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Genetic Variability within the Population of the Vulnerable Mindanao Endemic Blue-Capped Kingfisher (*Actenoides hombroni*) Using Polymorphic DNA Markers

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Abstract - The restricted range and small population size of Mindanao endemic blue-capped kingfisher (*Actenoides hombroni*) and the degradation of its habitat provide an ideal case to study the genetics of this species. DNA samples were taken from *A. hombroni* netted from Nursery and Gabunan, Rogongon, Iligan City and Kimangkil, Bukidnon. Random amplified polymorphic DNA (RAPD) analysis and agarose gel electrophoresis were used to assess the genetic variability within its population. The random primer used had generated 25 reproducible RAPD markers showing three distinct banding patterns with low polymorphism. Shared or specific fragments were counted and genetic similarity within population was calculated. The similarity coefficient (F) estimation revealed that there were more pairs of individuals with F values equal to 1 (high genetic similarity) and having no F value equal to 0 (low genetic similarity).

Results suggested that there is a considerable low genetic diversity that existed within the population of *A. hombroni*, which could be explained by their geographic proximity. Findings of this study are significant most specially in the formulation of conservation strategies for the Mindanao endemic and vulnerable *A. hombroni.*

Keywords - *Actenoides hombroni*, genetic variability, PCR, RAPD markers, similarity coefficient, Rogongon, Iligan City, Mt. Kimangkil, Bukidnon

INTRODUCTION

Blue-capped Kingfisher (*Actenoides hombroni* Bonaparte) is a rare species that has a restricted range of distribution (Kennedy, et al. 2000). This species is a Mindanao endemic and is included in the list of globally threatened species categorized as vulnerable (Bird Life International 2000). Kingfishers are strictly woodland forms and never found in the open (Collar, Mallari, and Tabaranza 1999). Thus, forest destruction particularly at lower elevations has been the main threat to this species (Calizo 2000).

The destruction of habitats and the exploitation on the wild population are among the most damaging effects of human on biodiversity. While species extinction has become an issue of global concern, relatively little consideration has been given to the effects of exploitation on genetic diversity within species despite the large body of scientific literature demonstrating the importance of genetic variability for survival, adaptability and long-term persistence (Hauser, Adcock, and Carvalho n.d.).

It is important to point out that the genetic variation that the population of organism possesses is the fuel that allows them to be able to change or evolve in the response to changing environmental conditions. Therefore if long-term persistence of species is the goal that conservation biology is intending to achieve, genetic variability is of the utmost importance to preserve (Smith 1998). Hence, determination of the genetic diversity within population of a particular species helps identify those populations that should receive priority for conservation

efforts. Since species will ultimately go extinct if conservation action is not taken and since extinction is irreversible, this threatened species is in greatest danger and must, therefore, be given the highest priority.

This study was conducted mainly to provide basic information on the genetic variability of the globally vulnerable Mindanao endemic Blue-capped Kingfisher (*A. hombroni*) collected from Rogongon, Iligan City and Kimangkil, Bukidnon. Specifically, it aimed to determine the genetic variability within the population *A. hombroni* by Random Amplified Polymorphic DNA (RAPD) analysis and agarose gel electrophoresis, establish basic information on its population structure, and provide genetic data applicable to the long-term management and conservation of this species.

MATERIALS AND METHODS

Study Area

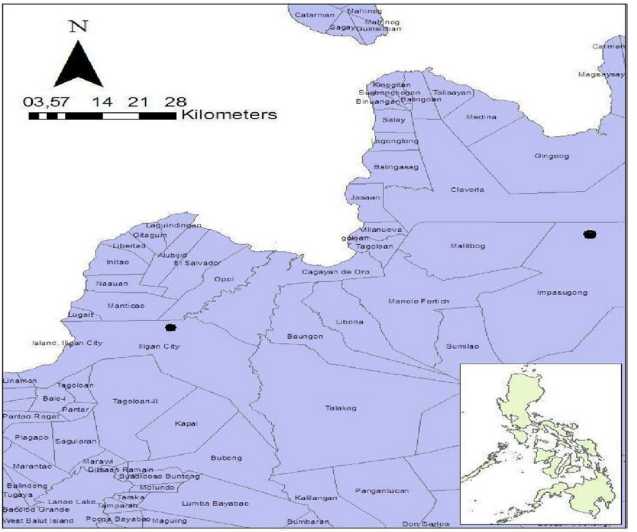

Fig. 1. Geographic location of the sampling sites.

Village Rogongon is the only remaining forest ecosystem in Iligan City (Fig. 1). It is situated in the northeastern part (08° 13'N to 08º 14'N and 124º 24'E to 124º 27'E) having an area of 38,000 (ha) and an

elevation of 1,020 meters above sea level (masl). The topography is rugged, from mountainous to hilly to semi-rolling.

Mt. Kimangkil Range in Mindanao Island, on the other hand, is located between 8º 29′ – 8º 36′ North latitude and 124º 59′ – 125 º 8′ East longitudes. It is approximately 100 aerial kilometers south of Mindanao Sea and it covers a total area of 8,079.072 hectares. The range stands home to a diversified species of flora and fauna and is classified within the Central Mindanao Cordillera that serves as the physiographic feature of the eastern portion of Bukidnon. The base of Mt. Kimangkil Range crosses the Bukidnon-Claveria, Misamis Oriental Province.

Sampling was done in Rogongon, Iligan City and Mt. Kimangkil, Bukidnon. The first two sites were established in Rogongon with Nursery as site 1 with an elevation of 950-980 masl, and Mt. Gabunan as site 2, that has an elevation of 1036 masl. Site 3 was established in Mt. Kimangkil, situated in a lower montane forest at 1300-1350 masl.

Preliminary sampling and ecological assessment was done to determine if the species in consideration still exists in the study areas with the corresponding ecological requirements. Site 1 which is dominated mainly by tall trees and large ferns (Fig. 2) was observed to have more on-site disturbance than site 2. This area was slightly fragmented due to the construction of the new Iligan-Bukidnon road, which may lead further to human encroachment and eventually habitat destruction.

Fig. 2. Sampling site 1, Nursery, Rogongon, Iligan City (950-980 masl).

Site 2, Mt. Gabunan with diversified floral and faunal species was observed to have the least on-site disturbance (Fig. 2). There were few number of settlers noted when compared to the first site. A stream and a small clearing that was planted with corn were noted in the area. This area, which abounds with mollusks, insects and seeds, could be probable feeding sites of *A. hombroni.*

Fig. 3. Sampling site 2, Mt. Gabunan, Rogongon, Iligan City (1036 masl).

Site 3 is a montane forest that originally possesses a rich biological diversity (Fig. 4) but is now slowly declining. Among the three sampling sites, this area was observed to have the most on-site disturbance where indiscriminate logging was noted. During the sampling, it was observed that villagers come and cut down trees to get rattan as their source of income. They also practice slash and burn farming.

Fig. 4. Sampling site 3, Kimangkil, Bukidnon (1300-1350 masl).

Collection of *A. hombroni*

Fig. 5. Photograph of the blue-capped kingfisher (*A. hombroni),* source of blood sample for genomic DNA extraction.

A. hombroni (Fig. 5) was captured using mist nets with mesh size of 37 mm and overall size of 2 m x 12 m. The mist nets were set at ground level, across flyways and forest trails, near feeding areas, water bodies and high up the canopy trees. Ten mist nets were set in each sampling site with three sub-sampling sites chosen. Nets were opened for capture from 6:00 AM till 5PM and were checked once every hour to avoid subjecting the netted birds to stress and to prevent damage to mist nets.

Intensive mist netting of these birds was conducted on December 2002 to January 2003, April to May 2003, and December 2003 to January 2004. A total of 42 field days or 420 net days was spent for the whole duration of the study having 140 net days per sampling site to meet the standard netting procedure (Heaney and Heideman 1989).

Prior to blood collection, actual measurements of body parts and morphological descriptions of each sample were taken. The captured

birds were then identified up to the species level using the Guide to the Birds of the Philippines by (Kennedy et al. 2000), Philippine Birds by du Pont (1971), and a Photographic Guide to Birds of the Philippines (Fisher and Hicks 2000).

Blood Sample Collection, Preservation, and DNA Extraction

One ml of whole blood samples from the captured individuals of *A. hombroni* was drawn from the largest brachial vein using a sterile syringe. After blood samples were taken from the specimens, wounds were treated with 10% betadine solution and the under parts of the wings were marked to prevent recapturing of the same species. Collected blood samples were stored in pre-sterilized 1.5-ml eppendorf tubes containing 0.5 ml of absolute ethyl alcohol. The eppendorf tubes with the blood samples were placed in the mini bucket with crushed ice to prevent desiccation. Further preservation was done by adding PBS/EDTA buffer to the samples. Samples were transferred immediately to the freezer at -20^0 C upon arrival at the MSU-IIT laboratory.

Of the blood samples taken from 24 specimens of *A. hombroni*, only nine samples proved useful for genomic DNA isolation, although all samples were subjected to the same extraction and amplification procedure. This can be attributed to the blood samples collected that were in small volumes ranging from 200-280μL. As reported by (Salleh et al. 1999), appreciable amount of DNA could be extracted from blood samples of 300 to 500 μL.

Genomic DNA isolation was performed according to the FBI protocol (Roe, 1998) as modified by Dr. Teves (pers. com.).

Random Amplified Polymorphic DNA (RAPD-PCR) Analysis

The generation of the RAPD markers employing the polymerase chain reaction (PCR) was carried out in 25 total reaction volume. The random primer was diluted to get 2.5M concentration required in the reaction. Two microliters of the random primer (3g/l) was diluted with 2,498 sterile triple distilled water to make 2,500 total volume of the random primer 2.5M concentration. Master mix (100 total volume) of all common PCR reagents (except DNA) for the number of reactions

carried out was prepared first. Master mix was used to minimize any errors caused by pipetting small volumes. The master mix contained the following PCR reagents (PCR Core Kit System II, Promega, USA) in the correct order of adding: 50 of sterile triple distilled water, 12.5 of 10X PCR buffer $MgCl_2$ – free, 10 L of 10 nM PCR nucleotide mix (dNTPs), 15 of 25 nM of $MgCl_2$, 10 of 2.5 M random primer, and 2.5 Taq DNA polymerase. Twenty microliters of master mix was aliquoted into the PCR tube and added with 5 of the template DNA. Amplifications were carried out using a Thermal Cycler.

The PCR products were resolved by electrophoresis at 90 V in 0.8% agarose (Promega, USA) gels, ran with 1X TAE buffer. A molecular size marker (DNA digested with Hind III) of known concentration was run with the PCR products. Initial checking for the presence of possible DNA bands was performed through a preliminary run of the PCR products without the markers on the agarose gel electrophoresis.

Data Analysis

The RAPD profiles were determined by direct comparison of the amplified DNA electrophoresis profiles and the data obtained were analyzed based on the presence or absence of bands, which correspond to RAPD markers. Genetic variability was determined by the banding patterns (RAPD markers) and further by estimation of similarity coefficient (F) of Nei and Li (1979), comparing the number of shared and unique bands between profiles.

$$F = \frac{2\,(\text{No. of common bands})}{(\text{No. of bands A}) + (\text{No. of bands in B})}$$

Where:

F= 0 means no band in common (low genetic similarity)

F=1 indicates identical bands (high genetic similarity)

Computation for Percentage of RAPD Polymorphism:

$$\text{Percentage of RAPD Polymorphism} = \frac{\text{No. of polymorphic markers}}{\text{Total number of RAPD markers}} \times 100\%$$

RESULTS AND DISCUSSION

RAPD Profile of Samples

Across all individuals tested, the random primer produced 25 scorable bands (RAPD markers) in total with number of fragments per individual ranging from two to five. Fig. 6 shows the RAPD profile of *Actenoides hombroni.* Lanes 1 and 2 are sample numbers 2 and 4 taken from site 1 (Nursery, 950-980 masl) while lanes 3 to 6 are sample numbers 7, 15, 16 and 17 from site 2 (Mt. Gabunan, 1036 masl) and lanes 7 to 9 are sample numbers 18, 19, and 20 from sampling site 3 (Kimangkil, 1300-1350 masl). RAPD markers showed three different banding patterns: the first banding pattern in lanes 1,2 5, and 8; the second banding pattern in lanes 4, 6,7,9; and the third banding pattern in lane 3. The first banding pattern is found in three sampling sites, specifically in individuals from site 1 of sample numbers 2 and 4; from site 2 of sample number 16 and from site 3 of sample number 19. The second banding pattern is found both in sampling site 2 of sample numbers 15 and 17 and in sampling site 3 of sample numbers 18 and 20. On the other hand, the third banding pattern is exhibited only on lane 3 of sample number 7 from sampling site 2.

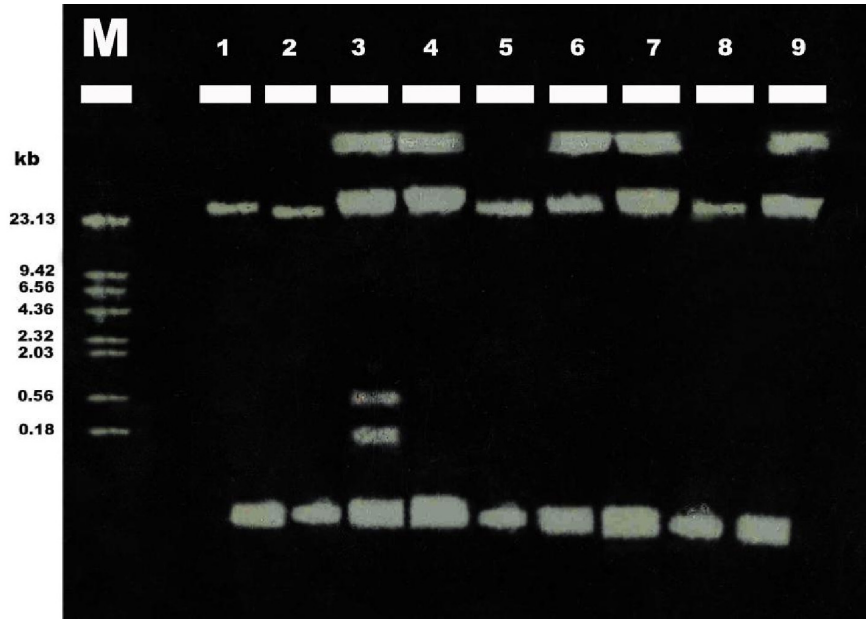

Fig. 7. RAPD profile of *A. hombroni.* M represents the DNA molecular size marker (λ DNA digested with Hind III). Lanes 1and 2 are sample numbers 2 and 4 from sampling site 1 (Nursery); lanes 3 to 6 represent sample numbers 7,15,16, and 17 from sampling site 2 (Gabunan); lanes 7 to 9 represent sample numbers 18, 19, and 20 from sampling site 3 (Kimangkil, Bukidnon).

As observed, RAPD analysis revealed a low percentage of polymorphism (28%) within the population of *A. hombroni* as indicated by the polymorphic markers, above the 23.13 kb and at the 0.56 and 0.18 kb. Of the 25 RAPD markers, only seven markers are polymorphic while the rest are all monomorphic.

In scoring RAPD polymorphism, RAPD markers that are present in some individuals but not in others are said to be polymorphic while those markers that appear in all individuals are considered monomorphic (Ferraris and Palumbi 1996). Polymorphic markers are generated as a result of variation in the DNA sequence of the region between the priming sites, or a variation in, or lack of, the primer sequence (Saunders and Parkes 1999). A distinct polymorphism

is exhibited in lane 3 although all blood samples were taken from individuals that were characterized and identified as *A. hombroni* based on the actual measurements and morphological descriptions noted during the time of sampling. This distinct polymorphism (the high number of polymorphic markers) found in lane 3 suggests that the base pair sequence of the random primer matched with most genome parts of this individual , An alternative hypothesis to explain these distinct markers observed at 0.56 and 0.18 kb of lane 3 could be the emergence of a subspecies of *A. hombroni.* It could not be a different species since all of the samples were identified up to the species level and they were found to possess the same phenotypic characteristics. Moreover, detection of a subspecies of *A. hombroni* is possible since the RAPD technique used in this study is so sensitive to detect similarities between individuals and can even differentiate close relatives at the subspecies level (Saunders and Parkes 1999).

Table 1. Similarity matrix showing the F values, similarity coefficient of Nei and Li (1979) to assess the similarity between profiles.

			LANES						
LANES	1	2	3	4	5	6	7	8	9
1	-	1	0.57	0.80	1	0.80	0.80	1	0.80
2		-	0.57	0.80	1	0.80	0.80	1	0.80
3			-	0.75	0.57	0.75	0.75	0.57	0.75
4				-	0.80	1	1	0.80	1
5					-	0.80	0.80	1	0.80
6						-	1	0.80	1
7							-	0.80	1
8								-	0.80
9									-

Similarity assessment based on the RAPD profiles of *A. hombroni* revealed a high degree of genetic similarity (Table 1). They are said to have high genetic similarity because when the similarity coefficient values between individuals were rounded off to the nearest ones, all pairs of individuals have a coefficient value of 1. The similarity coefficient of Nei and Li (1979) could only have a value between 0 and 1, where 0 indicates that there were no markers in common (low

genetic similarity) and 1 indicates identical marker patterns (high genetic similarity) (Saunders and Parkes, 1999). This result suggests that the population of *A. hombroni* consists of related individuals as demonstrated by the high number of F values equal to 1 and having no F values equal to 0. The high genetic similarity observed between individuals in the population could be due to the short distances between areas separating these individuals.

A study conducted by (Triggs et al., 1992) on the genetic structure of Blue duck population showed that Blue ducks separated by short geographic distance have high genetic similarity, which decreases with increasing geographic separation between samples. Thus, individuals from within the same river showed high similarity than those individuals from different rivers.

Bader (1998) also reported that geography plays an important role in the process of exchange of alleles since the fewer the barriers and the closer two populations are geographically; the more likely they are to exchange individuals and their alleles. When the rate of the exchange of alleles is very low or nil, drift, selection, and even mutation may lead to genetic differentiation (Slatkin 1987). On the other hand, if there is greater rate of the exchange of alleles between populations, the more homogeneous those populations will become (Bader 1998).

Considering the small population size, rare abundance, and the restricted range *A. hombroni* there is a possibility that inbreeding occurs among individuals in the population that might have caused the high genetic similarity observed. Rave (1994) in her study on analysis of wild population of Hawaiian geese using DNA fingerprinting indicated that inbreeding caused the high genetic similarity on the population found at Puwaawaa and Kauai.

In a large and randomly breeding population, it is likely that an individual will be able to successfully find a mate who is almost completely unrelated to himself or herself (Adcock and Ramirez, 1998). However, in the case of *A. hombroni* the likelihood that an individual will mate with another who is completely unrelated decreases dramatically. Mating of these related individuals in a population increases the proportion of homozygous individuals and correspondingly decreases the frequency of heterozygotes (Carpen et al. 1993). As this occurs, the probability that deleterious alleles will come together in one individual increases which will then result the

reduced average fitness of the population as a consequence of a higher mortality rate, lower fecundity and lesser ability to survive throughout the life stages (Smith 1998).

In the present study, the low RAPD polymorphism and the high genetic similarity indicate that there is a considerable low genetic variability that exists among individuals of *A. hombroni* in Rogongon, Iligan City and Kimangkil, Bukidnon. As we have known, low genetic variability may adversely affect individual fitness whereas high genetic variability may increase the potential for future evolutionary adaptation (Frankel and Soule 1981). If all individuals in the population were genetically similar, it is likely that they would all be equally susceptible to biological forces, human intervention, and natural phenomena such as drought, floods, or drastic fluctuation in the temperature. Individuals within populations must be able to adapt to the changing environments for them to survive (Bader 1998). Therefore, for *A. hombroni* to survive, its population must exhibit sufficient genetic variability to adapt to the constantly changing stresses found in Rongongon, Iligan City and Mt Kimangkil, Bukidnon.

Implications on long-term Management and Conservation of A. hombroni

Findings of this study have several implications for the conservation of the vulnerable Mindanao endemic *A. hombroni.* The higher genetic similarity between individuals of this species suggests that they are closely related and based on the banding patterns their population is not structured. Thus, conservation action for *A. hombroni* appears urgent.

The most practical and perhaps the best option is to save this vulnerable species is to restore its habitats to allow for natural population growth. Hence, increasing population size without losing genetic variation. By conserving genetic variation, the species may maintain the evolutionary potential to colonize, through a natural way, new natural habitat patches (Galbusera et al. 2000). As stated by Lande and Barrowclough (1987), the number of individuals is a crucial parameter in determining the amount of genetic variability that can be maintained in a population. It influences the probability of long-

term survival of a population because genetic variation is requisite for evolutionary adaptation to a changing environment. Thus, maintaining population numbers and genetic variation must be a central theme of plans for long-term population management.

CONCLUSIONS

Of the 24 individuals of *A. hombroni* that were captured only nine individuals successfully yielded sufficient genomic DNA for RAPD analysis. PCR runs of these samples produced 25 scorable bands (RAPD markers).

Application of the Random Amplified Polymorphic DNA technique revealed three different banding patterns exhibiting low RAPD polymorphism as indicated by the polymorphic bands. No apparent population structuring of *A. hombroni* in the area as far as these banding patterns are concerned, which is reinforced by the F values, indicating low level of genetic variability. These results may suggest that geographic proximity allows individuals of *A. hombroni* in the study areas to interbreed, thus, homogenizes genetic variation through them. Furthermore, the observed habitat destruction in the sampling areas could also have contributed to the low genetic variability of the individuals in the population.

RECOMMENDATIONS

Based on the results, it is recommended that acrylamide gel be used instead of the agarose gel to have a better resolution and silver staining or radioactive labeling to visualize PCR products. Moreover, extensive studies employing further molecular analysis and the use of other types of random primers should be carried out on the different geographically distant population of *A hombroni* to determine if the result of the present study holds for other populations. It is also recommended that the result of this study be used as basis for conservation program and the restoration of habitats in the sampling areas to enable the retention, if not increase, the genetic diversity of this vulnerable and Mindanao endemic *A. hombroni.*

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