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Short Communication: Genetic identification of local pigs, and imported pigs (Landrace and Duroc) based on cytochrome b sequence analysis

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Abstract. *Hartatik T, Soewandi BDP, Volkandari SD, Tabun AC, Sumadi, Widodo. 2016. Genetic identification of local pigs, and imported pigs (Landrace and Duroc) based on cytochrome b sequence analysis. Biodiversitas 17: 270-274*. The aim of this study was to identify the genetics of local pigs and imported pigs (Landrace and Duroc) based on qualitative analysis. Thirty-eight pigs were used in this study and consisted of 11 local pigs (from Bali), six Landrace pigs and four Duroc pigs (from Malang), nine Landrace pigs (from Bali) and eight local pigs (from Kupang). Qualitative traits in pigs such as coat color, body shape (back shape, belly shape, and ears) and hair cover were observed. The cytochrome b (*Cyt* b) gene of mitochondrial DNA was analyzed using PCR-restriction fragment length polymorphism. The PCR analysis resulted in a 464 base pairs (bp) amplified band, and this was digested using *Taq*I restriction enzyme. The PCR-RFLP analysis resulted in two bands, 246 and 218 bp (monomorphic). The alignment analysis showed four points of single nucleotide polymorphisms. Bali and Kupang pigs had a specific pattern on exterior characteristics such as curved back shape, belly hanging shape, small ears and thick hair, and had many variations on coat color such as black, cream, spotted and mottled. The differences in coat color and body shape, and the corresponding mtDNA *Cyt* b sequence (with four SNPs) is a marker for genetic variation in pigs.

Keywords: Coat color, mtDNA, cytochrome b, qualitative analysis, genetic variation

INTRODUCTION

Indonesia has a great variety of domestic animals such as native chickens, ducks, goats, sheep, cattle and pigs. There are five species of local pig in Indonesia, *Sus barbatus* (Babi Berjanggut), *Sus celebenis* (Babi Sulawesi Berkutil), *Sus verrucous* (Babi Jawa Berkutil), *Sus scorfa* (Babi Alang-Alang) and *Babyroussa babyrussa* (Babirusa) (Rothschild et al. 2011). Genetic variations of local pigs in Indonesia are important to investigate. Since these pigs have important roles for cultural activities in the society, it is necessary to maintain their sustainability. The local Kupang pigs were used for traditional ceremonies such as weddings and religious ceremonies (Johns et al. 2010). For Bali cultural activities, pigs were used as the oblation for the religious activities. The oblation pig was a young intact boar up to eight or nine months old which had been fattened for five to six months (Soewandi 2013). The study of genetic diversity based on mitochondrial DNA (mtDNA) as a maternal line has been a reportedly useful tool as a molecular marker. Mitochondrial DNA is maternally inherited and experiences nucleotide changes faster than DNA (Brown et al. 1979), which makes mtDNA an ideal tool for studying population genetics (Bailey et al. 2000). Mitochondrial DNA is a useful genetic marker for both intra- and interspecies studies (Brown et al. 1979; Kikkawa et al. 1995).

Continental of wild boars and domestic pigs were clearly divided into eastern and western clades (Larson et al. 2005; Wu et al. 2007; Leutkemeier et al. 2010) using d-loop mitochondrial DNA. A previous study based on Single Nucleotide Polymorphism (SNP) revealed that population of wild boars from Near Eastern Asia (Turkey, Iran and Armenia) and Europe (Spain, Belgium and Russia) are genetically different (Manunza et al. 2013). Asian pig populations were comprised of three groups. One group is represented by Erhualian and Meishan breed, while the second represented by Lanyu pigs and the third represented by the Asian wild boars. The Asian domestic populations were derived from multiple Asian ancestral origins whereas the European domestic populations represent a single ancestral European lineage (Leutkemeier et al. 2010).

Most genetic studies on wild boars in East Asia were carried out using mtDNA sequence analysis, which revealed several subclades (Larson et al. 2005; Hongo et al. 2002; Cho et al. 2009; Ramayo et al. 2010; Ji et al. 2011; Larson et al. 2010). Previous studies based on both mtDNA and nuclear genes demonstrated no population substructures exists in neither wild boars nor domestic pigs

in East Asia and showed a very high level of admixture between them (Ji et al. 2011). Korean wild boars were clearly clustered within Asian wild boar groups, sharing the same cluster with populations from Myanmar and Thailand (Cho et al. 2009) and the Vietnamese wild pig haplotype (Hongo et al. 2002). On the other hand, Larson et al. (2010) ascertained that wild boars in South Korea belong to groups unique within East Asia, and remain differentiated from domestic pigs.

Based on the mitochondrial DNA, the Vietnamese wild boars were clustered into two groups, group I was genetically distinct to Asian wild boars and group II that was genetically close to Asian wild boars. There are three types of haplotype in Vietnamese domestic pigs and two types of haplotypes in Vietnamese wild boars in Central Highland (Long et al. 2014). Chinese pig breeds were originally from the wild boars in the South China and the Yangtze River Region (Yu et al. 2013). The others studies showed that Chinese native pig breeds had a single origin (Lan and Shin 1993; Huang et al. 1999).

Study on the cytochrome b gene of indigenous pigs with PCR-SSCP methods had previously identified four SNPs located at positions 47 (T/C),49 (G/A), 52 (C/T) and 56 (G/A) and revealed the Asian origin of the Indigenous pigs (Ghungroo, Meghalaya local and Nagaland local) with A1 haplotype which revealed absence of mixed haplotype (Saikia et al. 2015). Other study in India, based on the analysis of sequence generated from the partial fragment (421 bp) mtDNA cytochrome b (*Cyt* b) gene exhibited unambiguous (>3%) genetic variation between Indian wild and domestic pigs. They observed nine forensically informative nucleotide sequence (FINS) variations between Indian wild and domestic pigs (Gupta et al. 2013).

This study was conducted to determine the genetic variation of local pigs (Bali and Kupang) compared with imported pigs (Landrace and Duroc) based on coat color, body shape and mtDNA *Cyt* b by using PCR-RFLP and sequence analysis.

MATERIALS AND METHODS

Samples and DNA extraction

Thirty-eight pigs were studied, consisting of 11 local pigs (Bali), eight local pigs (Kupang, NTT), 15 Landrace pigs and four Duroc pigs. Exterior characteristics of the pigs such as coat color, body shape (back shape, belly shape, and ears) and hair cover were observed. Blood and ear tissue samples were collected for DNA analysis. Blood samples were taken through jugular venipuncture and preserved in K_3EDTA solution tubes. Samples were stored frozen (-20 °C) until needed. The DNA from blood or ear tissue samples was extracted by using the standard SDS/Proteinase K modified method according to Sambrook et al. (1989).

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

The 464 base pairs (bp) fragment of the mtDNA *Cyt* b gene was amplified by polymerase chain reaction (PCR) using forward and reverse primers according to Wolf et al. (1999): L14735 (5'-AAA AAC CAC CGT TGT TAT TCA ACTA-3') and H15149 (5'-GCC CCT CAG AAT GAT ATT TGT CCT CA-3'). Polymerase chain reaction was performed with a final volume of 20 µL of reaction mixture containing 1 µL of DNA sample (10-100 ng), 1 µL of each primer (10 pmol/ µL), 10 µL PCR KIT (Kappa, Biosystem), and 7 µL of double distilled water. The amplification process was performed using Thermocycler (Infinigen, TC-25/H) with the following conditions: initial denaturation at 94°C for 2 min, followed by 35 thermal cycles of denaturation at 95°C for 36 sec, annealing at 51°C for 73 sec, extension at 72°C for 84 sec and the final extension at 72°C for 3 min (Prado et al. 2005). The PCR product was visualized on 1% agarose gels buffered with 1X Tris-Boric-EDTA buffer (1XTBE), stained with ethidium bromide and visualised under ultraviolet (UV) light. The PCR-amplified DNA fragment of the *Cyt* b gene was digested using the *Taq*I restriction enzyme to identify genetic patterns. The total volume of digestion was 12 µL containing 3 µL PCR product, 0.2 µL *Taq*I (Fermentas) enzyme (1U), 1.2 µL Tango buffer and 7.6 µL aquabidest sterile. The enzymatic digestion was conducted at 65 °C for two hours by the *Taq*I enzyme. The digestion products were separated on 10% polyacrylamid gels in 1XTBE buffer and run with 50 V for three hours for separation of the DNA fragments. The bands were stained with ethidium bromide before visualization under UV light. The size of the amplified bands was compared with DNA marker ϕX174 DNA/*Bsu*RI (*Hae*III) (Fermentas).

Sequencing and analysis

A total volume of 30 µL for each PCR product and 10 µL *Cyt* b primer (10 pmol/µL) was prepared for sequencing. Sequencing the amplified bands of PCR products was performed by BioSM Macrogen (Korea). The DNA sequences were aligned by using BioEdit version 7.7 for identification of the single nucleotide polymorphism.

RESULTS AND DISCUSSION

The exterior characteristics of the pigs were determined based on expert judgment and included coat color and body shape. Landrace pigs had 100% white color while Duroc pigs had 100% reddish brown color. Bali and Kupang pigs had a huge diversity of color: 63.6% of Bali pigs had black coat color and 36.4% had mottled color, whereas 37.5% Kupang pigs had reddish brown color, 25% had cream color and 12.5% had spotted (white and black) color. Local pigs had specific characteristics of body shape at back and belly whereas imported pigs (Landrace and Duroc) had a straight body shape between back and belly. In addition, Bali and Kupang pigs had small, upright ears and thick hair. This is different from the Landrace and Duroc pigs that had sparse hair and big ears, with 50% having ears folded forward (Figure 1).

The specific DNA fragment of the mtDNA *Cyt* b gene in local pigs, Landrace pigs and Duroc pigs was amplified by using L14735 and H15149 primers. The PCR product of

the mtDNA *Cyt* b gene was 464 bp (Figure 2a). The product size of PCR-RFLP using the *Taq*I enzyme showed the same restriction pattern. There were two fragments of DNA, 246 bp and 218 bp (Figure 2b), which indicates that the sample population was monomorphic. In total of 38 samples, one haplotype of cytochrome b sequence was observed based on PCR-RFLP.

The sequence analysis of the mtDNA *Cyt* b gene of local pigs (Bali), Landrace pigs and Duroc pigs was compared to the complete mtDNA of *Cyt* b gene database available at GenBank (DQ.534707.2/*Sus scrofa* breed Taoyuan; NC. 014692.1/*Sus scrofa taiwanensis*; NC.012095.1/*Sus scrofa domesticus*; and GQ. 338965.1/*Sus scrofa*). Based on mtDNA *Cyt* b sequence alignment analysis (Figure 3), we found four points of single nucleotide polymorphism (SNP) which changed the nucleotides from C to T and G to A. However the local, Landrace and Duroc pigs had a similar sequence of mtDNA *Cyt* b in this study.

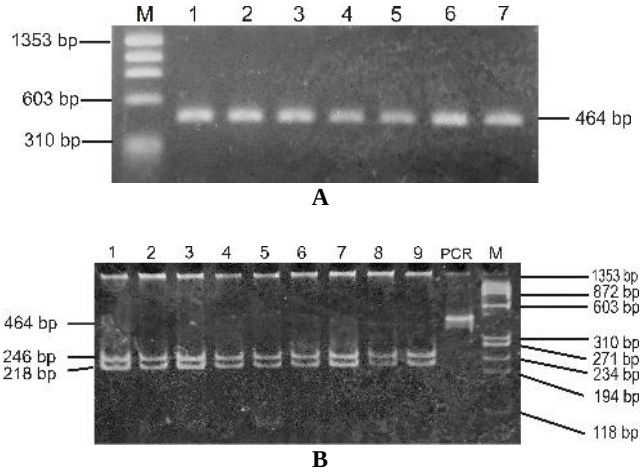

Figure 2. PCR product and RFLP with *Taq*I enzyme. A. PCR product of cyt b gene (464 bp); B. PCR-RFLP product using the *Taq*I enzyme: lanes 1-2: Duroc, lanes 3-5: Landrace, lanes 6-9: Bali pig, PCR: product, M: Marker ΦX174 DNA/*Bsu*RI (*Hae*III) (Fermentas).

Figure 1. Coat color and body shape of pigs. A. Bali pig, B. Kupang pig, C. Duroc pig and D. Landrace pig

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DQ534707.2          TACACATCAGACACAACAACAGCTTTCTCATCAGTTACACACATCTGTCG 150
NC_014692.1         TACACATCAGACACAACAACAGCTTTCTCATCAGTTACACACATCTGTCG 150
LANDRACE (BALI)     TACACATCAGACACAACAACAGCTTTCTCATCAGTTACACACATCTGTCG 150
DUROC (MALANG)      TACACATCAGACACAACAACAGCTTTCTCATCAGTTACACACATCTGTCG 150
BALI (LOCAL)        TACACATCAGACACAACAACAGCTTTCTCATCAGTTACACACATCTGTCG 150
GQ338965.1          TACACATCAGACACAACAACAGCTTTCTCATCAGTTACACACATTTGTCG 150
NC_012095.1         TACACATCAGACACAACAACAGCTTTCTCATCAGTTACACACATTTGTCG 150
                    ******************************************* *****

DQ534707.2          AGACGTAAATTACGGATGAGTTATTCGCTACCTACATGCAAACGGAGCAT 200
NC_014692.1         AGACGTAAATTACGGATGAGTTATTCGCTACCTACATGCAAACGGAGCAT 200
LANDRACE (BALI)     AGACGTAAATTACGGATGAGTTATTCGCTACCTACATGCAAACGGAGCAT 200
DUROC (MALANG)      AGACGTAAATTACGGATGAGTTATTCGCTACCTACATGCAAACGGAGCAT 200
BALI (LOCAL)        AGACGTAAATTACGGATGAGTTATTCGCTACCTACATGCAAACGGAGCAT 200
GQ338965.1          AGACGTAAATTACGGATGAGTTATTCGCTATCTACATGCAAACGGAGCAT 200
NC_012095.1         AGACGTAAATTACGGATGAGTTATTCGCTATCTACATGCAAACGGAGCAT 200
                    ****************************** ******************

DQ534707.2          CCATGTTCTTTATTTGCCTATTCATCCACGTAGGCCGAGGCCTATACTAC 250
NC_014692.1         CCATGTTCTTTATTTGCCTATTCATCCACGTAGGCCGAGGCCTATACTAC 250
LANDRACE (BALI)     CCATGTTCTTTATTTGCCTATTCATCCACGTAGGCCGAGGCCTATACTAC 250
DUROC (MALANG)      CCATGTTCTTTATTTGCCTATTCATCCACGTAGGCCGAGGCCTATACTAC 250
BALI (LOCAL)        CCATGTTCTTTATTTGCCTATTCATCCACGTAGGCCGAGGCCTATACTAC 250
GQ338965.1          CCATATTCTTTATTTGCCTATTCATCCACGTAGGCCGAGGTCTATACTAC 250
NC_012095.1         CCATATTCTTTATTTGCCTATTCATCCACGTAGGCCGAGGTCTATACTAC 250
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Figure 3. Multiple sequence alignment of partial mtDNA cyt b in pigs

The sequenced DNA data showed that Bali pig ancestors were from China, which possibly were similar to the *Sus scrofa taiwanensis* ancestor. In four positions of SNPs, the same nucleotide between Bali pig and DQ.534707.2 (Breed Taoyuan) and NC. 014692.1 (Breed Taiwanesis) was observed (Figure 3). A previous study by Sihombing (1997) revealed that the Bali pig ancestor came from a cross between a second type of *Sus scorfa vittatus* and the South China pig. This finding supports our data that Bali pigs have a genetic similarity to *Sus scrofa taiwanensis* and the Taoyuan pig. The mitochondria of the pigs was divided into two types (Asian type and Europe type), Taoyuan pig included to Asian type (Chang et al. 2008).

A previous study by Clop et al. (2004) proved that Landrace and Duroc breeds had an Asian allele. It was likely due to an introduction process of Landrace and Duroc pigs with the Asian allele. Evidence showed that there was an introduction of Asian pig breeds to commercial pig breeds (Clop et al. 2004; Giuffra et al. 2000). The introduction processes did not result in any differences in their performances, rather their mtDNA showed high genetic similarity. Mitochondrial DNA has been widely used to unravel evolutionary studies, due to its greater diversity compared to nuclear DNA.

To conclude, local pigs, Landrace and Duroc pigs have the same sequence of cytochrome b gene. The sequence analysis of seven breed of pigs shows four position of single nucleotide polymorphism.

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