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Research Article

CYTOCHROME B BASED GENETIC RELATIONSHIP OF WILD BOARS FROM DAK NONG PROVINCE OF VIETNAM

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ABSTRACT

This study aimed to estimate the genetic relationship of wild boars from Dak Nong province by cytochrome b. The sequence alignment showed 26 single-nucleotide polymorphisms (SNPs) between haplotypes from Dak Nong with others from Asia and Europe. A highly variable region was from the position at 15029 to 15045. The haplotype DKN3 and Vietnamese native wild boar haplotypes showed identical SNPs (TATG), while the others (DKN1 and DKN4-DKN7) and Vietnamese hybrid wild boar haplotypes exposed two SNPs (CATA and CATG). The haplotypes DKN1, DKN4-DKN7, and Vietnamese hybrid wild boar haplotypes had a close genetic relationship with Asian wild boar haplotypes, whereas the haplotype DKN3 and Vietnamese native wild boar haplotypes were located in the separated clade of the phylogenetic tree. These results indicated that there were two wild boar populations in Dak Nong. This reveals that the Vietnamese native wild boars and Vietnamese hybrid wild boars have been distributed throughout the Central Highlands.

Keywords: cytochrome b; phylogenetics; variable position; wild boars

1. Introduction

Previous studies of molecular phylogeny have reported that wild boars are genetically separated into Asian and European clusters (Giuffra et al., 2000; Larson et al., 2005; Scandura et al., 2011). mtDNA analysis demonstrated that the amounts of genetic variation of wild boars from South-Eastern Asia generally were greater than European wild boars (Vilaca et al., 2014).

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Most other *Sus* species can be found in Southeast Asia, which is a biodiversity hotspot and has been considered to be the phylogenetic origin of wild boars (*Sus scrofa*) (Amills et al., 2018). In comparison to wild boars from other parts of Asia, Southeast Asian wild boars showed substantial genetic diversity in nearly all major East Asian lineages (Wu et al., 2007).

Mitochondrial DNA (mtDNA) of pigs is a 16 kb circular molecule, including 13 protein-coding genes, 22 tRNA, and genes responsible for 12S and 16S rRNA (Tsai et al., 2016; Niu et al., 2019). Genetic variability at the cytochrome b (*Cyt b*) region has been used as a tool for dissecting the genetic relationships between different breeds and populations due to its roles as control region sequences where the variations are more than the other regions of mtDNA (Jadav et al., 2014; Pradhan et al., 2018). This means that the evolution in Cyt b is the fastest among animal genomes, making it the most favorable to be accessed the evolutionary information of closely related groups or determined intraspecific phylogenies. The cytochrome b haplotypes of Asia's pigs have been used for determining the appeared frequencies among domestic pigs in Spain (Clop et al. 2004) and evaluating the genetic characterization, phylogeography on the European native pigs which has been crossbred with immigration ones (Garcias et al. 2011) also revealed by Cyt b region. Here, in Vietnam, researches on the phylogenetic relationships of the pig's group were conducted and also got some significant results providing evidence about the domestication or geographical distribution of these pig populations in the north region of Vietnam (Ishiguro et al. 2008).

The Vietnam Central Highlands is an area with high biodiversity of wild boars in Vietnam. In our previous study, we have assessed the genetic relationship of wild boars from four provinces in central highlands, including Kon Tum province, Gia Lai Province, Dak Lak Province, and Lam Dong Province. However, the genetic diversity of wild boars in Dak Nong province has not been well characterized, northeir genetic relationship with other wild boar populations in the Central Highlands. This study, therefore,was conducted to determine classification and the genetic relationship of wild boars from Dak Nong province assessed by cytochrome b.

2. Materials and methods

2.1. Sample collection

Ear tissue samples were collected from wild boars in Dak Nong province. Sample DKN1 was collected from Đăk Mil District, Dak Nong; sample DKN3 was collected from Tuy Duc District, Dak Nong; and samples DKN4-DKN4 were collected from Gia Nghia, Dak Nong. All the specimens were permitted from the local authorities. A small piece of each sample was washed with 0.9% saline and put in different zip-lock bags. These bags were placed in ice containers and instantly delivered to the laboratory. The samples were then washed with distilled water and stored at -20°C.

2.2. DNA isolation and PCR

Total DNA was extracted by GeneJET Genomic DNA Purification Kit (K0721, Thermo Scientific) according to the manufacturer's instructions. Each PCR reaction was

carried out with the final volume of 25 µl containing 2.5 µl Master Mix, 1 µl DNA template, 1 µl Primer (Forward and Reverse), 20.5 µl distilled water (iProof HF Master Mix (1725310, Biorad). Primers specific for *Cyt b* gene were F: 5'-CAC GAC CAA TGA CAT GAA AAA TC-3' and R: 5'-GCT GCG AGG GCG GTA AT-3' (Fernández et al., 2008). The thermal cycle was performed under the following conditions: one cycle at 94°C for 5 min; 40 cycles at 94°C for 30 sec, 55°C for 45 sec, 72°C for 45 sec; and one cycle at 72°C for 10 min. Amplified products were visualized following electrophoresis in 1% agarose gel.

2.3. Sequence analysis

The PCR products were purified by ExoSAP-IT™ Product PCR Cleanup reagent (Thermo Fisher Scientific, US) and used as sequencing templates. These templates were sequenced at Macrogen (Seoul, Korea). Both ends of obtained sequences were trimmed off to remove misleading data. The size of *Cyt b* sequences after trimming was 552 bp. *Cyt b* sequences from Vietnamese wild boars and other wild boars from Genbank were compared. Phylogenetic and molecular evolutionary were analyzed by MEGA version 6 (Tamura et al., 2013).

The wild boar sequences of Dak Nong (DKN1 and DKN3-DKN7) were estimated the relationship with the sequences of Asian wild boars and European wild boars from Genbank (AF136541 - AF136553), Vietnamese native wild boars (VN_native_WB1 - VN_native_WB5), and Vietnamese hybrid wild boars (VN_hybrid_WB1 - VN_hybrid_WB5) (Long et al., 2014). The wild boar sequence with accession number NC_000845 was used as a reference. The sequence alignment was performed with CLUSTAL W (Tamura et al., 2011). Genetic distances were accessed by Tamura & Nei model. The neighbor-joining method was applied for phylogenetic construction (Saitou & Nei, 1987). Bootstrap analysis (using 1000 replications) was used to acquire confidence in branching order.

3. Results and discussion

3.1. Results

In this study, 6 *Cyt b* sequences of Dak Nong wild boars (DKN1, DKN3-DKN7) were compared with 24 other sequences. Sequence alignment displayed 26 single-nucleotide polymorphisms (SNPs), accounting for 5.4% of the total sequence (483 bp). A highly variable region from position 15029 to 15045 was detected, which had 35.3% of the substitution rate, 6.5 times than the overall substitution rate (5.4%). The SNPs analysis show that all obtained haplotypes had a close relationship with the Asian wild boar, expressed at positions 15038 (G→A), 15041 (C→T), and 15200 (G→A) (Figure 1) Haplotypes DKN1, DKN4-DKN7 had characteristic variable positions of Vietnamese hybrid wild boar, including positions 14855 (A→G) and 15002 (T→C); while haplotype DKN3 carried the typical changes of Vietnamese native wild boar at positions 14780 (C→T) and 14963 (T→C). This result indicated that wild boar haplotypes obtained from the study were divided into two groups: group 1 (DKN1, DKN4-DKN7) had a close relationship with Vietnamese hybrid wild boars and Asian wild boars, and group 2 (DKN3) was related to Vietnamese native wild boars.

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                                    1111111111 1111111111 111111
                                    4444444444 4555555555 555555
                                    7177788999 9000000000 111222
                                    4768905689 9012333446 017001
                                    5790575378 9279368150 239052
NC_000845                           GAACCAATAC TTTTGTGCGA ATAGTC
Me_{group=A1}                       ......G... .C...CATA. ...A..
AWB10_{group=A1}                    ......G... .C...CAT.. ...A..
LW1_{group=A2}                      ......G... .C...CAT.. ...A.T
AWB11_{group=A3}                    .....TG... .C....AT.. .C.A..
EWB1_{group=E1}                     .......... .......... G.....
EWB2_{group=E1}                     .......... .......... G.....
H1_{group=E1}                       .......... .......... G.....
H2_{group=E1}                       .......... .......... G.....
L1_{group=E1}                       .......... .......... G.....
L2_{group=E1}                       .......... ...C...... G.....
LW2_{group=E1}                      .......... .......... G.....
Ma_{group=E1}                       ....T..... ..C....... G.....
EWB3_{group=E2}                     ...T...... .......T.. ......
VN_hybrid_WB1_{group=VN_Hyrid}      ......G... .C...CATA. ...A..
VN_hybrid_WB2_{group=VN_Hyrid}      ......G... .C...CAT.. ...A..
VN_hybrid_WB3_{group=VN_Hyrid}      A.....G... .C...CATA. ...A..
VN_hybrid_WB4_{group=VN_Hyrid}      A.....G... .C...CAT.. ...A..
VN_hybrid_WB5_{group=VN_Hyrid}      ......G... .C...CATA. ...A..
DKN1_{group=DKN_group_1}            ......G... .C...CAT.. ..GA..
DKN4_{group=DKN_group_1}            ......G... .C...CAT.. ...A..
DKN5_{group=DKN_group_1}            ......G... .C...CAT.. ...A..
DKN6_{group=DKN_group_1}            ......G... .C...CATA. ...A..
DKN7_{group=DKN_group_1}            ......G..T ....ACAT.T ..GA..
DKN3_{group=DKN_group_2}            ...T...CG. C.....AT.. ...A..
VN_native_WB1_{group=VN_Native}     .GGT...C.. ......AT.. ...AC.
VN_native_WB2_{group=VN_Native}     ...T...C.. ......AT.. ...A..
VN_native_WB3_{group=VN_Native}     ...T...C.. ......AT.. ...AC.
VN_native_WB4_{group=VN_Native}     ...T...C.. ......AT.. ...A..
VN_native_WB5_{group=VN_Native}     ...T...C.. ......AT.. ...A..
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Figure 1. *Variable positions of the cytochrome b sequences. Sequence identities are indicated by dots. The Sus scrofa mitochondrion genome (accession number: NC_000845) was used as the reference sequence*

The within-group distance of the Vietnamese hybrid wild boars and Vietnamese native wild boars was 0.0043 ± 0.0017 and 0.0039 ± 0.0018, respectively, lower than that of the Asian group (0.0052 ± 0.0024) and higher than the European group (0.0028 ± 0.0011). This suggested that the European wild boars had the lowest diversity of the four groups, followed by the Vietnamese native wild boars, the Vietnamese hybrid wild boars, and the Asian wild boars.

The genetic distance between groups of native and hybrid wild boars in Vietnam had a closer relationship with Asian wild boars than with European wild boars (Table 1). The Vietnamese hybrid wild boars were almost similar to the Asian group with the genetic distance almost zero, while the genetic distance with the European group was 0.014 ± 0.005. Meanwhile, in the Vietnamese native wild boar group, the genetic distance with Asian wild boar was 0.010 ± 0.004 and with European wild boar was 0.011 ± 0.005. The genetic distance at 0.011 ± 0.005 between hybrid and native wild boars in Vietnam also showed the difference between these two groups.

Table 1. *Matrix of Tamura and Nei genetic distance among sequences. Lower triangular matrix values were mean genetic distances and upper triangular matrix values were standard errors*

	Asian	European	VN_hybrid	VN_native
Asian		0.005	0.000	0.004
European	0.013		0.005	0.005
VN_hybrid	0.000	0.014		0.005
VN_native	0.010	0.011	0.011	

The phylogenetic tree was built based on the genetic distance between wild boar groups, according to the Neighbor-joining algorithm (Figure 2). All haplotypes were split into three main clades. Clade E included two main subclades, E1 and E2 from the European group. Clade A was synonymous with the Asian group, divided into three subclades: A1, A2, and A3. Haplotypes DKN1, DKN4-DKN7 from this study were all located close to clade A1 and A2 of the Asian subspecies (bootstrap 49%). Meanwhile, haplotype DNK3 was classified into a separate group, located with the Vietnamese native wild boar in the previous study and isolated from the two main clades A and E of Asia and Europe (bootstrap 87%).

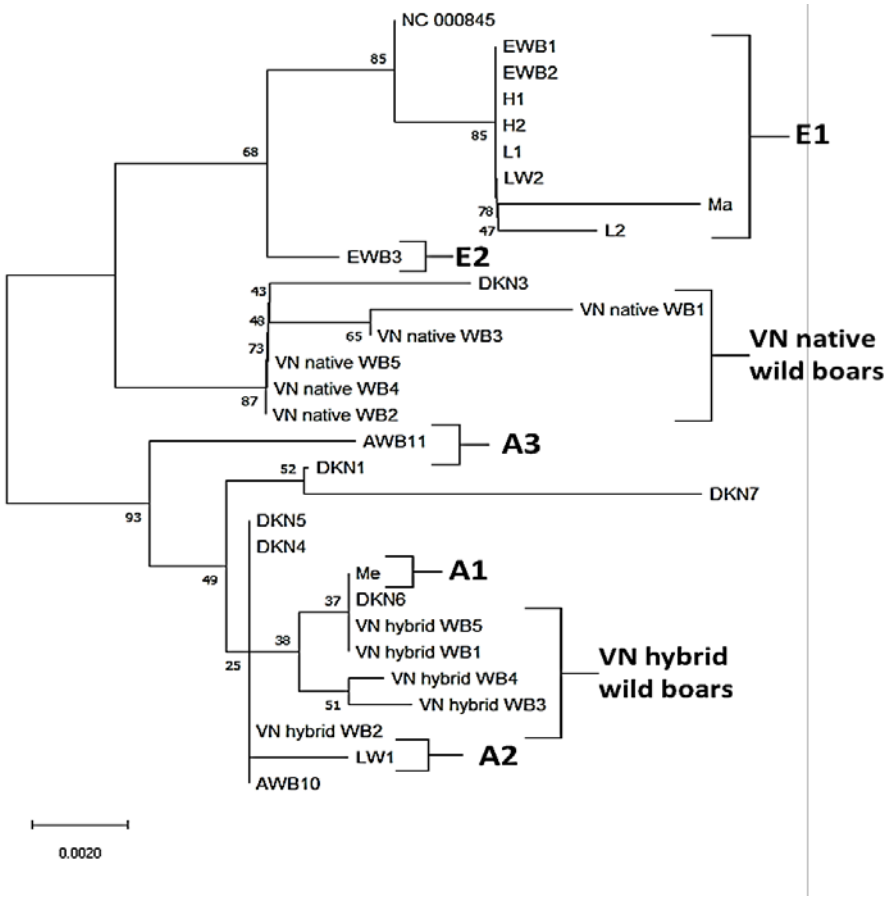

Figure 2. *Phylogenetic tree conducted from cytochrome b sequences by the neighbor-joining analysis method. Bootstrap resampling was done 1000 times, and resulting bootstrap values are shown on the corresponding branches*

3.2. Discussion

Vietnam is one of the hotspot regions in the Indo-Burma region which exhibited the remarkable peculiarity of pig populations. The Indo-Burma region has been considered the homeland of the *Sus* genus, demonstrated by six out of eight species that are endemic to this area (Mona et al., 2007). There are two wild boar species reported in Vietnam, concluding *Sus bucculentus* and *Sus scrofa*. However, *Sus bucculentus* was described more than a century ago but has not been reported since and has remained in the 'mystery' category (Groves et al., 1997). *Sus Suscrofa* was a popular wild boar in Vietnam. The wild boar populations in Vietnam Central Highland have been genetically characterized by using mitochondrial genes. In this investigation, cytochrome b was applied to estimate the genetic relationship of wild boars from Dak Nong.

Pig mitochondrial DNA contains four single nucleotide polymorphisms in the cytochrome b gene, including 15036 (T/C), 15038 (G/A), 15041 (C/T), and 15045 (G/A) (Clop et al., 2004). In this study, the European haplotypes E1 (TGCG) and E2 (TGTG) were not observed in Dak Nong wild boars and other wild boars from Vietnam (Jones, 1998; Giuffra et al., 2000). The haplotype DKN3 located in the Vietnamese native wild boar group and showed identical SNPs (TATG) while others (DKN1 and DKN4-DKN7) were located in the Vietnamese hybrid wild boar group with two SNPs (CATA and CATG). This result suggested that there were at least two wild boar populations in Dak Nong province.

In this study, the haplotypes DKN1 and DKN4-DKN7 were crossbred for production in various husbandry farms. These haplotypes have shared similar SNPs with Vietnamese hybrid wild boars and Asian wild boars. This revealed that the process of inter-specific or intra-specific hybridization of an invasion population with native or non-native populations was occurred in Dak Nong province, leading to the generation of novel genotypes (Lee, 2002). The haplotype DNK3 shared the same SNPs with Vietnamese native wild boars, suggesting that the distribution of Vietnamese native wild boars was extended to Dak Nong province. In the previous study, three pig populations were detected in Vietnam Central Highland (Gia Lai, Kon Tum, Lam Dong, and Dak Lak), concluding one domestic pig population and two wild boar populations (Long et al., 2014). This study also determined two wild boar populations in Dak Nong province, disclosing that Vietnam native wild boars and Vietnam hybrid wild boars have been distributed throughout the Central Highlands.

4. Conclusion

The cytochrome b analysis revealed two wild boar populations in Dak Nong province. This result indicated that the Vietnamese native wild boars and Vietnamese hybrid wild boars have a large distribution in Central Highlands.

❖ ***Conflict of Interest:*** *Authors have no conflict of interest to declare.*

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QUAN HỆ DI TRUYỀN DỰA TRÊN GENE CYTOCHROME B Ở HEO RỪNG VIỆT NAM TỈNH ĐẮK NÔNG

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TÓM TẮT

Nghiên cứu nhằm đánh giá mối quan hệ di truyền của heo rừng tỉnh Đắk Nông dựa trên trình tự gene cytochrome b. Kết quả sắp xếp trình tự cho thấy tồn tại 26 điểm đa hình đơn nucleotide (SNP) giữa các haplotype ở heo rừng Đắk Nông với các haplotype khác từ châu Á và châu Âu. Vùng biến đổi mạnh xuất hiện từ vị trí 15029 đến 15045. Haplotype DKN3 và nhóm heo rừng bản địa Việt Nam biểu hiện một vị trí các SNP giống hệt nhau là TATG; trong khi các haplotype DKN1, DKN4-DKN7 và nhóm heo rừng lai Việt Nam cùng biểu hiện hai vị trí SNP CATA và CATG. Các haplotype DKN1, DKN4-DKN7 và nhóm heo rừng lai Việt Nam có mối quan hệ di truyền chặt chẽ với lợn rừng châu Á; trong khi haplotype DKN3 và heo rừng bản địa Việt Nam nằm trong nhánh tách rời của cây phát sinh loài. Kết quả này cho thấy có hai quần thể heo rừng ở Đắk Nông, dẫn đến kết luận rằng heo rừng bản địa Việt Nam và heo rừng lai Việt Nam có sự phân bố trải rộng khắp Tây Nguyên.

Từ khóa: cytochrome b; quan hệ phát sinh loài; vị trí biến đổi; heo rừng