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Submission date: 27-Jan-2023 10:38AM (UTC+0800)
Submission ID: 2000251242
File name: Conservation_impact-Tidak_terakreditasi.pdf (871.49K)
Word count: 3195
Character count: 16943

Eco. Env. & Cons. 21 (2) : 2015; pp. (685-689)

ISSN 0971–765X

Conservation impact on molecular genetic changes on Java green peacock *(Pavo muticus)* through mitochondrial D-loop marker

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(Received 9 December, 2014; accepted 15 February, 2015)

ABSTRACT

The study of genetic diversity of species Java Green Peacock (*Pavo muticus*) captured from the wild and the conservation-bred in East Java is aimed at investigating the impact of conservation on the molecular genetic changes of Java Green Peacock (*Pavo muticus*). This study utilized samples from 10 (ten) wild Java Green Peacocks (*Pavo muticus*) from the forest and 6 (six) Java Green Peacocks (*Pavo muticus*) bred in the conservation in East Java. The materials examined were their feathers (calamus). The technique of *Polymerase Chain Reaction* (PCR) with D-loop mitochondrial based-designed primer was used to investigate the genetic diversity of species among Java Green Peacock (*Pavo muticus*). PCR products were analyzed using electrophoresis gel 1% . Then they were purified and labelled for data sequencing. The sequencing data obtained were in a form of base order that enabled the analysis of genetic diversity among species (Phylogenetic Analysis) by using a software program from Genetix Mac Ver 10.0. Green Peacock (*Afropavo congensis*) was used as the reference species with the code DQ 834507. The result showed that the 10 (ten) samples of wild Java Green Peacock *(Pavo muticus)* feathers were genetically different from the Green Peacock reported to the GenBank with code DQ 834507 as well as from the 6 (six) conservation-bred species Java Green Peacock *(Pavo muticus)*. Compared to wild Java Green Peacock (*Pavo muticus*) from the forest, the conservation-bred Java Green Peacock (*Pavo muticus*) experienced mutations since the result of phylogenetic analysis indicated that the conservation-bred Java Green Peacock species and the wild ones were not in the same cluster. Thus, peacocks kept in conservation, regardless the sophisticated and modern facilities, may lost their inherited genetic characteristics which in return would eliminate Indonesian superior germs for Java Green Peacock (*Pavo muticus*) in Indonesia

Key words : *Java Green Peacock, PCR, Mitochondrial D-Loop, Conservation, Indonesia*

Introduction

Genetic conservation is mainly used to support scientists to define, describe and prioritize scarce units for conservation (Carty *et al.*, 2009). Globally, the number of endangered species increased significantly in recent years (IUCN, 2008). According to Carty *et al* (2009) the percentage of hindrances will reach at least 18% by 2050 due to climate change. Adequate conservation measures must be taken to maximize the ability of species to adapt to rapid environmental change.

There are two types of peacocks in the world; the blue peacocks (*Pavo cristatus*) that spread over India and Sri Lanka, and the green peacocks (*Pavo muticus*) that spread in Burma, Thailand, Indochina,

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Malaysia and Java island. Green Peacock consists of three subspecies which are green peacocks of Burma (*Pavo muticus spicifer*) spread in Burma (extinct), Indochina Green Peacocks (*Pavo muticus imperator*) spread in Indochina, and Java Green Peacock (*Pavo muticus muticus*) spread in Malaysia (already extinct) and Java (Hernowo, 2011). Java Green Peacocks (*Pavo muticus muticus*) Linnaeus 1758, currently only live in Java. This bird spreads in a relatively small number of population in various habitat types. Java Green Peacocks can be found in some protected areas (Natural Reserves, Wildlife and National Parks) and unprotected areas (productive forests, plantations) (Hernowo, 2011).

On the other hand according to Wan *et al.* (2004), quoting from several sources, they stated that the conservation process led to some differences and similarities with its ancestors. This study is aimed at examining whether the peacocks bred in conservation experience genetic variations. It is very important to consider the genetic changes in order to keep the wealth of Indonesia superior germ plasma as well as to avoid genetic quality degradation.

Therefore, in a bid to maintain Java Green peacocks (*Pavo muticus*), the management factor is needed to keep the existence of the population through genetic enrichment programs, in which the basic information can be identified through phylogenetic reconstruction (Moritz *et al.*, 1996). High genetic variability enable the species to cope with the pressure from environmental changes (Avise, 1994; Hartl, 2000). In addition, the high heterozygosity of a population is a good basic for further developing the population. Appropriate molecular markers to reconstruct population phylogenetic are mitochondrial DNA sequence variations. The characteristics of mitochondrial DNA are: (1) the relatively high speed of evolution (5-10 times) compared to nuclear DNA; (2) Transmittable through the maternal line between generations without experiencing recombination, so that the whole molecules can be considered as a single genetic unit with many alleles (Sudoyo, 1995); and (3) the relatively small size that is easy to observe (Li and Graur, 1991; Taberlet, 1996). In observing the closely-related types, the use of nucleotide sequences of mtDNA control region (D-Loop) can provide good resolution, since the mtDNA D-loop sequences contain a variety of sequences which mutation rate are of 4-5 times faster compared to other parts of mtDNA (Horai *et al.*, 1993).

Materials and Methods

The samples were taken from the feather (Calamus) of 6 (six) Java Green Peacocks (Pavo muticus) from conservation areas and 10 (ten) Java Green Peacocks from the forest in Probolinggo, East Java,

DNA extraction was performed on 25 mg (3-4 Calamus), from the apex of the feathers by first adding 180 µl of lysis buffer (100 ml of 1 M Tris, 200 ml of 0.5 M EDTA, 2 ml of 5 M NaCl, 100 ml of 10% SDS), 25 µl of 100 mg/ml DTT (dithiothreitol) and 20 µl of 10 mg/ml proteinase K. The resulting mixture was further incubated at 50 ° C, to disolve all parts of the feathers (calamus) approximately (3-5 hours). Then 400 µl phenol (Tris-HCl pH 8.0) was added and the mixture was re-incubated for another 30 minutes. Next, it was centrifuged at 13,000 rpm speed for 3-5 minutes, and the top layer was removed to another vial which already contained 400 µl of chloroform Isoamyl alcohol (24: 1) which was then incubated for 10 minutes. After that, it was centrifuged at 13,000 rpm for 3-5 minutes and the layer formed at the bottom of vial was removed. Next, it was mixed with absolute ethanol and 40 µl of sodium acetate and incubated for 45 minutes at -20°C. Then it was centrifuged at13,000 rpm for 30 minutes at 4°C and then cleansed with 70% ethanol and re-centrifuged at the speed of 13,000 rpm for 10 minutes at 4°C. DNA obtained was re-dissolved with 100µl TBE buffer (10 mMTris-HCl pH 8.0, 1 mM EDTA) to determine the level of purity and spectrophotometer with a wavelength of 260 and 280 nm (Sefc *et al.*, 2003; Leeton *et al.*, 1993; Malago *et al.*, 2002; Kimball *et al.*, 1999).

PCR reaction was using the primer design already prepared which was GPDF (5'-GGGGGGTATACTATGCATAATC GTG -3') and GPDR (5'- AAAGAATG GGCCTGAAGCTAGT -3'). To amplify, the materials used were: 25µl total volume which consists of 1.5 µl of 10 × PCR buffer (Sigma), 1.5 µl25 mM MgCl 2, 100 ng for each primer, 200 µM of each dNTP, 0.5 U Taq DNA polymerase, and 250 ng sample DNA. PCR amplification conditions consist of stage pre-denaturation 94°C for 1 minute 30 seconds, 35 cycles that consist of 94°C for 30 seconds, 57°C for 30 1 minute, and 72°C for 31 minutes, and the final stage elongation 72°C for 10 minutes. The resulting PCR product was 330 bp (Wiwegwean and Meckvichai, 2011).

PCR result 330 bp was visualized by agarose gel 1% that contains ethidium bromide (Sefc *et al.*, 2003;

Leeton *et al*, 1993; Malago *et al*, 2002; Kimball *et al*, 1999). PCR products obtained were purified using some methods similar to the Qiagen kit (Wiwegwean and Meckvichai, 2011). After purification, the products were labeled, and followed by sequencing using the ABI Prism 310 (Wiwegwean and Meckvichai, 2011). Phylogenetic analysis on conservation-bred peacocks and wild forest ones was performed using sequence data obtained based on mitochondrial D-loop and data references in GenBank with code DQ 834 507. The results then were processed using a Genetix Mac Ver 10.0 software.

Results

Adult male Java green peacocks have a crest on the head and bluish-green chin, long ornamental feathers colored mix gold-green, and bronze-green which are big in size and the body length can reach 210 cm (Sativaningsih, 2005; Hernowo 1995; Hernowo, 2011) (Fig. 1).

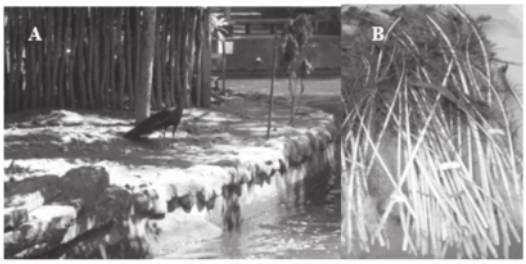

Fig. 1. A. Java Green Peacock; B. Calamus

The distal portion of feathers (calamus) is used as sample since it is suspected to contain lots of mitochondrial DNA. In this study to determine the genetic diversity of mtDNA control region, primer design PCR technique was used on mitochondrial D-loop region with a length of 330 bp (Fig. 2).

The results of this study showed that, in general, Java Green Peacocks (*Pavo muticus*) from the conservation in East Java have different genetic structure compared to the peacock's DNA reported in Genbank (code DQ 834507). It was suspected that despite their same species, the distance enables mix breeding among species and the condition on conservation also contributes to the formation of species with new genetic structure (Fig. 3). Further, the result regarding the population of Java green peacock

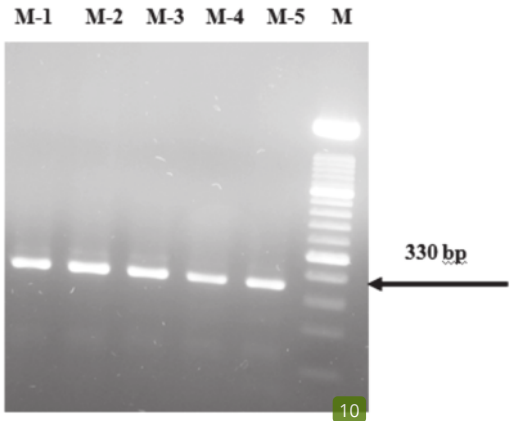

Fig. 2. PCR products. M (marker); M-1 (sample), M-2 (samples), M-3 (samples); M-4 (samples); M-5 (sample) (eletrophoresis gel 1%)

(*Pavo muticus*) living in the wild nature also showed difference compared to the peacock reported in Genbank (code DQ 834507) (Fig. 3). The difference also existed in the clusters of conservation-bred Java Green Peacock (*Pavo muticus*) and Java green peacock (*Pavo muticus*) from the wild (Fig. 3).

Regarding the hierarchy analysis of the F1 and F2 in this study, there is a need for further research. This is due to the fact that the samples are from adult and youngsters which do not carry F1 related information. Nevertheless, it appears that adult Java Green Peacock (*Pavo muticus*) forms separate clusters (MJD-1 and MJD2-1), while youngsters or young Java green peacock (*Pavo muticus*) also form

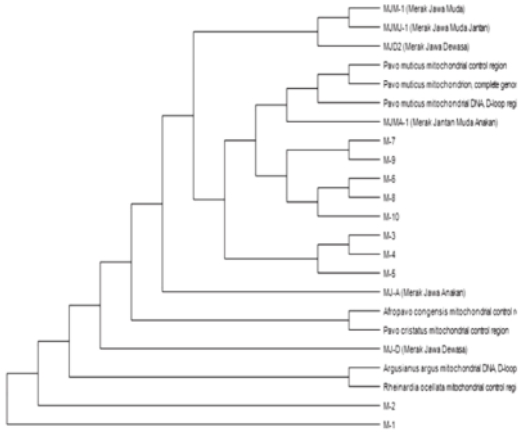

Fig. 3. Phylogeny Analysis of Java Green Peacocks (*Pavo muticus*) from conservation and from the wild

a separate cluster (MJM-1; MJMJ-1; MJA -1; MJMA-1). Similar condition also observed in the population of Java Green peacock (*Pavo muticus*) from the wild. Yet, despite unclear data identity, there is an assumption F1 and F2 are different, and that several clusters present which are first cluster (M), second cluster (M-2), third cluster (M-3, M-4 and M-5) and fourth cluster (M-6, M-7, M-8, M-9 and M-10) (Fig. 3).

Discussion

The theory of neutral molecular evolution (Wan *et al*, 2004) suggested that most of the changes in nucleic acid and protein sequences occur selectively. Although still controversial, this theory still highlights the need to consider the act of natural selection at the molecular level.

Along with the decline of peacocks population, especially the Java green peacocks, several attempts have been conducted including breeding in conservation efforts. According to Wiwegwean and Meckvichai (2011) many research have been carried out, especially on *Pavo muticus* in Thailand in relation to genetic variation caused by breeding in conservation. In this case, the genetic diversity of *Pavo muticus* breeding in conservation will vary among populations depending on the number and geographic range of the mothers. Differences in mitochondrial DNA (mtDNA) haplotypes in a population will illustrate some of the origins of the lineage of the female parent. Thus, the study of mtDNA variations may provide insight on the origin of the female parent of the peacocks bred in conservation and the genetic structure of natural or wild populations. In addition, the study can provide some evidences about the origin of the hybridization through mtDNA introgression between *Pavo muticus* and related species.

According to Tarwiningsih (2009), quoting from several sources, she suggested that to detect the molecular basis of genetic diversity may utilize mitochondrial DNA (mtDNA). This method is widely used to examine animal genetic diversity and systematic relationships at different levels of hierarchy (Lamb and Osentoski, 1995) due to the maternal nature of mtDNA, which is purely descended from the female parent. Mitochondrial genome also has a relatively small size, approximately ± 16,500 bp and has a rapid rate of evolution, especially in the control area (D-loop), giving rise to a high diversity in mtDNA sequences intra-species (Avise, 1994).

The existence of genetic variations that appear in an organism is a result of an evolutionary process in which genetic variation can occur due to a change in gene frequency. According to Tarwiningsih (2009), there are four basic factors that cause changes in gene frequency, i.e. natural selection, mutation, migration and genetic deviation. Natural selection is a natural process in which some individuals have genetic basis linkages that may improve live survival or reproduction to adapt and have offspring that can survive in the environment. The existence of migration causes individuals to move from one area to another. If these individuals survive and reproduce in the new place, they will pass on their genes to their new environment. According Tarwiningsih (2009), the assumption is that the isolated population does not affect changes in genes with neighboring groups. Many populations that are not fully isolated from other populations of the same species, experience some normal changes in genes.

In addition, this research was also expected to reveal the genetic structure of the green peacock from the conservation and the wild ones, including the genetic structure of the kinship hierarchy between F1 and F2. It is important since the regulation stated that the flora and fauna of protected breeding parents from natural habitat (W) is declared belong to the state. Wildlife breeding parents first generation (F1) on conservation are declared as state property. Parent specimens of protected wildlife that come from the natural habitat, and/or captive breeding first generation (F1) of protected wildlife are illegal to be sold and must be submitted to the state at any time.

The research results showed that 10 (ten) samples from wild Java Green Peacock (*Pavo muticus*) feathers were genetically different from the green peacock reported in GenBank with code DQ 834 507 and from 6 (six) samples of conservation-bred Java Green Peacock (*Pavo muticus*). Compared with the conservation-bred ones, it was obvious that the Java Green Peacocks experienced mutation which is indicated by the results of phylogenetic analysis in which both species were not in the same cluster.

Therefore, it should be considered that the peacocks kept in conservation, despite sophisticated and modern facilities provided, experience a decrease in any inherited genetic characters which may eliminate Indonesian superior germs in relation to Java Green Peacock (*Pavo muticus*) in Indonesia.

Acknowledgement

Our gratitude is extended to the Rector of Universitas Airlangga who has granted the researchers BOPTN fund to support the excellent research universities in year 2013 and 2014.

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