and cytochrome oxidase 1 genes at the population, species, and genus levels”, *Russian Journal of Genetics*, **42**(4), pp.341-362.
- [11] H.C. Dessauer, C.J. Cole, M.S. Hafner (1996), *Collection and Storage of Tissues, Molecular Systematics*, Sinauer Associates, 2nd edition, Sinauer Associates Inc., Sunderland, Massachusetts, pp.18-29.
- [12] J.A. Schulte II, J.B. Losos, F.B. Cruz, H. Núñez (2004), “The relationship between morphology, escape behaviour and microhabitat occupation in the lizard clade Liolaemus (Iguanidae: Tropidurinae: Liolaemini)”, *Journal of Evolutionary Biology*, **17**, pp.408-420.
- [13] S.D. Werman, M.S. Springer, R.J. Britten (1996), *Nucleic acids I: DNA-DNA hybridization, Molecular Systematics*, Sinauer Associates, 2nd edition, Sinauer Associates Inc., Sunderland, Massachusetts, pp.169-204.
- [14] J. Ye, G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen, T. Madden (2012), “Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction”, *BMC Bioinformatics*, **13**, DOI: 10.1186/1471-2105-13-134.
- [15] F. Sanger, A.R. Coulson (1975), “A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase”, *Journal of Molecular Biology*, **94**(3), pp.441-446.
- [16] T.A. Hall (1999), “BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT”, *Nucleic Acids Symposium Series*, **41**, pp.95-98.
- [17] S. Ratnasingham, P.D.N. Hebert (2007), “The barcode of life database”, *Molecular Ecology Notes*, **7**, pp.355-364.
- [18] Z. Zhang, S. Schwartz, L. Wagner, W. Miller (2000), “A greedy algorithm for aligning DNA sequences”, *Journal of Computational Biology*, **7**(1-2), pp.203-214.
- [19] N.D. Chung, T.T.M. Huong, T.D.V. Hung (2007), “Some biological characteristics of green water dragon (*Physignathus cocincinus* Cuvier, 1829) in Nam Dong, Thua Thien - Hue province”, *Journal of Research and Development, Thua Thien - Hue Department of Science and Technology*, **6**(65), pp.84-89.
- [20] S. Meiri (2010), “Length-weight allometries in lizards”, *Journal of Zoology*, **281**, pp.218-226.
- [21] M. Nelson-Tunley, M. Morgan-Richards, S.A. Trewick (2016), “Genetic diversity and gene flow in a rare New Zealand skink despite fragmented habitat in a volcanic landscape”, *Biological Journal of the Linnean Society*, **119**(1), pp.35-51.
- [22] M.J. Jilania, M. Raisb, M.A. Asadia, T. Mahmood (2018), “Comparison of morphometric and gravimetric measurements of common skittering frog (*Euphlyctis cyanophlyctis*) from paddy fields and urban wetlands”, *Journal of King Saud University - Science*, **30**, pp.404-411.
- [23] E.A. Chambers, P.D.N. Hebert (2016), “Assessing DNA barcodes for species identification in North American reptiles and amphibians in natural history collections”, *PLOS ONE*, **11**(4), DOI: 10.1371/journal.pone.0154363.
- [24] K.C.R. Kerr, D.A. Lijtmaer, A.S. Barreira, P.D.N. Hebert, P.L. Tubaro (2009), “Probing evolutionary patterns in neotropical birds through DNA barcodes”, *PLOS ONE*, **4**(2), DOI: 10.1371/journal.pone.0004379.
- [25] C.M. Francis, A.V. Borisenko, N.V. Ivanova, J.L. Eger, B.K. Lim, A. Guillén-Servent, S.V. Kruskop, L. Mackie, P.D.N. Hebert (2010), “The role of DNA barcodes in understanding and conservation of mammal diversity in Southeast Asia”, *PLOS ONE*, **5**(9), DOI: 10.1371/journal.pone.0012575.
- [26] D. Steinke, T.S. Zemlak, P.D.N. Hebert (2009), “Barcoding nemo: DNA-based identifications for the ornamental fish trade”, *PLOS ONE*, **4**(7), DOI: 10.1371/journal.pone.0006300.