Cytochrome B (Cyt B) mtDNA genetic diversity of the second filial (F2) of Banteng Bali cross in UPT-PTHPT Pucak, South Sulawesi Indonesia

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Abstract: Bali cattle are native to Indonesia and are the result of domestication from Banteng, (Bos banteng). The cytochrome B gene is widely used in systematic studies to solve differences at many taxonomic levels and has been considered as one of the genes used in phylogenetic action. Cytochrome B is also very useful for comparing species in the same genus or the same family. Genetic characterization tests were carried out to identify the characteristics of mtDNA specific identifiers of Bali cattle with Banteng Bali cross (F2). The edited nucleotide sequences are then aligned using the Clustal W program contained in the MEGA 6 software. Construction of the phylogeny tree is carried out using the MEGA 6 program based on p-distance values. Phylogeny tree construction uses bootstrap UPGMA method with 1000 replication. Based on genetic distance analysis, it shows that Bali cattle and Banteng Bali cross (F2) with Bos javanicus have a genetic distance that is relatively the same as the genetic distance value (0.000–0.001) but has a great genetic distance with the Bos taurus, Bos indicus and Bubalus bubalis with genetic distance values (0.050–0.094). This shows that in cytochrome B, Bali cattle and Banteng Bali cross F2 have identical genetic distances. Based on the UPGMA tree phylogeny method, the results show that the kinship group 1 (Bali cattle (CBB3 and CBB7), Banteng Bali cross (F2) (CBC1, CBC7, CBC8, and CBC9) with Bos javanicus) and group 2 (Bali cattle (CBB2, CBB4, CBB8, CBB9) and Banteng Bali cross (F2) CBC10) are in the same branch showing that the results are relatively similar and have a close kinship. It can be concluded that the characteristics of mtDNA cytochrome B in Bali cattle and Banteng Bali cross F2 were relatively the same and both have a very close relationship.

1. Introduction

Bali cattle are native to Indonesia and are the result of domestication from Banteng (Bos banteng) [1,2]. The distribution of the Bali cattle population has expanded to cover all parts of Indonesia, including the island of Sulawesi. The largest concentration of Bali cattle is in South Sulawesi, Timor island, Bali and Lombok. Bali cattle are excellent among beef cattle in Indonesia because they have high reproductive capacity, high...
percentage of carcass, low-fat meat, high positive heterosis in crossing, high adaptability to the harsh environments and birth rates can reach 80% [3,4].

The importance of developing, purifying and improving genetic quality of Bali cattle is caused by several factors, among others, because Bali cattle are native cattle and Indonesian germplasm, but Bali cattle have decreased genetic quality due to crossing with seedlings that are not superior. Bali cattle are now thought to experience a decline in performance compared to Bali cattle in the past.

Considerable attention to preservation of germplasm is by establishing a national breeding program for Bali cattle. The national program includes a purification program and genetic improvement for Bali cattle. The Bali cattle purification program is carried out by establishing a pure Bali cattle breeding area which includes Bali island, Sumbawa island in West Nusa Tenggara province, Flores island in East Nusa Tenggara province, Bone and Barru regency in South Sulawesi province at the same time the area was designated as a national source of Bali cattle breeds [5].

One of the programs that can be carried out to restore the genetic purity of Bali cattle is the introduction of genetic blood from their wild ancestors, namely *Bos banteng* or a cross between Bali cattle and Banteng (Banteng Bali cross). One of the existing Banteng Bali cross is Jaliteng cattle, which was successfully developed by the Singosari Artificial Insemination Center, East Java. The results of these crosses are reported to have better performance, where birth weights are in the range of 15─21 kg, live weight reaches 450 kg, higher than the average live weight of ordinary Bali cattle which only reaches around 300 kg [6].

Genetic characterization studies at the molecular level in local livestock in Indonesia are still rare. One of the DNA markers that can be used to determine genetic relationships among livestock is cytochrome B of mitochondrial DNA. The cytochrome B gene is widely used in systematic studies to solve differences at many taxonomic levels and has been considered as one of the genes used in phylogenetic work. The purpose of this study was to identify the genetic relationships between Bali cattle and their ancestor, Banteng (*Bos banteng*).

2. Materials and method

2.1. Sampling

The study was conducted by collecting blood samples from 10 heads of Banteng Bali cross (F2) and 9 heads of Bali cattle at UPT-PTHPT Pucak, Tomobobu Sub-district, Maros Regency (see table 1). Genetic characterization test was carried out at the Laboratory of Integrated Biotechnology, Faculty of Animal Science, Hasanuddin University to identify the characteristics of mtDNA of the specific identifiers of Bali cattle with Banteng Bali cross (F2).

| Breed type                  | Sex  | Number of animal (head) |
|-----------------------------|------|-------------------------|
| Bali cattle                 | Male | 5                       |
|                             | Female | 4                      |
| Banteng Bali cross (F2)     | Male | 4                       |
|                             | Female | 6                      |
| **Total**                   |      | **19**                  |

Blood sampling is done by collecting about 5 ml of blood samples through the jugular vein using a venojet and vacuttainer tubes with EDTA anticoagulant. The blood samples then were stored at -20°C for the next analysis.
2.2. DNA extraction and PCR analysis
A total of 200 μL blood samples were lysed by adding 200 μL of lysis buffer solution and 20 μL proteinase K (10 mg/mL), then incubated at 60°C for 60 minutes in a water bath shaker. After incubation, the solution is then added with 200 μL of 96% absolute Ethanol and centrifuged 15,000 g for one minute.

DNA extraction results were amplified by PCR (Polymerase Chain Reaction) method using forward primer (5’-TTCTTACATGGAATCTAACCATGA-3’) and reverse primer (5’-GGGAGGTTAGTTCTCCTTCT-3’) with a target of 1276 bp [7]. The composition of the PCR reaction was conditioned at a reaction volume of 25 μL consisting of 100 ng DNA, 0.25 mM for each forward and reverse primer, 10 mM dNTP, 50 mM MgCl₂, 50U/μL Taq DNA polymerase and 10× buffer.

PCR conditions begin with initial denaturation at a temperature of 94°C × 5 minutes, followed by 35 subsequent cycles of each denaturation of 94°C × 45 seconds, with annealing temperature of 57°C × 45 seconds, followed by an extension of 72°C × 60 seconds, which then ends with one final extension cycle at 72°C for 10 minutes using a PCR machine.

PCR products that show a single clear DNA band from each sample are then purified for further sequencing. The primers used for sequencing are the same primers used for DNA amplification by PCR and DNA sequencing was done by using the ABI Prism 3700-Avant Genetic Analyzer machine.

2.3. Computational analysis
The edited nucleotide sequences are then aligned using the Clustal W program contained in the MEGA 6 software. The construction of the phylogeny tree is carried out using the MEGA 6 (Molecular Evolutionary Genetic Analysis) program based on p-distance values ie the number of different nucleotides divided by the number of nucleotides total nucleotides are compared. The phylogeny tree construction uses bootstrap UPGMA method with 1000 replication [8].

3. Results and discussion

3.1. Cyt B amplification
The results of cytochrome B gene amplification using PCR techniques were analyzed by PCR machines and visualized in agarose gel can be seen in figure 1. The length of the cytochrome B gene amplification results was 1276 bp and the target sequencing in the cytochrome B gene was 1140 bp. This is consistent with the research of Kim et al (2007), that the length of fragments in the amplification of the cytochrome B gene used in the study was 1140 bp [9].

3.2. Analysis nucleotide compositions
Based on the size of the nucleotide sequences that have been aligned along 992 bp which can be analyzed, the results of the alignment of the Bali cattle nucleotide, Banteng Bali cross F2 and access from GenBank are the Bos javanicus (JN632606.1), the Bos taurus (MN200930.1), the Bos indicus (KX575711.1) and Bubalus bubalis (MK234704.1), so the average nucleotide composition can be seen in table 2.
Figure 1. Results of cytochrome B amplification on PCR machines, M: 100 bp line markers 1–3 : samples of Bali cattle; line 4–6: sample Banteng Bali cross F2, bp: base pair.

The average order of the frequency of Cytochrome B DNA nucleotides in Bali cattle, Banteng Bali cross F2 and access from GenBank are Bos javanicus (JN632606.1), Bos taurus (MN200930.1), Bos indicus (KX575711.1) and Bubalus bubalis (MK234704.1) at most high in nucleotides A (30.7%), then C (29.7%), T (26.2%) and G (13.4%). In Bali cattle the comparison of the average frequency of A and T (57.2%) was significantly higher than that of C and G (42.7%), as well as the Banteng Bali cross F2, A and T (57.1%) were significantly higher than C and G (42.8%). This is consistent with the opinion of Yuwono [10], which states that the bacteria that live at thermophilic temperatures have a high A + T composition, therefore Bali cattle have better endurance ability in tropical environments than Bos taurus or Bos indicus.

Table 2. Average frequency of cytochrome B in Bali cattle, Banteng Bali cross F2, Bos javanicus, Bos taurus, Bos indicus and Bubalus bubalis (size 992 bp).

| Breed type             | T (%) | C (%) | A (%) | G (%) | A+T (%) | C+G (%) |
|------------------------|-------|-------|-------|-------|---------|---------|
| CBB03                  | 26.4  | 29.4  | 30.7  | 13.4  | 57.1    | 42.8    |
| CBB04                  | 26.4  | 29.4  | 30.8  | 13.3  | 57.2    | 42.7    |
| CBB07                  | 26.4  | 29.4  | 30.7  | 13.4  | 57.1    | 42.8    |
| CBB08                  | 26.4  | 29.4  | 30.8  | 13.3  | 57.2    | 42.7    |
| CBB09                  | 26.4  | 29.4  | 30.8  | 13.3  | 57.2    | 42.7    |
| CBC01                  | 26.4  | 29.4  | 30.7  | 13.4  | 57.1    | 42.8    |
| CBC07                  | 26.4  | 29.4  | 30.7  | 13.4  | 57.1    | 42.8    |
| CBC08                  | 26.4  | 29.4  | 30.7  | 13.4  | 57.1    | 42.8    |
| CBC09                  | 26.4  | 29.4  | 30.7  | 13.4  | 57.1    | 42.8    |
| CBC10                  | 26.4  | 29.4  | 30.8  | 13.3  | 57.2    | 42.7    |
| Bos javanicus          | 26.4  | 29.4  | 30.7  | 13.4  | 57.1    | 42.8    |
| Bos taurus             | 25.2  | 30.9  | 30.1  | 13.7  | 55.3    | 44.6    |
| Bos indicus            | 25.7  | 30.4  | 30.5  | 13.3  | 56.2    | 43.7    |
| Bubalus bubalis        | 24.6  | 30.7  | 30.5  | 13.1  | 55.1    | 43.8    |

Note: CBB: Bali cattle, CBC: Banteng Bali cross F2, BJ: Bos javanicus, BT: Bos taurus, BI: Bos indicus, BU: Bubalus bubalis.
3.3. Genetic distance

Genetic distance is the level of gene differences between populations or species as measured by several numerical quantities [11]. Genetic distance is used to see the close genetic relationship of livestock. A small genetic distance indicates a close genetic relationship and vice versa, a large genetic distance indicates a far genetic relationship. Furthermore, genetic distance information can be used as an initial indication of population structure and differentiation of a group in making conservation program decisions [12]. The difference in the order of nucleotide bases in each living creature will determine the performance of living things by regulating chemical reactions in cells that are regulated by enzymes (proteins) which are translated from nucleotide bases in the constituent components of genes. The slightest genetic variation possessed by the nucleotide base sequences will affect the performance of these living things.

Based on the data in table 3, it shows that between Bali cattle, Banteng Bali cross and Bos javanicus the genetic relationship is close (0.000–0.001), but it is much different when compared to Bos taurus, Bos indicus, and bubalus bubalis with a range of genetic distance values (0.050–0.094). This shows that based on the mitochondrial gene marker cytochrome B in Bali cattle and Banteng Bali cross F2, they have a very close genetic relationship. This is in accordance with the results of Sawitri et al (2013), which stated that the genetic distance between haplotypes was identified to be around 0.000–0.009, this result shows that the genetic distance between the haplotypes is very small, so that from the genetic aspects of the F2 cross Banteng and Bali cattle have close genetic relationship [13]. Handayani et al (2011) stated that genetic distance can describe the closeness between species or in populations and also used to see the closeness of genetic relationships between individuals [14].

Table 3. Genetic distance based on the composition of cytochrome B.

| Genotype | CBB1 | CBB2 | CBB3 | CBB4 | CBB5 | CBB6 | CBC1 | CBC2 | CBC3 | CBC4 | CBC5 | BJ  | BT  | BI  |
|----------|------|------|------|------|------|------|------|------|------|------|------|-----|-----|-----|
| CBB2     | 0.001|      |      |      |      |      |      |      |      |      |      |     |     |     |
| CBB3     | 0.000| 0.001|      |      |      |      |      |      |      |      |      |     |     |     |
| CBB4     | 0.001| 0.001| 0.001|      |      |      |      |      |      |      |      |     |     |     |
| CBB5     | 0.000| 0.001| 0.000| 0.000| 0.001|      |      |      |      |      |      |     |     |     |
| CBB6     | 0.000| 0.001| 0.000| 0.001| 0.000| 0.000|      |      |      |      |      |     |     |     |
| CBC1     | 0.001| 0.000| 0.001| 0.000| 0.001| 0.001| 0.001|      |      |      |      |     |     |     |
| CBC2     | 0.001| 0.000| 0.001| 0.000| 0.001| 0.001| 0.001| 0.000|      |      |      |     |     |     |
| CBC3     | 0.001| 0.000| 0.001| 0.000| 0.001| 0.001| 0.001| 0.000| 0.000|      |      |     |     |     |
| CBC4     | 0.001| 0.000| 0.001| 0.000| 0.001| 0.001| 0.001| 0.000| 0.000| 0.000|      |     |     |     |
| CBC5     | 0.000| 0.001| 0.000| 0.001| 0.000| 0.000| 0.000| 0.000| 0.000| 0.001| 0.000|     |     |     |
| BJ       | 0.001| 0.000| 0.001| 0.000| 0.001| 0.001| 0.001| 0.000| 0.000| 0.000| 0.000| 0.000| 0.000| 0.000|
| BT       | 0.054| 0.055| 0.054| 0.050| 0.054| 0.054| 0.055| 0.055| 0.055| 0.054| 0.055|     |     |     |
| BI       | 0.050| 0.051| 0.050| 0.051| 0.050| 0.051| 0.051| 0.051| 0.051| 0.050| 0.051| 0.011|     |     |
| BU       | 0.094| 0.094| 0.094| 0.094| 0.094| 0.094| 0.094| 0.094| 0.094| 0.094| 0.094|     |     | 0.093|

Note: CBB: Bali cattle, CBC: Banteng Bali cross F2, BJ: Bos javanicus, BT: Bos taurus, BI: Bos indicus, BU: Bubalus bubalis.

The results of the phylogeny analysis were based on cytochrome B gene DNA sequences in the Bali cattle, Banteng Bali cross F2, Bos javanicus (JN632606.1), Bos taurus (MN200930.1), Bos indicus (KX575711.1) and Bubalus bubalis (MK234704.1) (see figure 2). Based on the UPGMA phylogeny method, the group 1 relationship of Bali cattle (CBB3 and CBB7) and Banteng F2 (CBC1, CBC7, CBC8, and CBC9) with Bos javanicus) and group 2 (Bali cattle (CBB2, CBB4, CBB8, CBB9) and Banteng F2 (CBC10) are in the same branch showing that the results are relatively the same and have a close kinship. This is in accordance with the opinion of [1,2,15], stated that Bali cattle were the result of direct domestication of a Banteng. Li and Graur [16] stated that phylogeny tree analysis is used because it can describe the exact kinship relationship between organisms. Lubis (2014) mentions the phylogeny tree as an
arrangement in which species are arranged in the form of branches that connect them based on evolutionary kinship [17]. To see the relationship of each species can be described using phylogeny tree.

**Figure 2.** The phylogeny tree of Bali cattle (CBB), Banteng Bali cross F2 (CBC) based on cyt B mitochondrial DNA.

4. **Conclusion**

Based on the results of the study showed that the results of crosses between Banteng and Bali cattle did not change the DNA composition of cyt B and showed a very close relationship based on the results of phylogenetic analysis and genetic distance. It can be concluded that Bali cattle raised in UPT-PTHPT Pucak South Sulawesi, based on genetic variations of the mtDNA cyt B gene are still the same as those of Banteng, so it can be said that their purity is still maintained from the introduction of genetic blood from other cows such as *Bos taurus* and *Bos indicus*.

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