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Genetic Diversity and Relative Abundance of Cebu Black Shama (*Copsychus cebuensis* Steere) in Fragmented Forests of Cebu Island, Philippines

ABSTRACT

This study determined the relative abundance of Cebu black shama (Copsychus cebuensis Steere) in selected isolated forest fragments in Cebu Island, Philippines and their genetic diversity based on 619 bp cytB gene. Mist nets were used to capture the bird in these forest fragments. Four contour feathers were plucked from the body of the caught birds, before they were released, and were stored in tubes with 70% ethanol before DNA extraction. Fifty-nine black shama (C. cebuensis) individuals were encountered from the visited territories. At least 13 black shama individuals were estimated to inhabit one hectare of forest habitat. For the first time, analyses of mitochondrial genes revealed that C. cebuensis had a long evolutionary history from an initially large and stable population that went through recent expansion resulting from a recent isolating or bottleneck event as indicated by high haplotype diversity (Hd) and nucleotide diversity (πn), i.e. Hd>0.50 and πn>0.005, and non-significant values of Tajima's D test, Fu and Li's D, and Fu's Fs statistics. It is hypothesized that this bottleneck event was habitat fragmentation. Furthermore, phylogenetic analyses of C. cebuensis supported its monophyly.*

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INTRODUCTION

The island of Cebu in central Philippines is a classic case of severe deforestation and the remaining forests on it are highly fragmented and quite isolated from one another (*Mallari et al. 2001; Jakosalem et al. 2005; Vandermeer 1967 in Bensel 2008; DENR-FMB 2010; Bagarinao 2010*). Conversely, it was found out that in these forest fragments, populations of bird species endemic to the island are thriving, which include the Endangered Cebu black shama (*Copsychus cebuensis* Steere) (*Collar et al. 1999; Birdlife International 2001*), among others. The continuous existence of these threatened bird species in a highly fragmented habitat is very much uncertain.

Habitat fragmentation is a serious condition especially for small and declining wildlife populations (*Weins 1997; van Dyck and Slotow 2003*). *Amos et al.* (*2012*) articulated that these small and declining populations lose fitness through inbreeding depression and loss of genetic diversity with fragmentation, thus reducing their adaptability to environmental change. Likewise, the degree of genetic variability of the bird populations, among and within the fragments, is equally affected considering the degree of isolation between fragments, which limits the movements of individuals.

Conversely, the genetic diversity, which is a measure of the level of intraspecific genetic variation, has provided valuable information on levels of genetic variation, gene flow, population subdivision, historical patterns of population fragmentation, and the evolutionary history of populations (*Moore et al. 1991; Ball & Avise 1992; Bermingham et al. 1992; Zink & Dittmann 1993; Zink 1994; Schneider et al. 1998; Gibbs 1999; Gill et al. 1999; Macey et al. 1999; Patton et al. 2000; Bates et al. 2004*). Furthermore, there is a growing concern over the conservation of many threatened species and it has highlighted the importance of genetic data.

The maintenance of genetic diversity has been the major goal for some biodiversity conservation programs (*Frankham et al. 2002; Fernandez et al. 2004*). It is widely accepted that genetic diversity is important for both the short- and long-term viability and future evolution of populations (*Landweber & Dobson 1999; Amos and Balmford 2001; Frankham 2005; Avise 2008 in Laikre et al. 2009*). In the short-term, genetic diversity provides a buffer against population crashes in events of extreme environmental fluctuations; and in the long-term, genetic diversity is the raw material for evolution and thus provides the biological capacity for populations to respond to future environmental changes (*Laikre et al. 2009*).

In this regard, the abundance and genetic diversity of endangered Cebu black shama, C. cebuensis, in the island of Cebu, particularly in selected fragmented and isolated forests, was determined in order to assess the extent of the effects of forest fragmentation towards biodiversity. Conversely, the genetic information from mitochondrial gene segments cytochrome B (cytB) and cytochrome c oxidase subunit I (COI) of *C. cebuensis* from this study is a new contribution to the species bioinformatics.

MATERIALS AND METHODS

Study Sites

The black shama (*C. cebuensis*) is endemic to the island of Cebu and were reportedly thriving in forest fragments located in the mountainous parts of the island. The black shama (*C. cebuensis*) is known to be territorial and one black shama territory was estimated to have an area of around 1,000 m^2 (*Pedro pers. comm.*). Four known black shama (*C. cebuensis*) forest habitats, namely Tabunan (Cebu City), Nug-as (Alcoy), Tabayag (Argao), and Casili (Mandaue City/ Consolacion), were visited and surveyed between September 2013-February 2014 after having approved by the Department of Environment and Natural Resources (DENR) Regional Office VII (**Figure 1**).

Population Size Estimate and Distribution of Cebu Black Shama

Forty-five territories were visited and surveyed (**Table 1**). All of the black shama (*C. cebuensis*) territories that were surveyed were characterized by dark understory and thick litterfall (*Malaki et al 2013*). The use of the

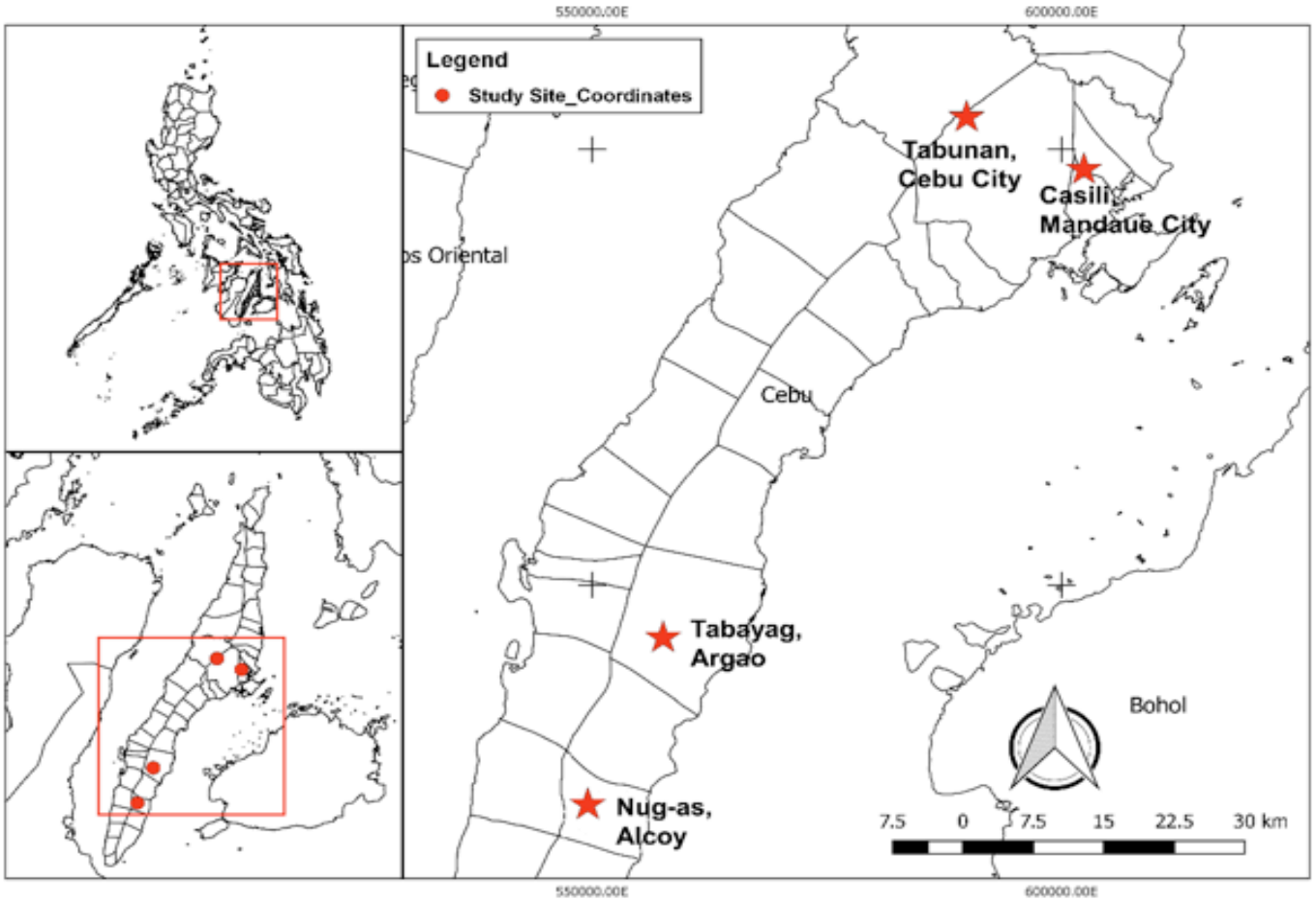

Figure 1. Location map of the four fragmented forests (study sites) in Cebu province.

Table 1. Number of black shama territories sampled per study site.

IBA/ Location	No. of Territories	Inclusive Dates	Net days
Tabunan	13	Jan. 10-12 and 24-26	52-78
Nug-as	14	Sep.16-20 and Dec.13-15	56-84
Tabayag	10	Oct. 14-18	40-60
Casili	8	Jan. 31 and Feb. 01-02	32-48
Total	45		180-270

bird's song playback (downloaded from www.xentocanto.org) helped in flushing out the shy but aggressive black shama. The number of encountered black shama (*C. cebuensis*) was recorded per territory.

Mist nets were set up along the bird's flyway in each territory. The mist nets were checked at least every 30 min and more frequently when it was very hot or raining following *Robertson and Liley* (*1998*) in *Bibby et al.* (*2000*). When mist nets in a given territory had caught at least one black shama (*C. cebuensis*), they were then transferred to another territory. Net success rates per bird habitat were determined by dividing the number of caught black shama (*C. cebuensis*) individuals with the number of mist nets that were set-up.

Genetic and Phylogenetic Analyses

At most, four contour feather samples from each of the caught black shama (*C. cebuensis*) individuals were collected following *Taberlet and Bouvet* (*1991*). The feathers were plucked with a single motion and stored in a sterile Falcon tubes containing 70% ethanol. Handling of the feather samples was observed with caution to avoid touching the base or calamus, i.e., to prevent contamination. Samples were stored in the freezer at 4°C before DNA extractions were performed.

DNA extraction and purification were carried out according to the technique of *Taberlet and Bouvet* (*1991*). The feathers were cut transversely 2 mm from the base. The samples were placed in 1.5 ml microcentrifuge tubes and processed following the Wizard® SV Genomic Purification System's quick protocol.

In preparing the samples for DNA extraction, 275 µl of digestion solution master mix were added in each tube, which contained 200 µl nuclei lysis solution, 50 µl of 0.5M EDTA (pH 8.0), 20 µl of proteinase K (20 mg ml^{-1}), and 5 µl RNase solution (4 mg ml^{-1}). The sample tubes were incubated overnight (18-24 hours) in a 55°C heat block. Then, 250 µl of Wizard® SV lysis buffer was added to each sample and was vortexed. As soon as the buffer was added, the lysate was processed. Each sample lysate was transferred from the 1.5 ml tubes to a separate Wizard® SV mini-column assembly. The assembly was centrifuged at 13,000 x g for 3 min. The mini-column was then removed from the assembly and the liquid from the collection tube was discarded, after which the mini-column was placed back into the collection tube. Into each reassembled mini-column assembly, 650 µl column wash assembly (CWA; with 95% ethanol added) was added. After a minute of being centrifuged at 13,000 x g, the liquid from the collection tube was discarded. Washing was repeated four times. After discarding the liquid and reassembling the mini-column assembly, it was centrifuged for 2 min at 13,000 x g to dry the binding matrix. The mini-column assembly was then transferred to a new 1.5 ml microcentrifuge tube and 250 µl of nuclease-free water was added. The tubes were incubated at room temperature for 2 min before they were centrifuged at 13,000 x g for 1 min. An additional 250 µl of nuclease-free water was added to the tubes, incubated at room temperature for 2 min and were centrifuged again at 13,000 x g for 2 min. The mini-column was removed and the purified DNA was stored at -20 to -70°C.

Mitochondrial DNA procedures were carried out following the procedures of *Lohman et al.* (*2010*). The improved primer pairs Copsy-cytb-F1 (5' CTA ATG RCC CTC AAT CTH CGT AA 3)' and Copsy-cytb-R1 (5'CCT GTY TCG TGT AGG AAG GTR AGG 3') (*Sheldon et al. 2009*) were used to amplify the 619 bp cytB gene.

Each polymerase chain reaction (PCR) consisted of 10 µl 2X Vivantis Taq Master Mix [Taq DNA polymerase (0.05 u/µl), 2X Vibuffer A, 0.4 mM dNTPs, and 3.0 mM MgCl2], 1.2 µl of each 10 µM primer, 7.6 µl nuclease-free water, with 5.0 µl template DNA, for a total reaction volume of 25 µl, and was amplified with the following thermal cycler conditions: 3 min at 95°C followed by 40 cycles of 1 min at 53°C, and 1.5 min at 72°C, and finally 5 min at 72°C. PCR amplification of feather DNA was repeated twice, in which the number of cycles was reduced to 20 cycles depending on the quality of extracted DNA (*Svobodova et al. 2011*). After purification, the PCR products were sent to 1st Base Singapore, Ltd. for gene sequencing.

In this study, the rapidly evolving protein-coding mitochondrial gene, cytochrome b (cytB), was chosen for analyses. It was considered as among the most variable protein-encoding gene (*Meier, unpub. in Lohman et al. 2008*), hence can be useful in giving insights as to how the gene in the population behave across space and time.

Genetic Diversity Indices and Phylogenetic Analyses

Genetic diversity of the black shama (*C. cebuensis*) was quantified separately for each location and together as a group for the entire island of Cebu. Identical sequences were not removed from the dataset. The following genetic diversity indices were calculated using DnaSP 5.10.01 (*Librado and Rozas 2009*): number of haplotypes (H), number of variable nucleotide sites (S), haplotype diversity (Hd) and its standard deviation, nucleotide diversity (πn) and its standard deviation, and the mean pairwise nucleotide differences (k), along with its total variance (including components of stochastic and sampling variance). The haplotype diversity of a sample indicates the probability that two randomly chosen haplotypes within a sample will be identical, while nucleotide diversity calculates the average proportion of nucleotide sites that differ in all pairwise comparisons (*Tajima 1983*). In addition, it was also determined whether there was selection in the island population evaluating departures from neutrality using Fu's F-test, Fu and Li's D*, Tajima's D-test, and R2 (*Rodrigues et al. 2013*). The recent demographic expansion was tested using pairwise mismatch distributions and the expected values in a stable population, i.e., population with constant population size and in growing or declining population. The raggedness statistic, r (*Harpending 1994*), was also estimated to test the smoothness of the observed pairwise differences distribution. Genetic differentiation was tested using analysis of molecular variance (AMOVA) and pairwise Fst values were compared among population performed with Arlequin 3.0 (*Excoffier et al. 2005*), along with the moment estimators of the time of expansion (τ), and indices of population sizes before and after the expansion, $\theta 0$ and $\theta 1$, respectively, which were calculated with a generalized non-linear square approach with confidence intervals approximated with 1,000 replicates of parametric bootstrapping.

Alignment of nucleotide sequences and subsequent analyses were conducted using MEGA version 6 (*Tamura et al. 2013*). Parsimony analyses were performed with TNT 1.1 (*Goloboff et al. 2008*) following *Lohman et al.* (*2009*). This is a free program for phylogenetic analysis which allows the user an enormous flexibility in phylogenetic analyses or simulations. After increasing the maximum number of saved trees to 3,000, a traditional tree bisection reconnection (TBR) heuristic search was implemented, performing 1,000 replicates and saving 10 trees per replication, replacing existing trees. To assess confidence in the resulting phylogenetic estimate, the data were subjected to a Bootstrap analysis using symmetric resampling implementing a traditional search with 33% change probability (1,000 replicates). The results were summarized as absolute frequencies. In addition, the data were resampled with the Jackknife technique using traditional search with a 36% removal probability replicated 1,000 times.

Phylogenetic analyses followed the protocols of *Lohman et al.* (*2009*). Bayesian phylogenetic analyses were performed with MrBayes (*Ronquist and Huelsenbeck 2003*). jModelTest 2 (*Darriba et al. 2012*) using Akaike's Information Criterion (*Akaike 1974*) were performed to select the block model for cytB partitions. Parameter values for the substitution model were estimated from the data and allowed to vary independently between genes. Four Markov chains, one cold and three heated, were run simultaneously for 100th generation. After completion of the analysis, the first 25,000 trees were discarded before a majority-rule consensus tree was calculated from the remaining 75,001 trees. Maximum likelihood analyses and maximum bootstrap values were performed with RaxML.

RESULTS AND DISCUSSION

Black Shama (*C. cebuensis*) and Individual to Territory Ratio Estimates

Among the mist net stations per study site, were Tabunan (n=13), Nug-as (n=14), Tabayag (n=10), and Casili (n=8) (**Figure 2**).Meanwhile, the successful mist net stations per study site were Tabunan (n=5), Nug-as (n=6), Tabayag (n=6), and Casili (n=6) (**Figure 3**). Successful stations were those wherein the mist nets were able to capture black shama (*C. cebuensis*). Similarly, the number of black shama (*C. cebuensis*) seen and heard in each territory was recorded to provide information of individual to territory ratio which was computed to be between 1.00 to 1.40 individuals per territory (**Table 2**).

It was observed that the number of C. cebuensis individuals that were encountered increases with the number of sampled territories (**Figure 4**). In this regard, the number of *C. cebuensis* individuals is highly correlated with the area of suitable habitat (r=0.9764). This observation was in similar to *Malaki et al.* (*2018*) in which they empirically demonstrated that forest canopy cover, one of the factors that measures habitat suitability, is highly correlated with the bird's population density. This indicates that to increase the number of *C. cebuensis* population, it needs to improve forest canopy cover. However, the black shama (*C. cebuensis*) is not selective in terms of the vegetation composition of anygiven habitat (*Parilla et al. 2016*).

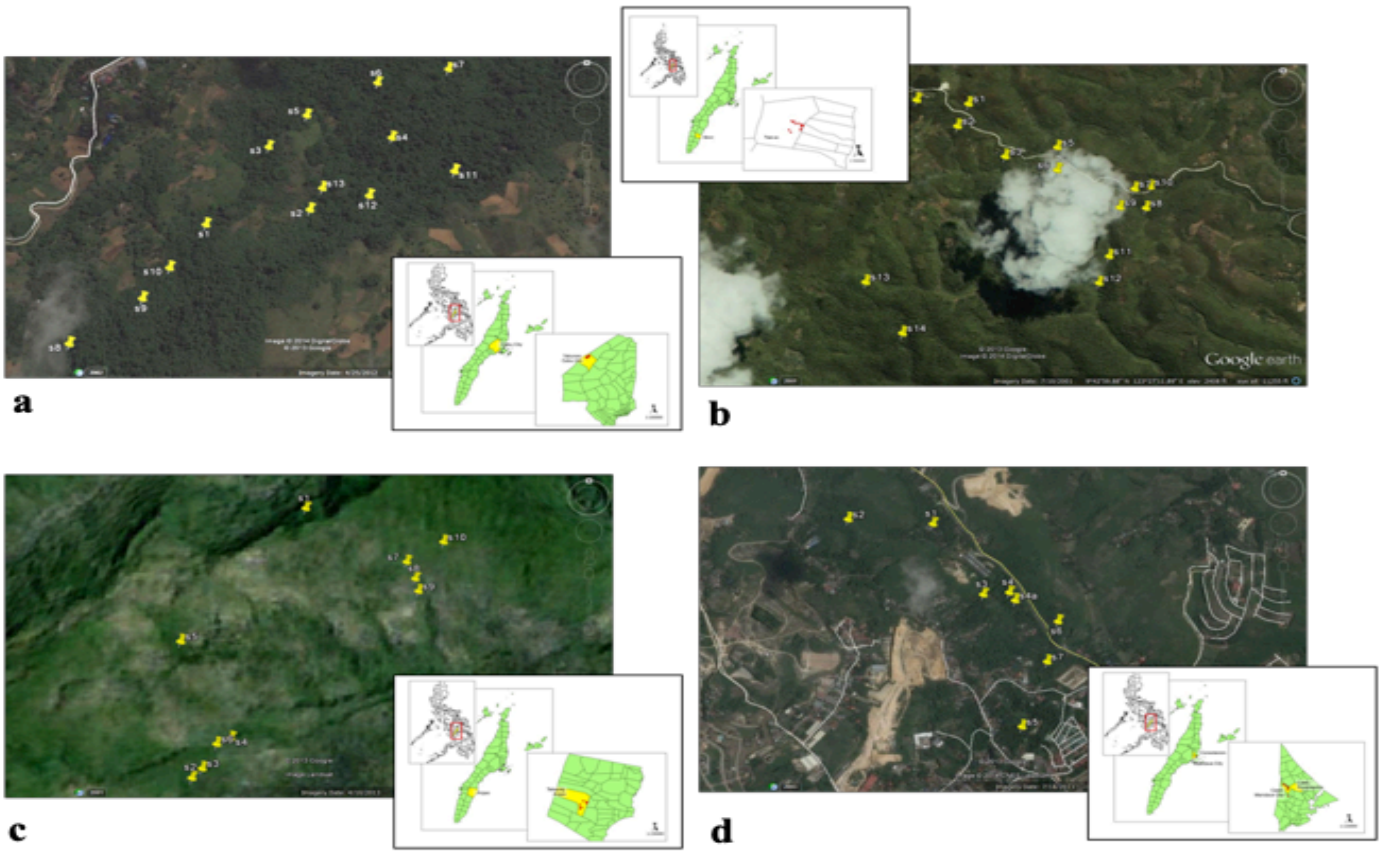

Figure 2. Location of mist net stations per study site. (a) Tabunan (n=13), (b) Nug-as (n=14), Tabayag (n=10), and Casili (n=8). (satellite image source: GoogleEarth).

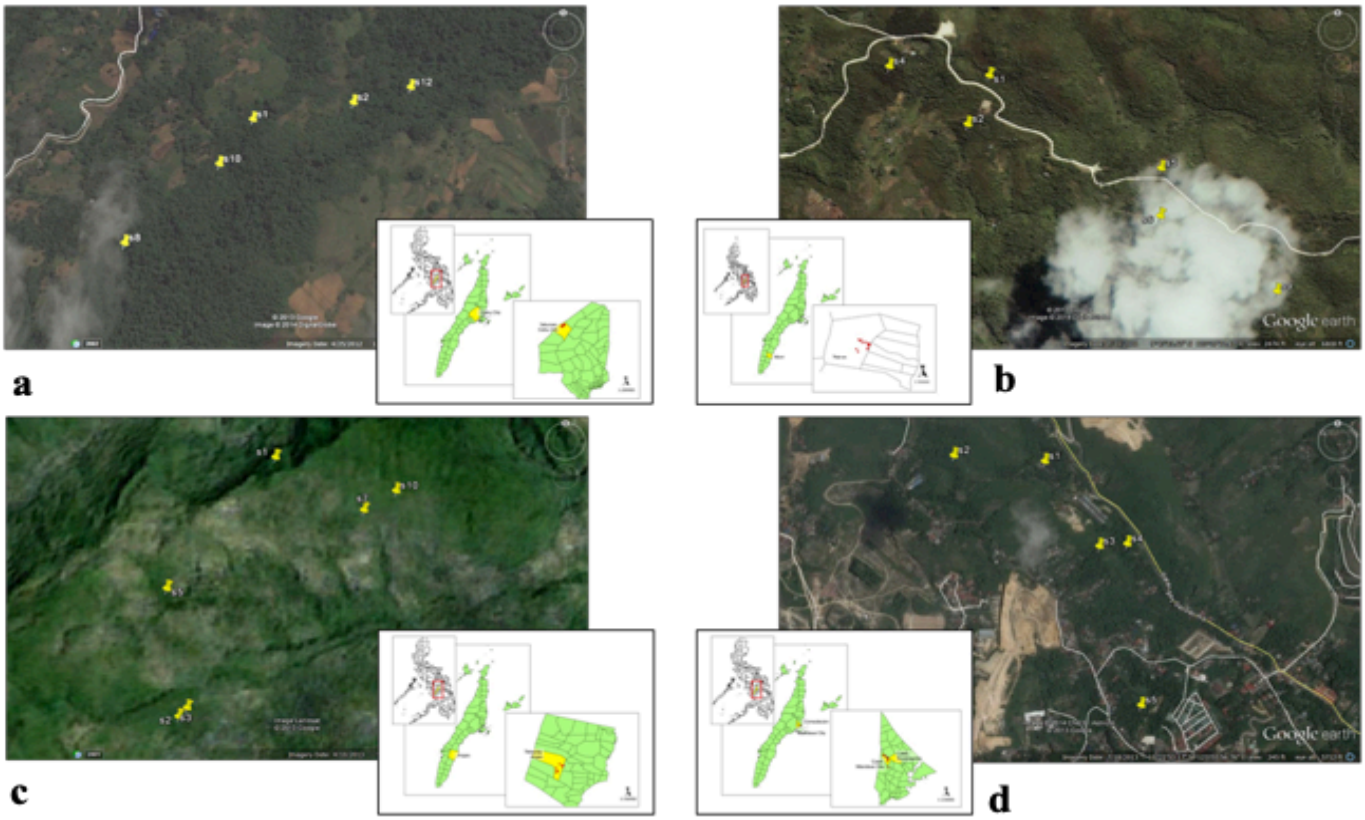

Figure 3. Location of successful mist net stations per study site. (a) Tabunan (n=5), (b) Nug-as (n =6), (c) Tabayag (n=6), and (d) Casili (n=6). (satellite image source: GoogleEarth).

Table 2. Number of black shama (*C. cebuensis*) individuals observed, captured, and released in Tabunan, Nug-as, Tabayag and Casili.

Location	Number of mist net stations	Number of successful mist net stations	Number of black shama seen	Individual to territory ratio	Number of black shama captured and released
Tabunan	13	5	18	1.38	6 (5 males and 1 female)
Nug-as	14	6	19	1.36	6 (1 male and 5 females)
Tabayag	10	6	14	1.40	6 (3 males and 3 females)
Casili	8	6	8	1.00	5 (all males)
TOTAL	45	23	59	1.31	23 (14 males and 9 females)

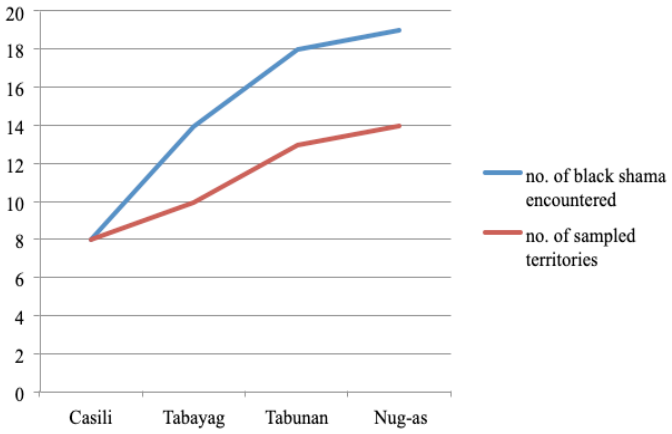

Figure 4. Number of encountered black shama (blue) and number of forest habitat/territories sampled (red) per IBA.

Conversely, based on the local's estimate of a black shama (*C. cebuensis*) territory, i.e., 1,000 m^2, sampling effort in this study has covered 45,000 m^2 or 4.5 ha forest. In this connection, it is estimated that 1 ha of *C. cebuensis* habitat supports 13 *C. cebuensis* individuals. This population size estimate in this study however is lower than that of *Malaki et al.* (*2013*), wherein at least 53 individuals were estimated to occupy 1 ha of forest. This difference can be accounted for the relatively higher degree of disturbances observed in the study sites. As reported by *Malaki and Buot* (*2011*), the disturbances observed in the black shama (*C. cebuensis*) habitat include human agricultural activities, fuelwood gathering, and wildlife hunting, among others. Similarly, it is known that *C. cebuensis* is a cryptic species which tend to shy away with the presence of humans (*Sheldon et al. 2009*).

Genetic Diversity

The DNA sequencing of cytB resulted to a useful nucleotide composition of 619 bp and the proportion of the bases is as follows: C=33.29%; T=25.17%; A=27.58%; and G=13.96%. The enrichment in cytosine of cytB and variation in the frequency of individual nucleotides may result from differential mutational pressures which is most probably caused by transcription-coupled mismatch repair. This particular event is also observed in mammalian genes and described to be not uncommon (*Louie et al. 2003*). Four cytB haplotypes are noted (**Table 3**). The most common haplotype is shared by 69.57% (n=16) of the 23 individuals, and was found across the four study sites, wherein 18.25% (n=3) was found in Tabunan, 31.25% (n=5) in Nug-as, 25.00% (n=4) in Tabayag, and another 25.00% (n=4) in Casili. One haplotype (4.35%) was unique to Nug-as indicating the role of this forest in sustaining bird's genetic diversity.

The cytB diversity parameters, such as number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (πn), substitution sites (S), and nucleotide differences (k) are higher in Tabayag and Tabunan while smaller in Casili and Nug-as (**Table 4**). Using the categories of *Grant and Bowen* (*1998*) (**Table 5**), which suggested that comparison of Hd and πn values within clades can provide information about patterns of historical population expansion and/or constriction, the values of Casili and Nug-as fall into category 1, with low Hd (<0.5) and low πn (<0.005), which may be attributed to recent population bottleneck or founder event by single or a few mtDNA lineages (*Grant and Bowen 1998*). On the other hand, the values of Hd and πn in Tabayag fall into category 4, with large values of Hd (> 0.5) and πn (> 0.005), characteristic of a large stable population with

Table 3. CytB haplotype frequency.

Haplotype	Frequency	Location/Code
1	16	Tabunan (n=3): Tab1M, Tab8M, Tab8F Nug-as (n=5): Alc1F, Alc2F, Alc4F, Alc5M, Alc6F Tabayag (n=4): Arg1M, Arg3F, Arg5F, Arg7M Casili (n=4): Cas2M, Cas3M, Cas4M, Cas5M
2	3	Tabunan (n=1): Tab10M Tabayag (n=2): Arg2F, Arg10M
3	3	Tabunan (n=2): Tab2M, Tab12M Casili (n=1): Cas 1M
4	1	Nug-as (n=1): Alc9F

Table 4. Sample sizes (N) and gene (cytB and COI) diversity estimates for the study.

Location/ Study Site	N	H	Hd + SD	πn ± SD	S	Tt:Tv	k
cytB (619 sites, 3 variable sites, 2 parsimony informative sites)							
Cebu	23	4	0.502±0.113	0.00534±0.00149	3	2:1	0.625
Tabunan	6	3	0.733±0.115	0.00490±0.00253	8	6:2	2.867
Nug-as	6	2	0.333±0.215	0.00199±0.00128	2	1:1	0.667
Tabayag	6	4	0.867±0.129	0.00642±0.00646	7	5:2	3.333
Casili	5	2	0.400±0.237	0.00213±0.00126	1	1:0	0.400

Number of haplotype (H), haplotype diversity (Hd) with standard deviation (Hd±SD), nucleotide diversity with standard deviation (πn±SD), number of substitutions (S) with ratio transitions versus transversions (Tt:Tv) and mean pairwise nucleotide difference (k)

Table 5. Interpreting haplotype and nucleotide diversities (*Grant and Bowen 1998*).

	Hd	
πn	**Small (<0.50%)**	**Large (>0.50%)**
Small (<1.0%)	1. Recent population bottleneck or founder event by single or a few mtDNA lineages.	2. Population bottleneck followed by rapid population growth and accumulation of mutations.
Large (>1.0%)	3. Divergence between geographically subdivided populations.	4. Large stable population with long evolutionary history or secondary contact between differentiated lineages.

long evolutionary history. Similarly, the values of Hd and πn in Tabunan fall into category 2, with high Hd (>0.5) and low πn (<0.005), indicating rapid expansion after a period of low effective population size and accumulation of mutations. As one population of the island of Cebu, the values Hd and πn fall into category 4, with high values of Hd and πn which are attributed to a long evolutionary history in a large stable population. In addition, Tajima's D test, Fu's Fs, and Fu and Li's D* are not significant (**Table 6**), indicating deviation from neutral evolution and suggestive of demographic expansion (*Lohman et al. 2008*). Likewise, none of the sums of squared deviations (SSD) was significant indicating that the curves fit the sudden expansion model tested.

The pairwise mismatch distribution for the cytB gene had a clear unimodal shape when all samples are taken together as one group belonging to the island of Cebu which is typical for recent population expansions resulting from coalescence of haplotypes to the same bottleneck event (*Lohman et al. 2008; Rodriques et al. 2013*) and deviations of the observed distributions of the nucleotide frequencies are not significantly different from those expected under a model of stepwise expansion. This trend was observed in the pairwise mismatch distribution of Casili, when the samples were examined separately. Conversely, the pairwise mismatch distribution of Tabunan, Nug-as, and Tabayag has bimodal shapes, suggesting that the initial population size of the black shama in these areas is larger before the expansion (**Figure 5**).

The AMOVA showed that variation among individuals within populations explained a significant proportion of the observed variance (**Table 7**). The *F*st (Fixation index) values between *C. cebuensis* populations from all groups of Cebu populations are not significant for the cytB gene (**Table 8**). These suggest that gene flow or exchange of genetic material through reproduction had existed between *C. cebuensis* individuals from

Table 6. Expansion indices for the black shama population: Expansion coefficient (S/k), Tajima's *D*, Fu's *Fs*, Fu and Li's *D**, Raggedness *r*, and Ramons-Onsins and Rozas *R2* values.

Location/ Study site	S/k	Tajima's *D*		Fu's *Fs*		*D**	Mismatch Distribution		*r*	*R2*
		D	*P*	*Fs*	*P*		SSD	P		
cytB										
Cebu	4.800	-0.591	0.244	-0.936	0.214	-0.1742	0.003	0.69	0.105	0.113
Tabunan	2.790	-1.072	0.142	1.720	0.820	-1.0627	0.122	0.32	0.240	0.328
Nug-as	2.999	-1.132	0.107	0.952	0.657	-1.1553	0.260	0.08	0.667	0.373
Tabayag	2.100	0.508	0.595	0.426	0.635	0.7198	0.103	0.20	0.204	0.202
Casili	2.500	-0.817	0.247	0.090	0.314	-0.8165	0.007	0.77	0.200	0.400

Significance level (P<0.05)

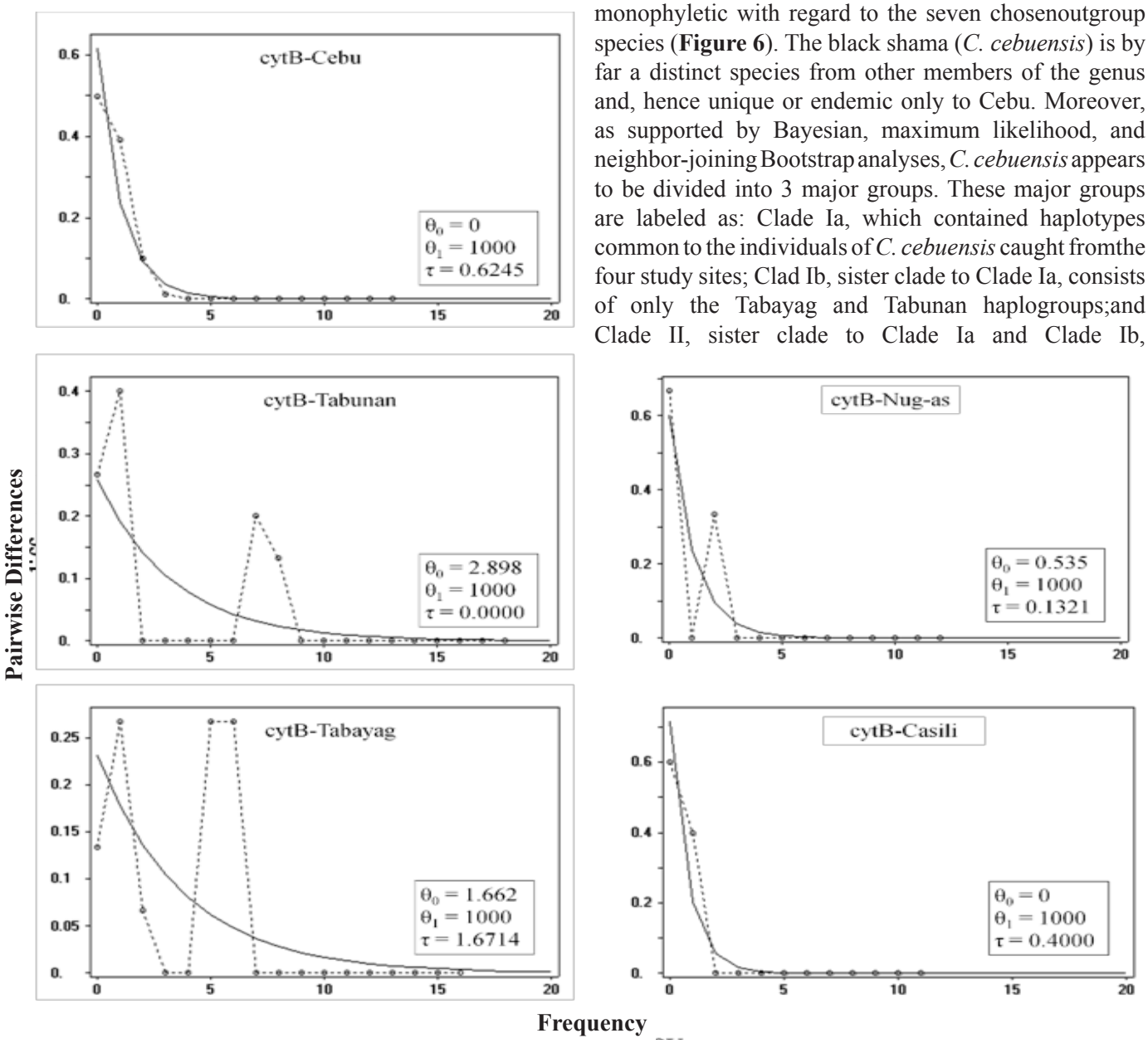

Figure 5. Pairwise mismatch distributions of cytB gene sequence of *Copsychus cebuensis* in the island of Cebu. Dashed lines indicate observed mismatch difference and solid lines represent the expected distribution in a constant population size. In the box, model for expected values in a population growth/decline: θ0 – Theta initial; θ1 – Theta final; τ – Tau.

different subpopulations in Cebu island. These results also suggest that *C. cebuensis* population in each study site a relatively stable population. On the other hand, these findings also imply that the populations were isolated very recently interms of evolutionary perspective, i.e. a sufficient time period has not passed for a possible separation of genetic lineages, leadingto a uniform genetic pattern (*Rodriques et al. 2013*).

Phylogenetic Analyses

Considering the cytB gene, *C. cebuensis* is monophyletic with regard to the seven chosenoutgroup species (**Figure 6**). The black shama (*C. cebuensis*) is by far a distinct species from other members of the genus and, hence unique or endemic only to Cebu. Moreover, as supported by Bayesian, maximum likelihood, and neighbor-joining Bootstrap analyses, *C. cebuensis* appears to be divided into 3 major groups. These major groups are labeled as: Clade Ia, which contained haplotypes common to the individuals of *C. cebuensis* caught fromthe four study sites; Clad Ib, sister clade to Clade Ia, consists of only the Tabayag and Tabunan haplogroups;and Clade II, sister clade to Clade Ia and Clade Ib, contained distinct haplotypes shared only by the black shama of Tabunan and Casili not found in Clade I.

The groupings of *C. cebuensis* form the constructed phylogenetic tree (**Figure 6**) showed that gene flow between populations existed as no clade is owned by a single haplogroup. Likewise, the tree also showed that the divergence of *C. cebuensis* is indicative of isolation. Hence, the populations of *C. cebuensis* in the four forest fragments being studied were once from a single population before the isolation event took place very recently. This isolation event is no other than

Table 7. Analysis of molecular variance (AMOVA) results.

Source of Variation	df	Sum of squares	Variance components	Percentage of variance	*F*st	P value
Cebu populations						
Among populations	3	1.457	-0.00253 Va	-0.51	-0.00508	1.000
Within populations	19	9.500	0.50000 Vb	100.51		
Total	22	10.957	0.49747			

Significance level (P<0.05)

Table 8. Pairwise genetic (cytB) differentiation statistics (Fst) among populations.

Location/ Study Site	Tabunan	P value	Nug-as	P value	Tabayag	P value	Casili
Tabunan	0.000	*					
Nug-as	0.000	0.99	0.000	*			
Tabayag	-0.029	0.99	0.000	0.99	0.000	*	
Casili	0.000	0.99	0.000	0.99	0.000	0.99	0.000

Significance level (P<0.05)

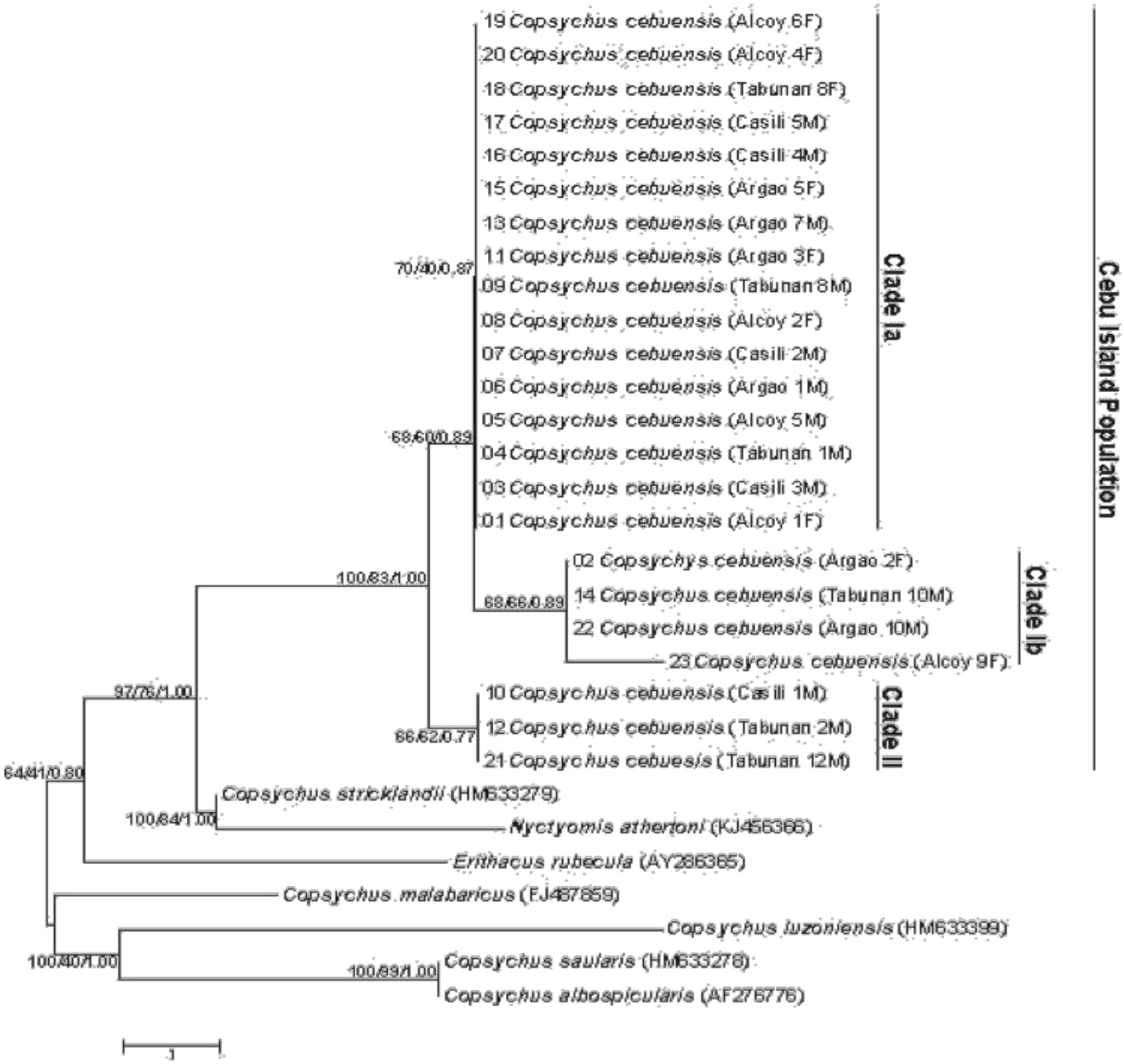

Figure 6. Consensus tree of *Copsychus cebuensis* cytB haplotypes Numbers above and below branches indicate maximum likelihood bootstrap support, neighbor-joining bootstrap support, and Bayesian inference, respectively.

deforestation. Historically, at the beginning of Spanish occupation in the Philippines in the 16th century, about 90% of land area was covered with forest (*Bankoff 2007 in Suarez and Sajise 2010*). However, logging under the Spanish rule for almost 300 years have reduced forest cover to 70% and by 1950, after the American and Japanese occupations, only about 50% forest cover was left (*Bankoff 2007 in Suarez and Sajise 2010*). Cebu was among the islands in the Philippines which was heavily deforested. In 1947, no patches of original forest in Cebu were present (*Rabor 1959*).

CONCLUSIONS AND RECOMMENDATIONS

Forty-five mist net stations were set-up in the black shama (*C. cebuensis*) territories in four selected forest fragments in the island of Cebu, Philippines wherein 59 *C. cebuensis* individuals were inhabiting the said territories. It was noted that number of individuals per territory for the whole island of Cebu was computed to be at 13.1 on the average. Based on the total area of territories covered in this study, the relative abundance of black shama (*C. cebuensis*) was estimated to be at 13 individuals per hectare.

Conversely, in terms of the genetic parameters such as the haplotype diversity (Hd) and nucleotide diversity (πn), the black shama population in the island of Cebu has a long evolutionary history from a large stable population. Independently, this trend was exhibited by the black shama in Tabayag's cytB gene (i.e., $Hd>0.50$ and $\pi n>0.005$). The cytB genetic parameters of the Tabunan's sample ($Hd>0.50$ and $\pi n<0.005$) demonstrated a population bottleneck followed by rapid population growth and accumulations of mutations. On the other hand, the genetic parameters of both Casili and Nug-as described recent population bottleneck or founder event by single or a few mtDNA lineages. Furthermore, three of the four cytB haplotypes were present in Tabunan samples while the samples from the three locations exhibit only two of the four cytB haplotypes.

Given the results of this study and some pertinent historical information, the most plausible explanation for the current genetic diversity and relative abundance of *C. cebuensis* in the forest fragments in Cebu island is the deforestation that took place in the recent past. Continuous efforts to rehabilitate the forests and the habitat of the *C. cebuensis* is seen to be an indispensable tool for the its continuing existence.

A black shama (*C. cebuensis*) habitat is characteristically a thickly vegetated area and that a hectare of black shama (*C. cebuensis*) habitat supports at least 13 individuals. It is therefore recommended that the black shama (*C. cebuensis*) habitats in the island of Cebu be enhanced or improved to increase their population. Similarly, a bird highway or habitat corridor must be built to connect the habitat fragments. This is to ensure continuous gene flow of *C. cebuensis* populations.

Among the forest fragments in island, Tabunan appeared as the center of *C. cebuenis* diversity. It is recommended that more management efforts be put in place in the area. However, the other three areas, namely Nug-as, Tabayag, and Casili must also be managed continuously to prevent the loss of *C. cebuensis* diversity.

Further genetic analyses using other genetic diversity parameters, such as microsatellites, be conducted to have a better understanding of the current state of the bird's genetic diversity. Likewise, legislators should initiate strict implementation of laws to protect the threatened bird's habitat.

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