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Nguyen Thi Phuong Mai
Tay Nguyen Institute of Scientific Research, VAST, Lam Dong, Vietnam

Ha Thanh Tung
Tay Nguyen Institute of Scientific Research, VAST, Lam Dong, Vietnam

Le Thi Chau
Tay Nguyen Institute of Scientific Research, VAST, Lam Dong, Vietnam

Nguyen Thien Tao
Vietnam National Museum of Nature, VAST, Ha Noi City, Vietnam

Hoang Nghia Son
Animal Biotechnology Department, Institute of Tropical Biology, VAST, Ho Chi Minh City, Vietnam

Correspondence
Nguyen Thi Phuong Mai
Tay Nguyen Institute of Scientific Research, VAST, Lam Dong, Vietnam

Genetic relationship of otters from Vietnam central highland assessed by cytochrome b gene

Nguyen Thi Phuong Mai, Ha Thanh Tung, Le Thi Chau, Nguyen Thien Tao and Hoang Nghia Son

Abstract
The present study was aimed to evaluate the genetic relationship of otters from Vietnam Central Highland assessed by cytochrome b gene. The results showed that there are 189 polymorphic sites in all haplotypes, representing 20.1% of the total analyzed-DNA sequence (940 bp). The phylogenic analysis revealed that the otters from Vietnam Central Highland were separated into three groups, including *Aonyx cinerea* (AC group) (samples LD1, LD2, LD3, LD4), *Lutrogale perspicillata* (LP group) (samples LD5 and LD6), and *Lutra lutra* (LL group) (samples LD7 and LD8). The otters from VN group 1 showed 8 variable positions (at position 102, 120, 209, 406, 588, 678, 688, and 906) comparing to *Aonyx cinerea*. The otters of VN group 2 showed 20 variable positions comparing to *Lutrogale perspicillata*. The otters from VN group 3 showed 2 variable positions (at position 72 and 993) comparing to *Lutra lutra*. The otters from from Vietnam Central Highland are less variable than other groups such as LL group (0.035 ± 0.005), LP group (0.001 ± 0.001), AC group (0.004 ± 0.001).

Keywords: Cytochrome b, otter, phylogenetics, vietnam central highland

Introduction
Otter (Lutrinae) is a group of carnivorous mammals that live in the water or ocean, part of the Mustelidae including weasels, brown weasels, badgers, as well as several other species. 13 species of distributed throughout the world excepting Antarctica and Australasia [1]. Research on morphological and molecular biology classification to distinguish species, identify new species has been studied by many studies in the world [1, 6]. Comparing the mitochondrial DNA sequences plays an important role in the study of taxonomy and genotyping, it allows biologists to clarify relationships and evolution between species. It also allows the examination of populations, and as such it is very important in the field of anthropology and biology, through the reconstruction of the family tree. Therefore, mitochondrial sequences are used to evaluate genotypes arising on many different objects. The research on phylogenetic relationships based on a number of mitochondrial sequences has also been carried out on a number of otters [7].
In Vietnam there are 4 species of otters: common otter (*Lutra lutra*), small clawed otter (*Aonyx cinereus*), smooth coated otter (*Lutrogale perspicillata*), and hairy-nosed otter (*Lutra sumatrana*). However, the identification of otters is only based on external morphological characteristics, not using molecular data. Thus, this study was carried out to estimate the genetic relationship of otters from Vietnam Central Highland assessed by cytochrome b gene.

Materials and Methods
DNA Sampling
The ear samples of otters were used for this study. These samples were washed by phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1. 8 mM KH_2PO_4). All collected samples were got permission from the local authorities and relative permits. The samples were transferred to laboratory by keeping in -20 ˚C.

DNA Extraction
Total DNA was extracted from ear samples. Tissue samples were collected and transferred to 1.5 ml tube, then minced and incubated with digestion buffer 200 µl (10 mM Tris-HCl, 10 mM NaCl, 25 mM EDTA and 1% sodium dodecyl sulfate) supplemented proteinase K (1 mg/ml). DNA was extracted using phenol: chloroform: isoamyl alcohol at ratio of 25:24:1 and

Precipitated by ethanol. Total DNA was re-suspended with TE buffer (0.1 mM Tris-HCl and 0.1 mM EDTA) and preserved at -20 ˚C.

PCR Conditions

The specific primers were applied to amplify cytochrome b of otters [7]. PCR was performed in a final volume of 50 µl containing 5 µl 10X PCR Buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 0.05 units/µl Taq DNA Polymerase, 0.5 µM Forward and Reverse Primer. PCR cycle was performed under the following conditions: 94 ˚C in 5 min; 40 cycles at 95 ˚C in 30 s, 56 ˚C in 30 s, 72 ˚C in 60 s; 72 ˚C in 10 min. PCR products were electrophoresed on a 1% agarose gel and stained with Gel Red. The gel was exposed to UV light and the picture taken with a gel documentation system.

Sequence analysis

The PCR products were purified and used as sequencing templates. The nucleotide sequences were directly sequenced (Macrogen, Seoul, Korea). The other of sequences were derived from Gen Bank. The misleading data from the ends of sequencing fragments were removed by the sequence trimming. The Clustal W was applied to perform the sequences alignment [8]. The Tamura & Nei model was used as a genetic distance model. The phylogenetic tree was constructed by the neighbor-joining method [9].

Results and Discussion

In this study, 8 samples of otters were used to analyze variable positions in cytochrome gene (Figure 1). A fragment of 1131 base pairs was amplified, however a nucleotide sequence of 940 base pairs was used for the analysis due to the higher quality and reliability of sequencing after manual edition. These sequences showed 189 polymorphic sites, representing 20.1% of the total DNA sequence analyzed (940 bp). These otters from Vietnam Central Highland were separated into three groups, including VN group 1 (samples LD1, LD2, LD3, LD4), VN group 2 (samples LD5 and LD6), and VN group 3 (samples LD7 and LD8).

The otters from VN group 1 showed the homologous positions to *Aonyx cinerea*, however, 8 variable positions (at position 102, 120, 209, 406, 588, 678, 688, and 906) were found in VN group 1 comparing to *Aonyx cinerea*. The otters of VN group 2 showed the homologous positions to *Lutrogale perspicillata*, however, 20 variable positions were found in VN group 2 Comparing to *Lutrogale perspicillata*.

The otters from VN group 3 showed the homologous positions to *Lutra lutra*, however, 2 variable positions (at position 72 and 993) were found in VN group 3 comparing to *Lutra lutra*.

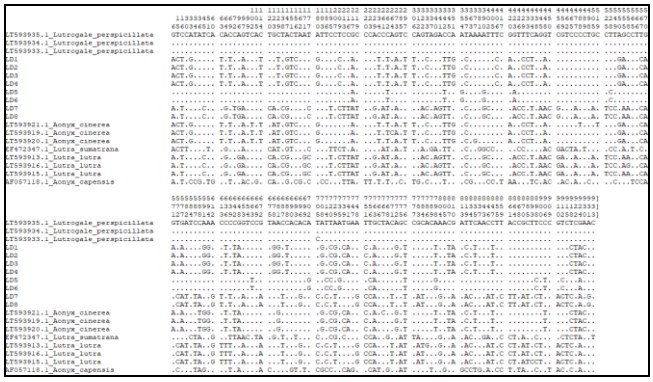

Fig 1: Variable positions of the cytochrome b gene in otters. Sequence identities are indicated by dots.

The genetic distance between groups was calculated based on the maximum-likelihood method showed that all haplotypes of VN group 1 (mean ± S.E., 0.000 ± 0.000 within group), all haplotypes of VN group 2 (0.000 ± 0.000 within group), all haplotypes of VN group 3 (0.000 ± 0.000 within group) (Table 1). This suggested that these groups from Vietnam Central Highland are less variable than other groups such as LL group (0.035 ± 0.005), LP group (0.001 ± 0.001), AC group (0.004 ± 0.001).

The VN group 1 showed lowest distance with AC groups (0.010 ± 0.003) comparing to LL (0.117 ± 0.016) group and LP group (0.073 ± 0.011) (Table 2). The VN group 2 showed lowest distance with LP groups (0.022 ± 0.005) comparing to LL (0.102 ± 0.014) group and AC group (0.078 ± 0.012). The VN group 3 showed lowest distance with LL groups (0.024 ± 0.004) comparing to LP (0.106 ± 0.015) group and AC group (0.121 ± 0.017).

Table 1: Mean distance within otter groups

Groups	Mean distance (within group)	Standard error
VN group 3	0.000	0.000
VN group 2	0.000	0.000
VN group 1	0.000	0.000
LL group	0.035	0.005
LP group	0.001	0.001
AC group	0.004	0.001

Table 2: Matrix of Tamura & Nei genetic distance among other groups. Lower triangular matrix values were mean genetic distances, Upper triangular matrix values were standard errors.

Groups	VN group 3	VN group 2	VN group 1	LL group	LP group	AC group
VN group 3		0.014	0.017	0.004	0.015	0.017
VN group 2	0.098		0.012	0.014	0.005	0.012
VN group 1	0.120	0.079		0.016	0.011	0.003
LL group	0.024	0.102	0.117		0.015	0.016
LP group	0.106	0.022	0.073	0.108		0.012
AC group	0.121	0.078	0.010	0.118	0.075	

The neighbor-joining tree was applied to assess the phylogenetic relationship of otters from Vietnam Central Highland and other otters (Figure 2). Haplotypes clustered into three lineages: VN group 1 and *Aonyx cinerea* with 100% bootstrap probability, VN group 2 and *Lutrogale perspicillata* with 100% bootstrap probability, VN group 3 and *Lutra lutra* With 100% bootstrap probability. However, the otters of VN group 1 were clustered in separated branch with 99% bootstrap probability and the otters of VN group 2 also were clustered in separated branch with 100% bootstrap probability. Moreover, the otters of VN group 3 also were clustered in separated branch with 99% bootstrap probability.

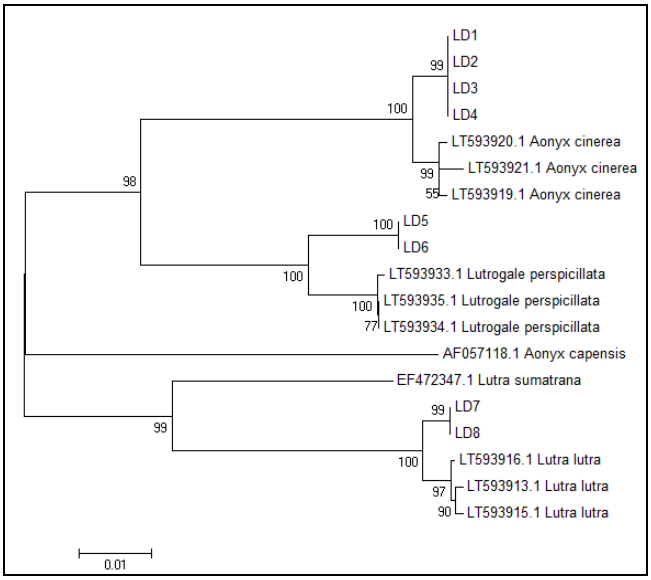

Fig 2: Phylogenetic tree constructed from cytochrome b sequences of otters by the neighbor-joining analysis method. Bootstrap resampling was done 1000 times, and resulting bootstrap values are shown on the corresponding branches.

In this study, we found that 3 species of otters have distributed in Vietnam Central Highland, demonstrated by cytochrome b gene analysis. These otters are river otters and belong to Eurasia group including *Lutra lutra* (Eurasian otter), *Aonyx cinereus* (Asian small-clawed otter), and *Lutrogale perspicillata* (smooth-coated otter). *Lutrogale perspicillata* showed the large distribution from India, Nepal across to Indochina to Malaysian Peninsula, Sumatra, and Java Island [7]. The large distribution is also observed in the small-clawed otter (*Aonyx cinerea*) which the world's smallest otter is weighing less than 5 kg [10]. They live in salt marshes and freshwater wetlands in Bangladesh, Myanmar, India, South China, Taiwan, Laos, Malaysia, Indonesia, the Philippines, Thailand and Vietnam. The previous study has not showed the specific area for otter sample collection in Vietnam. In the present study, the otter's samples were collected from Vietnam Central Highland and assessed the phylogenetic relationship by cytochrome b. Three other species were found in this area, suggesting that there is a diversity of otters in Vietnam Central Highland. However, in the past 30 years, the otters have been globally declining, many parts of the world rarely notice the distribution of otters or they are facing extinction [11, 12]. There are many reasons for this decline as their habitat is fragmented or lost, water pollution, hunting, and illegal trade. This declining has also appeared in Central Highland.

Conclusion

The present study assessed the variable position and genetic relationship of otters from Vietnam Central Highland. In the further research, we will be carried out to evaluate the distribution and genetic relationship of otters in other areas of Vietnam, and the results of these studies could contribute to the preservation of otters.

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