General diversity of marbled eel (*Anguilla marmorata*) population in central of Vietnam, based on *16S rRNA* sequences by barcode DNA

Huyen Kieu Thi[1*], Long Dang Thanh[2] and Linh Nguyen Quang[1,3]
1. Faculty of Fisheries, University of Agriculture and Forestry, Hue University, 102 Phung Hung, Hue City 490000, VIETNAM
2. Institute of Biotechnology, Hue University, Phu Thuong, Hue city, Thua Thien Hue 490000, VIETNAM
3. Faculty of Animal Sciences and Veterinary Medicine, University of Agriculture and Forestry, Hue University, 102 Phung Hung, Hue City 490000, VIETNAM
*Kieuthihuyen@hueuni.edu.vn

Abstract
The marbled eel has a distribution with great profitable, ecological and conservative values. Studies on the biology, population structure and phylogenetic of marbled eel are still incomplete. The research uses the large subunit ribosomal RNA (16S rRNA) of mitochondrial sequences to evaluate the genetic diversity populations of marbled eels living in the different ecosystems the regions in Central Vietnam.

There are 7 polymorphic sites. 8 haplotypes, haplotype diversity (Hd) = 0.534 ± 0.079 and nucleotide diversity (π) = 99 x 10^{-3} were identified. The negative values are based on analysis of the neutrality tests, Strobeck's S (S) values between individuals in populations = 0.00 to 1.000 and the genetic intraspecific distance between (Fst) individuals are in the population = 0.0000 – 0.0032 (mean = 0.0010). The individuals in the population signs of recent population expansion. A random pattern of genetic evolution with a high probability of occurrence of rare alleles as populations expand geographically has been found in the population. All marbled eels collected from Central Vietnam were genetically closely related. This result lays the foundation for the conservation strategy of the species germplasm resources and develops this species in Central Vietnam.

Keywords: Marbled eels, genetic diversity, Central Vietnam, *16S rRNA.*

Introduction
Marbled eel is a big species and distributed over a wide geographical area in *Anguilla* genus[27]. This species is mainly appearing in Central Vietnam (from Thanh Hoa to Binh Thuan belonging to the central region)[25]. In Thua Thien Hue, Vietnam, previous studies have confirmed the presence of *A. marmorata* in freshwater ecosystems as a widespread species[18,19]. They are widely distributed from near estuaries to upstream rivers and serve as an important predator of the food chain in freshwater bodies together with their catadromous life cycle, commercial and aquacultural roles, *A. marmorata* can be proposed as a comprehensive indicator and representative species for the freshwater important and biodiversity conservation programs[17]. Diversion and river management practices, overfishing and habitat pollution have affected the movement between upstream and downstream of eels[36] leading to the species population being reduced in the freshwater. Therefore, it is necessary to develop strategies to conserve natural resources[18].

The population genetics demonstrated genetic differentiation between geographical locations based on various molecular markers such as *cytochrome b (Cyt b)*[7], *mitochondrial* (*mt*) DNA, *16S rRNA* gene[16], *mitochondria control region* (*mtCR*)[5] and *COI*[2].

Recently, using short, standardized gene regions in DNA barcoding technical to species identification, evaluating the ecology and evolution of natural systems are believed to be a viablemethods[13,15,20]. For animals, *cytochrome c oxidase* 1 (*COI*) gene primers are thought to be universal that can allow amplification of the 5'-terminal fragment from most species[13] and can indeed be used as a standard for many higher animal taxa[20].

However, the use of *COI* fragments in vertebrate genetic studies is not the only option. 16S rRNA is a highly conserved gene in animal *mitochondrial* structure and is less variable than *COI*[13,35]. The studies on *16S rRNA* fragments are superior to *COI* for vertebrate clades[35]. In this study, we used *16S rRNA* gene sequencing by DNA barcode technique to analyze the genetic diversity of the giant mottled eel populations in Central Vietnam and the Indo-Pacific region to provide necessary data for conservation, protection and development of wild eel resources.

Material and Methods
Materials: *A. marmotara* eels were collected with sizes 169.0 – 1,080.0 mm (W = 7.0 – 3,200.0 g) at different locations in Thua Thien Hue from November 2018 to November 2019 (Fig. 1 and table 1). Muscle tissues were dissected from fresh specimens of eels to be used for DNA extraction, preserved in 95% ethanol and frozen at -80 °C before.

DNA extraction PCR amplification and sequencing: Genomic DNA of the all individual of eels was extracted from muscle tissue following the protocol described by Kumar et al[27].

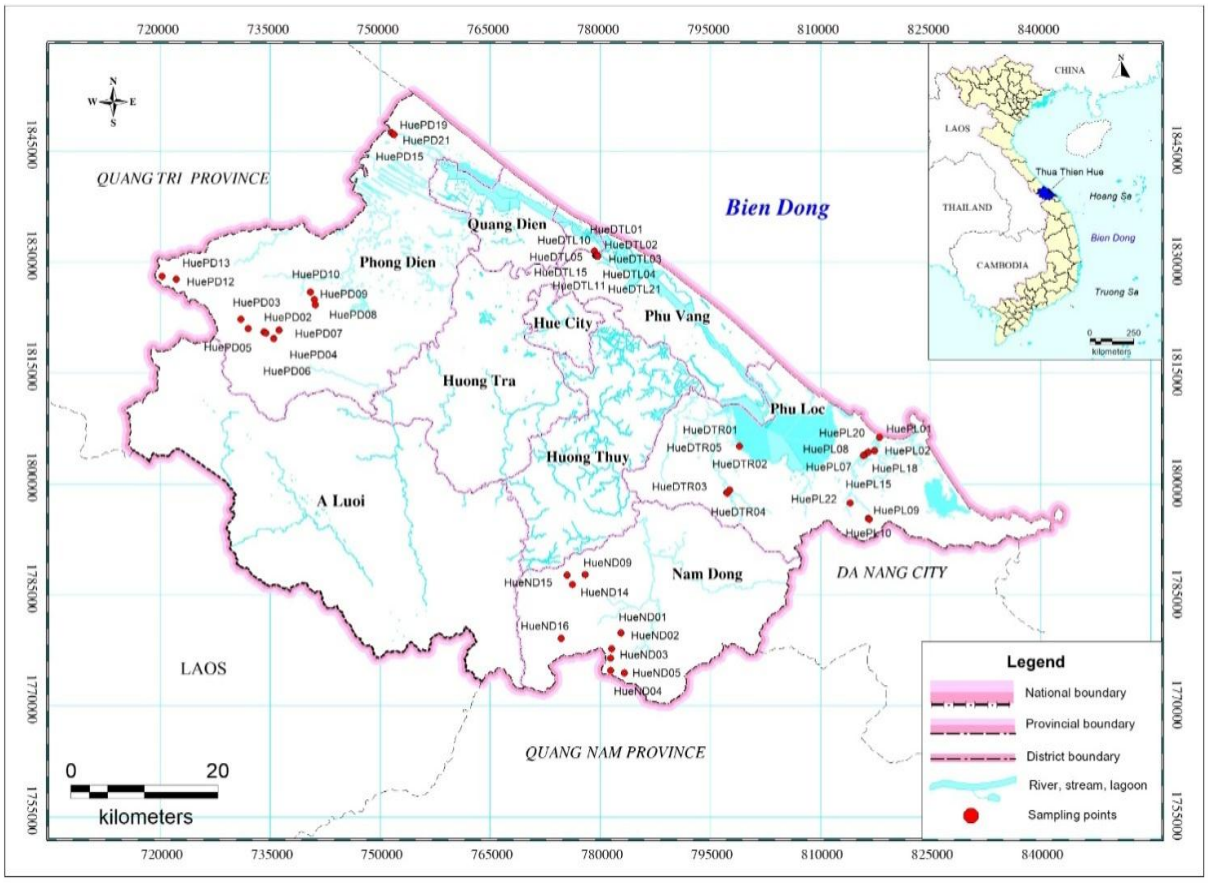

Figure 1: Location of sample sites in Thua Thien Hue, Viet Nam

Table 1
The number and characteristics of research samples

Population	Name of sample location	No. of sample	Genbank accessions	Source
Vietnam (VN)	Thao Long dam (downstream of Huong river) – HueDTL	10	MN633308 - MN633317	This study
	Truoi Dam (downstream of Truoi river) – HueDTR	05	MN633318 - MN633322	
	Nam Dong distrisc (upstream of Huong river and Truoi river) – HueND	09	MN633323 - MN633331	
	Phong Dien district (O Lau river and upstream of Huong river) – HuePD	14	MN633332 - MN633345	
	Lang Co lagoon – HueLC	02	MN633354 - MN633355	
	Bu Lu river – HueBL	08	MN633346 - MN633353	
Nouth of Pacific Ocean (nPacific)		23	AY735146,AB278811-AB278814, AB278817, AB278819 - AB278825, AB278829 - AB278835, AB278868 - AB278871	https://blast.ncbi.nlm.nih.gov/Blast.cgi
South of Pacific Ocean (sPacific)		17	AB278836 - AB278839, AB278841, AB278843, AB278847 - AB278854	
East of Indian Ocean (eIndo)		6	AB278855 - AB278857, AB278859 - AB278861	
West of Indian Ocean (wIndo)		6	AB278862 - AB278867	

The amplification reaction solution for the *16S rRNA* gene consists of 7 µL PCR buffer (10X), 5 mM dNTP; 10 µM of L2510 primer, 10 µL of H3080 primer; 100 ng DNA sample (50 ng/µL), 0.2 µL Taq DNA Polymerase enzyme (5 UI/µL) (Biolabs Inc., New England) and sterile distilled water added to a total volume of 35 µl. The sequence of primers is L2510 (5′-CGC CTG TTT ATC AAA AAC AT- 3′) and H3080 (5′ -CCG GTC TGA ACT CAG ATC ACG T- 3′)[26] used to amplify the *16S rRNA* gene region and the following thermal cycle is used: 95°C/5 minutes; 30 cycles (95°C/45 seconds; 55°C/30 seconds; 72°C/ 1 minute); 72°C/7minutes on MJ-MiniTM Persanol Thermal Cycle, Bio-Rad.

The PCR products of the amplified gene region were examined by electrophoresis (1% agarose gel in 1X TAE buffer with Ethidium bromide dye) and read by UV-transilluminator (Model: DyNa Light) before being sequenced in both directions with the PCR primers by the dideoxy-terminal method on the ABI PRISM® 3100 Avant Genetic Analyzer (Applied Biosystems) at Macrogen Co., Korea.

Data analysis: The *16S rRNA* gene fragment final sequences were assembled using BioEdit 7.2.5 and the non-overlapping sequence regions at the 5th and 3rd ends were trimmed and aligned using the ClustalW tool in MEGA 11[32]. All sequences have been updated and provided access codes on the NCBI (National Center for Biotechnology Information) database from MN633308 to MN633355. The number of separate polymorphic sites (S), the number of mutant sites (Eta), the number of haplotypes (h), haplotype diversity (Hd), the number of nucleotide differences(k) and nucleotide diversity (π) were six parameters used to measure the population polymorphic. Neutrality is tested based on five methods namely, Tajima's D test[9], Fs, Fu's statistic, D* and F*, Fu and Li's statistics[8] were used for DNAsp 6.0 software[29].

The Neighbor-Joining method was used to inferrinferlutionary history[30] with bootstrap 1000 replicates. The Maximum Composite Likelihood method[32] and the units of the number of base substitutions per site are used to compute the evolutionary distances. All ambiguous positions were removed by pairwise deletion option for each sequence pair.

Evolutionary analyses were conducted for 90 nucleotide sequences in MEGA 11[33]. A haplotype network was established to illustrate genetic relationships among individuals in the eel population based on *16S rRNA* sequences on Network 10.2 software. The F statistic (Fst) was calculated to estimate the genetic distance, the genetic differentiation and migration rate among the populations (significance testing with 1000 permutations) on DNAsp 6.0.

The Nm value is calculated by the formula: Nm = (1 - Fst)/4Fst for the gene segment located on the Diploid-Autosome[41]. The nucleotide and amino acid compositions of the sequences were compared with 01 other individuals: *A. marmorata* - AB278871.1 taken from GenBank and *16S rRNA* sequences of the Indo-Pacific populations were obtained from the Genbank data and were used to evaluate the genetic diversity and population evolutionary trends (Table 1).

Results and Discussion

Results: A high success rate (100%) for PCR amplification and sequencing of *16S rRNA* gene of this species using a single primer pair by DNA barcode was shown. The results of sequences for the *16SrRNA* gene region have shown 622 bp in the 48 sequences analyzed in table 2. The BLAST results on NCBI also showed the nucleotides sequenced and obtained at the region Thua Thien Hue, were similar to *A. marmorata* (accession number: AB278871 .1) with a coverage rate of 100%. The intraspecific distance of the *16S rRNA* sequence of the population in Vietnam, has ranged from 0.0000 to 0.0032 (mean = 0.0010).

The composition of each nucleotide type on the isolated gene included the highest value and Adenine (A) proportion of 33.47% (208 nucleotides), Cysteine (C) accounting for 24.60% (153 nucleotides), Guanidine (G) accounts for 21.83% (136 nucleotides) and the lowest is Timin (Uracil) with 20.10% (125 nucleotides). The component (G + C) reached 289 nucleotides (46.43%) as in table 2 showing a diversity of genes in the population[28,33].

There were 7 different nucleotide positions between 48 sequences of *16S rRNA* isolated in Vietnam on the population, 157, 199, 294, 388 are singlets on variable sites and 245, 289, 502 are Parsimony Informative sites containing two variants as mentioned by Gholamhosseini et al[11]. This number of nucleotide difference positions is higher than that of the *16SrRNA* sequences of the eels in the nPacific, sPacific, eIndo, wIndo regions.

Table 2
The characteristics of *16S rRNA* gene region of VN population

Number of nucleotides (%)				Total of base pairs	G+C (%)	Number of monomorphic sites	Variable sites (%)	Intraspecific distance (mean)
T	C	A	G					
125 (20.10)	153 (24.60)	208 (33.47)	136 (21.83)	622	289 (46.43)	615	1.40	0.0000-0.0032 (0.0010)

However, this difference rate is lower when comparing them with the Indo-Pacific region which accounts for 50% of the total. The *16S rRNA* gene region in VN population (48 individuals) has the total number of mutations (S) = 7, the average number of nucleotide differences is (k) = 0.61348, the nucleotide diversity coefficient is (π) = 99 x 10^{-3} creating 7 haplotype types (h) with haplotype diversity coefficient (Hd) = 0.534 ± 0.079. The analysis results show that individuals from the population eels in Central Viet Nam have a great difference with the eel population collected in the nPacific, sPacific, eIndo, wIndo and Indo-Pacific region when performing the analysis on DNAsp 6.0 software ($p < 0.05$) as in table 3.

In the marbled eel population collected in Vietnam, nPacific, sPacific, eIndo and Indo-Pacific showed the negative values for almost neutral tests (except for wIndo population, Fs = 0). Strobeck's S, (S = 0.00 to 1.000) rate was high among individuals in populations at table 4. The highest value of Fst is shown in the pairs of eel populations VN and wlndo (Fst = 0.0798, $p < 0.05$) and the lowest is in eel populations Vitenam and sPacific (Fst = 0.0420, $p < 0.05$). Between different populations, the Fst value based on *16S rRNA* haplotype is also different, frequencies ranged from 0.04199 to 0.0798 ($p < 0.05$) as in table 5.

The haplotype network was built based on median-joining analysis of polymorphic sites including the number and frequency of haplotypes for *16S rRNA* sequences (Figure 2). The haplotype network of the *16S rRNA* gene segment resembles radials with a large number of dominant haplotypes closely related to a central haplotype (H1) compared with other satellite haplotypes (73.45 % of Indo – Pacific, 66.67 % of VN, 95.23% of nPacific, 52.94% of sPacific, 100% wIndo and 83.33% of eIndo). This study also indicates that the haplotype (H1) is the ancestral haplotype. The marbled eel species *A. marmorata* distributed in Viet Nam has a strong correlation with populations in the Indo-Pacific region. Besides the central haplotype (H1), there is also the appearance of satellite haplotypes scattered in many directions compared to the H1 central haplotype.

In other words, there is appearance of eel individuals that are genetically different from eel individuals distributed in the ancestral haplotype. The evolutionary tree based on *16S rRNA* gene region allele frequencies was constructed by the Neighbor-Joining method[30] in MEGA 11[33].

Table 3
Genetic diversity of Marbled eel populations based on *16S rRNA* sequences

Population	n	S	Eta	H	Hd	k	π (x10^{-3})
VN	48	7	7	8	0.534 ± 0.079	0.61348	99
nPacific	21	1	1	2	0.095 ± 0.084	0.09524	15
sPacific	11	3	3	4	0.491 ± 0.175	0.54545	88
eIndo	6	1	1	2	0.333 ± 0.215	0.33333	54
wIndo	5	0	0	1	0	0	0
Indo-Pacific	91	12	12	13	0.407 ± 0.065	0.45169	73

Table 4
Neutral test results of *16S rRNA*sequences of *A. marmorata* populations

Population	n	Tajima's D test	Fu and Li's D* test	Fu and Li's F* test *	Fu's Fs statistic	Strobeck's S statistic
VN	48	-1.62974 **	-1.84556 *	-2.08557**	-5.307	0.999
nPacific	21	-1.16356*	-1.55770*	-1.66344*	-0.919	0.960
sPacific	11	-1.59996**	-1.87398	-2.03086 *	-2.042	0.977
eIndo	6	-0.93302 *	-0.95015 *	-0.96473 *	-0.003	0.882
wIndo	5	0	0	0	0	0
Indo-Pacific	91	-2.18090***	-4.06094 ***	-4.03690***	-15.335	1.000

Note: *: $P > 0.10$; **: $0.10 > P > 0.05$; ***: $P < 0.01$

Table 5
Fst value and gene flow among *A. marmorata* populations

Population	VN	nPacific	wlndo	elNDO	sPacific
VN	**	0.0162	0.0184	0.0126	0.0101
nPacific	0.0698	**	0	0	0
Wlndo	0.0798	0	**	0	0
elNDO	0.0532	0	0	**	0
sPacific	0.0420	0	0	0	**

Note: F_{st} values are above and Nm values are below of the diagonal.

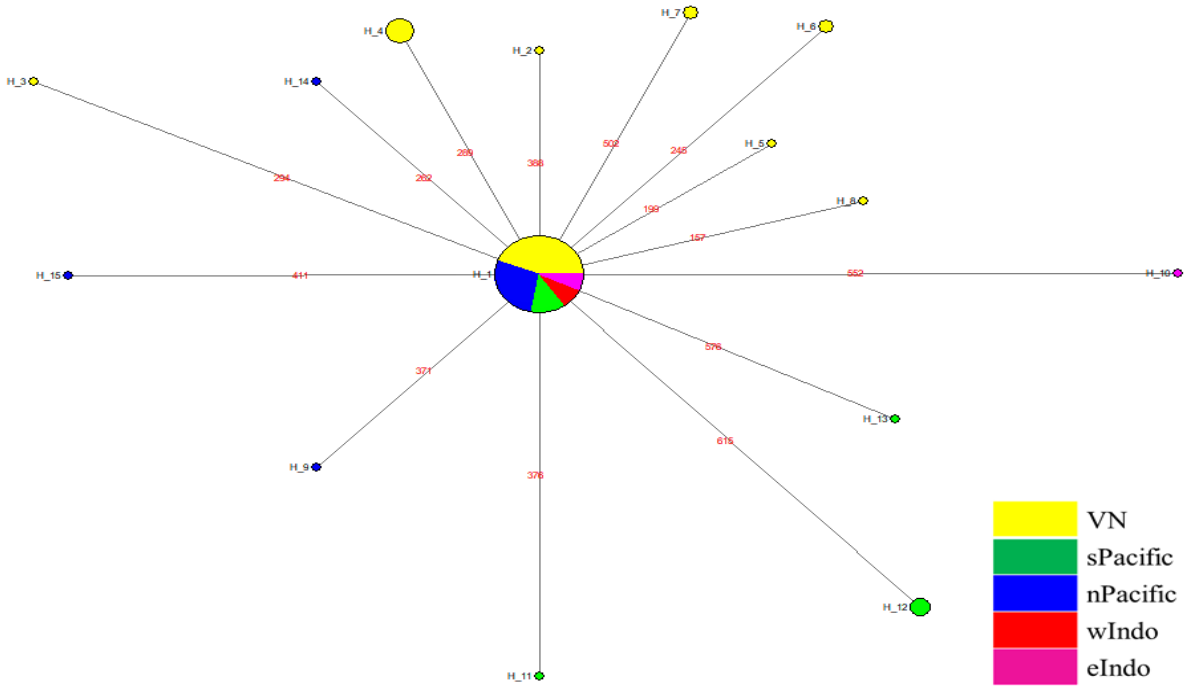

Figure 2: Median-joining haplotype network 16SrRNA nucleotide sequence variation in 5 populations of the *A. marmorata*. The red numbers on the connecting lines present the variable sites between each haplotype pair. Yellow, Green, Blue, Red and Pink colors represented for VN - Vietnam, sPacific-South of Pacific Ocean, nPacific - Nouth of Pacific ocean, wIndo-West of Indian Ocean and eIndo – East of Indian Ocean populations in the network.

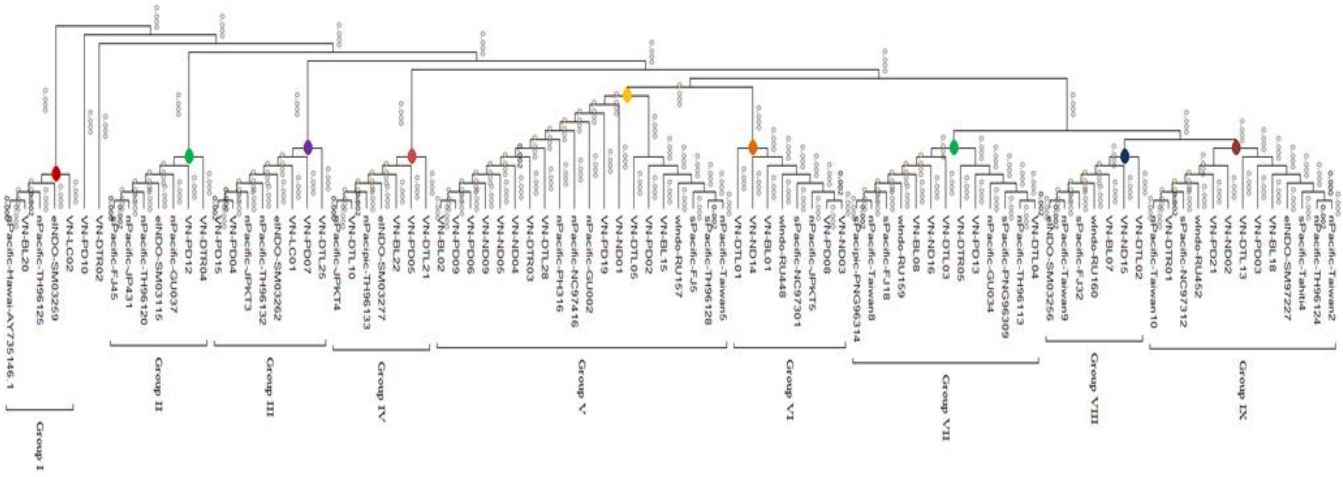

Figure 3: The phylogenetic relationship of *A. marmorata* among the haplotypes was determined using16S rRNA sequences. The figures include population code, haplotype and the number of individuals of the haplotype. The branch lengths on the scale bar are the evolutionary distances used to infer the phylogenetic tree

The results presented in fig. 3 show that the 9 groups are present with individuals of *A. marmorata* eels collected from Thua Thien Hue, Vietnam compared to the main individuals distributed in the Indo - Pacific region although they are genetic far apart.

In addition, the tendency of some of these individuals to separate to form new genetic variants in the population including eight individuals, HueDTL28, HueDTR03, HueND04, HueND05, HueND09, HuePD06, HuePD09 and HueBL02 always converges to form an independent group with the appearance of the haplotype H4 genetic variant and two individuals HueDTR04, HuePD12 (H5) and HueBL20 (H8) (Fig. 2 and fig. 3).

The Marbled eel has a geographical distribution[6] such as Sri Lanka, India, the Indo-Pacific region and the islands in the central South Pacific[37,38]. In the warm productive habitats, the eel growth stage may be as short as two to three years, while in the more northerly, latitudes range from six to twenty years or more[27]. In continental growth stages, it is found in freshwater, brackish and saline habitats[23]. The

mature eels and leptocephalus larvaes were captured in the depths of 150–200m[34] in the same ocean area such as the western Mariana Islands[4] in the Northern Equatorial region of the Northwest Pacific Ocean[22].

Based on genetic characteristics and morphology, it is thought that there are at least four different subpopulations of *A. marmorata* in the Indo-Pacific region (North Pacific, South Pacific, Indian Ocean, Guam area)[24,38]. Evidence for the existence of 2 independent populations: Sumatran and southwestern Indian Ocean (F_{ST} = 0.025 to 0.039) was provided by Gagnaire et al[10] while refuting the claims. The hypothesis of genetic ancestral polymorphism and differentiation with gene flow and support for a secondary association with unidirectional migration after a period of isolation of *A. marmorata* was based on analysis of 2 mitochondrial *16S rRNA* single-nucleotide polymorphisms (SNPs) and 10 nuclear microsatellite loci.

The *A. marmorata* population in the western Pacific Ocean is believed to be stable at both spatial and temporal scales based on PCR analysis of *mt*DNA structures[3]. Arai and Hussein[1] also confirmed the panmictic population formed of *A. marmorata* from the Indo-Pacific region based on genetic analysis of regional eel populations using *COI* markers by DNA barcode.

In Vietnam, *A. marmorata* has distributed river basins in the central provinces from Quang Binh to Phu Yen, especially in Chau Truc lagoon in Binh Dinh province, Hương river (Thua Thien Hue), Ba river (Phu Yen) and Tra Khuc river (Quang Ngai) or Giang river (Quang Binh) (Ministry of Science Technology and Environment, 2007). In the river basins in the north of Vietnam, this fish is rarely found. *A. marmorata* eel usually lives in most water basins bodies, concentrated in the upper reaches of rivers basins in places near rocky mountains, with many caves and downstream with a strong flow of water. In the Quang Binh to Khanh Hoa provinces are the river basins, where the eel is widely distributed and economically significant in wild[25].

In Central Vietnam, previous studies have confirmed the presence of *A. marmorata* in the freshwater body as a widespread species. The analysis of the relationship between giant mottled eel samples in countries in Southeast Asian waters such as Malaysia, Thailand and Vietnam using the *COI* sequences base on DNA barcode, shows that the *A. marmorata* populations in Malaysia, Thailand and Vietnam may have been transported from the Pacific Northwest breeding area and suggested possible dispersal and migration of *A. marmorata* into Southeast Asian waters[2,18]. In addition, some new genetic variants found in Vietnam have shown the important role of this region in the dispersal strategy and population expansion of *A. marmorata*[1,2].

In this study, we used part of the nucleotide sequence of the *16S rRNA* gene region to identify species and analyze the genetic diversity of eels collection in different river basins in Thua Thien Hue, Vietnam. The negative values of D, Fu and Li's D* and F* between these populations (Table 4) indicate an excess of recently derived haplotypes and suggest that either eel population expansion or background selection has occurred[8,9,31] of *A. marmorata* in Thua Thien Hue, Vietnam. Fst reflects the level of inbreeding within populations[40] or the extent to which populations are differentiated[12]. According to research of Weir[39], Fst values below 0.05 indicate negligible genetic differentiation whereas values greater than 0.25 indicate high genetic differentiation within the analyzed population.

The results in table 5 show that the eel population collection in Thua Thien Hue, Viet Nam has indicated weak genetic differentiation with eel populations Indo – Pacific region (value Fst ranging from 0.0420 to 0.0798 with $p < 0.05$) and extremely low gene flow among the populations (Pairwise Nm values ranged from 0.0101 to 0.0184) shows that the conservative role of the *16S rRNA* gene is very high in the genetic structure of the *A. marmorata* eel population in Thua Thien Hue. Haplotype network and phylogenetic (Fig. 2 and fig. 3) indicated genetically closely related and located close to *A. marmorata* eels were collected from nPacific and wlndo. In addition, the tendency of some haplotypes is to separate to form new genetic variants in the population (H4, H5 and H8) (Fig. 2 and fig. 3). These may be individuals that are specific to long-lived individuals and are influenced by inland environmental factors in Thua Thien Hue or are variations introduced from other geographical sources and higher conservative of *16S rRNA* in the genetic structure of *A. marmorata*.

In the study, we provide accurate barcode information of eel population collection in different ecosystem regions in Thua Thien Hue, Vietnam for researchers. It lays the foundation for the conservation, evaluation and protection of *A. marmorata* species germplasm resources.

Conclusion

The research results confirmed the close relationship between the marbled population in Central Vietnam and the populations in the Indo - Pacific region. The genetic variations and high levels of diversity were detected in the population structure in the region (Thua Thien Hue) and indicated the ecological role of Vietnam in the conservation and development strategy of the marbled eel in the Indo - Pacific region. Vietnam should be considered an important geographical area to develop a conservation strategy for the species. Furthermore, studies on the ecological, genetic and life cycle relationships of eels in this area need to be promoted.

Acknowledgement

This work was supported by Hue University [No. DHH2019-02-113] - 03 Le Loi str., Hue city, Viet Nam; Kieu Thi Huyen was granted by Vingroup joint-stock company and supported by the Domestic Master/Ph.D. scholarship program of Vingroup innovation foundation

(VINIF), Vingroup Big Data Institute (VINBIGDATA) [No. VINIF.2020.TS.132]; and The Ph.D. scholarship 2018 of Southeast Asian Regional Center for Graduate Study and Research in Agriculture (SEARCA) - College, Los Baños, Laguna 4031 Philippines.

References

1. Arai T. and Hussein T., Contrasting patterns of genetic population structure in tropical freshwater EELS of genus *Anguilla* in the Indo-Pacific, *Heliyon*, **7(5)**, e07097 **(2021)**

2. Arai T., Taha H., Mohd-Riduan M.N. and Mokti S.S.A., Molecular and morphological evidence for the identity of the giant mottled EEL, *Anguilla marmorata* in Southeast Asia, *Trop Ecol*., **61(3)**, 429–436 **(2020)**

3. Cheng C.C., Leander N.J., Shen K.N. and Tzeng W.N., Spatial and temporal population genetic structure of the giant mottled eel *Anguilla marmorata* in the Northwestern Pacific, *J. Fish. Soc. Taiwan*, **39(4)**, 269–282 **(2012)**

4. Chow S., Kurogi H., Mochioka N., Kaji S., Okazaki M. and Tsukamoto K., Discovery of mature freshwater eels in the open ocean, *Fisheries Science*, **75(1)**, 257-259 **(2009)**

5. Donovan S., Pezold F., Chen Y. and Lynch B., Phylogeography of *Anguilla marmorata* (Teleostei: Anguilliformes) from the eastern Caroline Islands, *Ichthyol. Res.*, **59**, 70–76 **(2012)**

6. Ege V., A revision of the genus *Anguilla* Shaw, a systematic, phylogenetic and geographical study, *Dana Rep.* **16**, 1-256 **(1939)**

7. Fahmi M.R., Solihin D.D., Soewwardi K., Pouyaud I. and Berrebi P., Molecular phylogeny and genetic diversity of freshwater *Anguilla* eels in Indonesian waters based on *mitochondrial* sequences, *Life and Environment*, **65(3)**, 139-150 **(2015)**

8. Fu Y.X. and Li W.H., Statistical tests of neutrality of mutations, *Genetics*, **133**, 693–709 **(1993)**

9. Fu Y.X., Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection, *Genetics*, **147**, 915-925 **(1997)**

10. Gagnaire P.A., Minegishi Y., Aoyama J., Réveillac E., Robinet T., Bosc P., Tsukamoto K., Feunteun E. and Berrebi P., Ocean currents drive secondary contact between *Anguilla marmorata*populations in the Indian ocean, *Marine Ecology Progress Series*, **379**, 267–278 **(2009)**

11. Gholamhosseini A., Razbanian M., Esmaeili H.R. and Eagderi S., Molecular systematics and morphological variation in the Mesopotamian spiny EEL *Mastacembelus mastacembelus* (Teleostei: Mastacembelidae), *The European Zoological Journal*, **89(1)**, 546-555 **(2022)**

12. Hartl D.L. and Clark A.G., Principles of population genetics, 4th edn, Sunderland, MA, USA, Sinauer Associates **(2007)**

13. Hebert P.D.N., Cywinska A., Ball S.L. and de Waard J.R., Biological identification through DNA barcodes, *Proc R Soc Lond Ser B.*, **270**, 313-321 **(2003)**

14. Hebert P.D.N., Penton E.H., Burns J.M., Janzen D.H. and Hallwachs W., Ten species in one: DNA barcoding reveals cryptic species in the Neotropical skipper butterfly *Astraptes fulgerator*, Proc Natl Acad Sci., USA **(2004)**

15. Hubert N., Hanner R., Holm E., Mandrak N.E., Taylor E., Burridge M., Watkinson D., Dumont P., Curry A., Bentzen P., Zhang J., April J. and Bernatchez L., Identifying Canadian Freshwater Fishes through DNA Barcodes, *PLoS ONE*, **3(6)**, e2490 **(2008)**

16. Ishikawa S., Tsukamoto K. and Nishida M., Genetic evidence for multiple geographic populations of the giant mottled eel *Anguilla marmorata* in the Pacific and Indian oceans, *Ichthyological Research*, **51**, 343-353 **(2004)**

17. Itakura H., Wakiya R., Gollock M. and Kaifu K., *Anguillid* eels as a surrogate species for conservation of freshwater biodiversity in Japan, *Sci. Rep.*, **10**, 8790 **(2020)**

18. Kieu T.H. and Nguyen Q.L., Phylogenetic analysis of *Anguilla marmorata* population in Thua Thien Hue, Vietnam based on the *cytochrome C oxidase* I (*CO*I) gene fragments, *AMB Expr*, **10**, 122 **(2020)**

19. Kieu T.H., Vo D.N., Tran N.N., Truong V.D., Vo V.P., Tran Q.D. and Nguyen Q.L., Using DNA barcodes based on mitochondrial *CO*I and 16*S rRNA* genes to identify *Anguilla* eels in Thua Thien Hue province, Vietnam, *Genet. Mol. Res*., **19(4)**, gmr18722 **(2020)**

20. Kress W.J., Garcia-Robledo C., Uriarte M. and Erickson D.L., DNA barcodes for ecology, evolution and conservation, *Trends in Ecology & Evolution* **30**, 25–35 **(2015)**

21. Kumar R., Singh P.J., Nagpure N.S., Kushwaha B., Srivastava S.K. and Lakra W.S., A Non-Invasive technique for rapid extraction of DNA from fish scales, *Indian Journal of Experimental Biology*, **45**, 992-997 **(2007)**

22. Kuroki M., Aoyama J., Kim H., Miller M.J., Kimura S. and Tsukamoto K., Migration scales of catadromous eels: Diversity and evolution of larval migration based on their distribution, morphology and early life history, *American Fisheries Society Symposium*, **69**, 887–89 **(2009)**

23. Lin Y.J., Jessop B.M., Weyl O.L.F., Iizuka Y., Lin S.H., Tzeng W.N. and Sun C.L., Regional variation in otolith Sr:Ca ratios of African longfinned eel *Anguilla mossambica* and mottled eel *Anguilla marmorata*: A challenge to the classic tool for reconstructing migratory histories of fishe, *Journal of Fish Biology*, **81(2)**, 427-441 **(2012)**

24. Minegishi Y., Aoyama J. and Tsukamoto K., Multiple population structure of the giant mottled eel *Anguilla marmorata*, *Molec Ecol.*, **17**, 3109–3122 **(2008)**

25. Nguyen A.T., Tsukamoto K. and Lokman P.M., Composition and distribution of freshwater eels *Anguilla spp.* in Vietnam, *Fisheries Science*, **84(8)**, 987–994 **(2018)**

26. Palumbi S. et al, The simple fool's guide to PCR, Version 2, Department of Zoology and Kewalo Marine Laboratory, https://rldcat.org/title/simple-fools-guide-to-pcr/oclc/700266456?referer=di&ht=edition **(1991)**

27. Pike C., Crook V., Gollock M. and Jacoby D., *Anguilla marmorata* (Errata Version Published in 2020), The IUCN Red List of Threatened Species 2019: E.T166189A167699312, https://doi.org/https://dx.doi.org/10.2305/IUCN.UK.2019-3.RLTS.T166189A167699312.en **(2019)**

28. Pradhan Prakash, Testing equivalency of interpolation derived bioclimatic variables with actual precipitation: A step towards selecting more realistic explanatory variables for Species Distribution Modelling, *Res. J. Chem. Environ*., **23(12)**, 38-41 **(2019)**

29. Rozas J., Ferrer-Mata A., Sánchez-DelBarrio J.C., Guirao-Rico S., Librado P., Ramos-Onsins S.E. and Sánchez-Gracia A., DNAsp 6: DNA Sequence Polymorphism Analysis of Large Data Sets, *Molecular Biology and Evolution*, **34(12)**, 3299-3302 **(2017)**

30. Saitou N. and Nei M., The neighbor-joining method: A new method for reconstructing phylogenetic trees, *Molecular Biology and Evolution*, **4**, 406-425 **(1987)**

31. Tajima F., Statistical method for testing the neutral mutation hypothesis by DNA polymorphism, *Genetics*, **123(3)**, 585–595 **(1989)**

32. Tamura K., Nei M. and Kumar S., Prospects for inferring very large phylogenies by using the neighbor-joining method, Proceedings of the National Academy of Sciences (USA), https://doi.org/101:11030-11035 **(2004)**

33. Tamura K., Stecher G. and Kumar S., MEGA 11: Molecular Evolutionary Genetics Analysis Version 11, Molecular Biology and Evolution **(2021)**

34. Tsukamoto K., Chow S., Otake T., Kurogi H., Mochioka N., Miller M.J., Shinoda A. Aoyama J., Kimura S., Watanabe S., Yoshinaga T., Kuroki N.M., Oya M., Watanabe T., Hata K., Ijiri S., Kazeto Y. and Tanaka H.K., Oceanic spawning ecology of freshwater eels in the Western North Pacifica, *Acific. Nat Commun.*, **2**, 179 **(2011)**

35. Vences M. et al, Comparative performance of the *16S rRNA* gene in DNA barcoding of amphibians, *Front Zool.*, **2**, 5 **(2005)**

36. Wasserman R.J., Weyl O.L.F. and Strydom N.A., The effects of instream barriers on the distribution of migratory marine-spawned fishes in the lower reaches of the Sundays river, South Africa, *Water South Africa*, **37(4)**, 1-10 **(2011)**

37. Watanabe S., Aoyama J., Miller M.J., Ishikawa S., Feunteun E. and Tsukamoto K., Evidence of population structure in the giant mottled eel, *Anguilla marmorata*, using total number of vertebrae, *Copeia*, **2008(3)**, 680–688 **(2008)**

38. Watanabe S., Miller M.J., Aoyama J. and Tsukamoto K., Morphological and meristic evaluation of the population structure of *Anguilla marmorata* across its range, *Journal of Fish Biology*, **74(9)**, 2069–2009 **(2009)**

39. Weir B.S., Genetic Data Analysis II: Methods for discrete population genetic data, Sinauer Associates, Inc., Sunderland **(1996)**

40. Wright S., Evolution and the genetics of populations, Variability within and among natural populations, University of Chicago Press **(1984)**

41. Wright S., The genitival structure of populations, *Ann. Eugenics*, **15**, 323-354 **(1951)**.

(Received 08th July 2022, accepted 10th September 2022)