Faculty and Staff Publication:

Adam McLain, PhD

Post-Print Final Draft

Citation for Final Published Work:

Brenneman, R. A., McLain, A. T., Taylor, J. M., Zaonarivelo, J. R., Lei, R., McGuire, S. M., ... & Louis, E. E. (2016). Genetic Analysis of the Indri Reveals No Evidence of Distinct Subspecies. *International Journal of Primatology*, 37(3), 460-477. DOI 10.1007/s10764-016-9911-3

Link to Publisher Site:

<http://link.springer.com/article/10.1007/s10764-016-9911-3>

Genetic Analysis of the *Indri* Reveals No Evidence of Distinct Subspecies

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Received: 24 September 2015 / Accepted: 9 June 2016
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Abstract Subspecies were traditionally defined by identifying gaps between phenotypes across the geographic range of a species, and may represent important units in the development of conservation strategies focused on preserving genetic diversity. Previous taxonomic research proposed that phenotypic variation between scattered *Indri indri* populations warranted the naming of two distinct subspecies, *I. i. indri* and *I. i. variegatus*. We tested these subspecific designations using mitochondrial sequence data generated from the control region or D-loop (569 bp) and a large section (2362 bp) of multiple genes and tRNAs known as Pastorini's fragment and nuclear microsatellite markers. This study used 114 samples of *I. indri* from 12 rainforest sites in eastern Madagascar, encompassing the entire range of the species. These genetic samples represent multiple populations from low- and high-elevation forests from both putative subspecies. Molecular analyses of the mitochondrial sequence data did not support the

Electronic supplementary material The online version of this article (doi:10.1007/s10764-016-9911-3) contains supplementary material, which is available to authorized users.

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two proposed subspecies. Furthermore, the microsatellite analyses showed no significant differences across the range beyond population level differentiation. This study demonstrates the utility of incorporating multiple lines of evidence in addition to phenotypic traits to define species or subspecies.

Keywords Lemur · Madagascar · Microsatellites · Mitochondrial DNA · Systematics · Taxonomy

Introduction

Traditionally, subspecies were morphologically assigned based on characters such as pelage, size, and behavior (Barton and Hewitt 1985); these intraspecific units may be important in the development of conservation strategies focused on preserving genetic diversity (Reed and Frankham 2003). Phenotypic variation such as coat color differences may lead to incorrect identification of some populations as independent species or subspecies. Genetic analyses now provide a methodology for testing historical species and subspecies designations previously based solely on aspects of phenotype. This synthesis of morphological and molecular analyses allows for a more comprehensive assessment of lineages (Prada *et al.* 2014).

Malagasy biodiversity displays a remarkable rate of endemism, and because of this there is an increased awareness and concern over the alarming rate of deforestation (Allnutt *et al.* 2008; Green and Sussman 1990; Harper *et al.* 2007; Jenkins 1990; Nicoll and Lagrand 1993). Since the early 20th century anthropogenic deforestation of Madagascar has increased substantially and remaining forested areas are under intense pressure. Forest-dwelling species are thus in great danger from continued habitat degradation and fragmentation (Allnutt *et al.* 2004).

Indri indri (Gmelin 1788) is the largest extant species of lemur and is known locally throughout its range in eastern Madagascar by its vernacular names of indri or babakoto (Powzyk and Thalmann 2003). *I. indri* is threatened by the reduction and fragmentation of habitat and from hunting (Golden 2005). The species' density range is sensitive to habitat quality and degradation (Glessner and Britt 2005). Designated as Critically Endangered according to the IUCN Red List of Threatened Species (IUCN 2015), *I. indri* is currently split into two subspecies with phenotypic variation (Groves 2001) that appears to be clinal (Louis *et al.*, unpublished). This clinal change from solid black to an admixture of black and white occurs from north to south through the range of the two suggested subspecies. *Indri indri indri* (Gmelin 1788) is distinguished by a predominantly black coat (Fig. 1a, b) often with white fur along the edges of the abdomen, a white pygal patch, and a white face ring (Thalmann *et al.* 1993), while *I. i. variegatus* (Gray 1872) has a black and white coat along with a white head cap (Fig. 1c–h). The type specimens held in European museums are the black variant and are all from the northern extent of the species' range (Groves 2001).

Thalmann *et al.* (1993) described the distribution of *Indri indri indri* as located in the northern part of the species range (above S15°15'30") and *I. i. variegatus* in the southern part of the range, with an area of possible overlap in Mananara Nord (S16°23') based on mixing of pelage patterns (Groves 2001). These distributions place *I. i. indri* in the northernmost extent of the range, between Anjanaharibe-Sud and Mananara Nord and

Fig. 1 Photographs of individual *Indri indri* representing populations from the northern to the southern portions of the species range from (a) Anjanaharibe-Sud Special Reserve, (b) Marotandrano Special Reserve, (c) Mananara Nord National Park, (d) Zahamena Special Reserve and National Park, (e) Analamazoatra Special Reserve (Andasibe), (f, g) Mantadia National Park, and (h) Maromizaha Classified Forest.

I. i. variegatus from Mananara Nord south to Anosibe an'ala (Fig. 2). Subfossil evidence indicates that *Indri* were once found as far north as the Ankarana Massif cave systems and the highlands of Ampasambazimba (Goodman and Jungers 2014; Jungers *et al.* 1995).

Fig. 2 Map of the forest fragments in eastern Madagascar harboring populations of *Indri* sampled in this study (Zaonarivelo *et al.* 2007b).

Some have expressed doubt about the validity of this subspecific classification due to overlap of pelage patterns in many forest fragments (Mittermeier *et al.* 2010). To date, aside from the systematic placement of the genus in the family Indriidae by karyotype analysis (Rumpler *et al.* 1988) and cytochrome *b* sequencing (Del Pero *et al.* 1995; Razafindraibe *et al.* 2000), little information is available on the genetics of the genus *Indri*. A previous population genetics study on *I. indri* indicated that similar genetic diversity existed within 10 populations and does not differ substantially from the genetic diversity estimated in other lemur species (Zaonarivelo *et al.* 2010). Zaonarivelo *et al.* (2007a) found no morphological evidence to suggest a subspecies differentiation, with the only observable differences between populations of *I. indri* being found in coat color and pattern. In addition, the Zaonarivelo *et al.* (2007a) study found no significant differences in physical measurements to support that *I. i. indri* is larger than *I. i. variegatus*, as was suggested by Groves (2001).

Here, we analyzed mitochondrial sequence data gathered from 114 individuals of *Indri indri* from 12 populations across the species' range. In addition, we analyzed nuclear microsatellite multilocus genotypes from 106 individuals from 10 of those sites. We use these datasets to test previous subspecies designations within the genus *Indri* and predict that genetic isolation will be greatest at the extremes of the geographic range. These results can resolve the taxonomic status of this flagship conservation species.

Methods

Sampling Locations

We sampled 114 individuals of *Indri indri* from 12 sites across the geographical range of the species from 2000 to 2005 (Fig. 2; Electronic Supplementary Material [ESM] Appendix S1). In addition to the sites reported in Zaonarivelo *et al.* (2010), we immobilized lemurs in Mananara Nord National Park and Mangerivola Special Reserve. The sampling sites ranged from lowland forests (Anosibe An'ala, 125 m asl) to highland forests (Anjozorobe, 1358 m asl).

Immobilization and Collection

We immobilized free-ranging lemurs using a CO₂-powered DAN-INJECT (Børkop, Denmark) Model JM rifle propelling Pneu-Darts (Williamsport, PA) loaded with 10 mg/kg estimated body weight of Telazol® (Fort Dodge). We collected whole blood (1.0 cc/kg) and 2.0-mm skin biopsies from the ear pinnae of each sedated lemur (Junge and Louis 2002) and placed a HomeAgain® (Schering-Plough Home Again LLC, Whitehouse Station, NJ) microchip subcutaneously between their scapulae. The microchip provides a unique recognition code to identify recaptured individuals during future immobilizations. Following data and sample collection, we administered a subcutaneous injection of lactated Ringer's solution to dissipate the effect of the anesthetic and to support the lemur's maintenance requirements. After a 3-h period of post recovery monitoring, we released each individual according to the capture coordinates taken at the time of immobilization.

Data Generation

We dissected ear punches into quarters and extracted the DNA using standard PCI/chloroform procedures (Sambrook *et al.* 1989). For all samples, we amplified the following regions of the mitochondrial genome: the displacement loop or mitochondrial control region (D-loop) (Baker *et al.* 1993; Wyner *et al.* 1999) and the PAST fragment (Pastorini *et al.* 2000) consisting of the cytochrome oxidase subunit III gene (*COIII*); NADH-dehydrogenase subunits 3, 4 L, and 4 (ND3, ND4L and ND4); as well as the tRNA^{Gly}, tRNA^{Arg}, tRNA^{His}, tRNA^{Ser}, and partial tRNA^{Leu} genes. We amplified the D-loop and PAST fragments using 50 ng of genomic DNA under the following conditions: 94 °C for 30 s, 47 °C (D-loop) and 50 °C (PAST) for 45 s, and 72 °C for 45 s for 34 cycles. Since potential nuclear insertions or mitochondrial pseudogenes within the nuclear genome can be amplified inadvertently, we minimized the likelihood by amplifying mitochondrial DNA regions as intersecting or overlapping segments and confirming these segments with

the degenerate oligonucleotide-primed polymerase chain reaction (PCR) methodology (Telenius *et al.* 1992; Zhang and Hewitt 1996). Thus, we amplified and sequenced three 1000-bp fragments with approximately 200 overlapping base pairs. We electrophoresed the amplified products on a 1.2 % agarose gel to confirm successful amplification and then purified them using QIAquick® PCR purification kits (Qiagen; Valencia, CA).

We cycle sequenced the purified products using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Using an Applied Biosystems Prism 3100 genetic analyzer, we generated the sequences by capillary electrophoresis. Sequencing and PCR primers specific for *Indri* were designed for the D-loop fragment (DLPMT4F: GACGGGATGAGCCTTACAAG; DLPMT4R: TCCACATCCTAGCA AATCGC). We used a suite of internal sequencing primers from Pastorini *et al.* (2000, 2001) to generate the PAST fragment, along with specific primers designed for *Indri* (ESM Appendix S2). Sequences are available in GenBank (ESM Appendix S1). We selected four individuals of additional species (*Avahi occidentalis*, *A. peyrierasi*, *Propithecus edwardsi*, and *P. verreauxi*) as outgroups for the phylogenetic tree reconstructions because of their familial relationships (Andriantompohavana *et al.* 2007).

Phylogenetic Analysis

We constructed consensus sequences from assembled sequence fragments using Sequencher v5.0 (Gene Corp; Ann Arbor, MI) and aligned the consensus sequences with ClustalX (Thompson *et al.* 1997). We compared sequences, estimated measures of variability, and calculated the number of parsimony informative sites and uncorrected p distances for D-loop and PAST fragments using MEGA v4.0 (Tamura *et al.* 2007). Using the Akaike information criterion as implemented in Modeltest v3.7 (Posada and Crandall, 1998), we selected the optimal nucleotide substitution models for each data set. We performed the maximum likelihood (ML) analyses for each data set under the best model selected by Modeltest v3.7 (GTR + I + G for PAST, HKY + I + G for D-loop) with PhyML v3.0 (Guindon *et al.* 2010). The best scoring trees were searched and saved in PAUP* v4.0b10 (Swofford 2001). We conducted Bayesian inference analyses of both sets of sequence data using MrBayes v3.0b4 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) with the best model of evolution (GTR + I + G for PAST and HKY + I + G for D-loop) as selected by MrModeltest v2.2 (Nylander 2004). We performed two Markov Chain Monte Carlo (MCMC) runs with four simultaneous chains and 1,000,000 generations for the D-loop and PAST fragments. We tested for convergence of the Bayesian run replicates with Tracer v1.5 (Rambaut and Drummond 2009), saving the tree with the best likelihood score every hundredth generation. Using PAUP*, we condensed the trees into a majority rule consensus tree for each fragment and computed clade posterior probabilities.

We estimated the number of haplotypes, segregating sites and haplotype diversity ($\hat{h}$) and the standard deviation (SD) from mitochondrial D-loop fragment sequences (Nei 1987). Following Nei *et al.* (1979), we estimated uncorrected nucleotide diversity (π), or the mean of pairwise sequence differences (k) and the SD for each population, using Arlequin v3.0 (Excoffier *et al.* 2005). We assigned portions of total genetic variation to divergence either among or within haplotype groupings. This approach incorporated information on the absolute number of differences among haplotypes as well as on haplotype frequencies. The significance of variance components and F -statistic analogues, designated Φ -statistics, was tested by multiple (1000) random permutations using

Arlequin. We estimated the evolutionary relationships of mitochondrial haplotypes by conducting a minimum spanning network using NETWORK 4.6.1.0 (Bandelt *et al.* 1999).

To test the correlations between log genetic similarity (M) and the geographic distance (km) we used the Isolation by Distance Web Service (IBDWS) v3.23 (Jensen *et al.* 2005; <http://ibdws.sdsu.edu/~ibdws/>). To calculate the average geographical distances between populations we used Google Earth (Google, Mountain View, CA). We analyzed the mtDNA data with Φ_{ST} (estimated from nucleic data) to allow incorporation of sequence distance information. Following Rousset's distance, defined as $R = (\Phi_{ST}/1 - \Phi_{ST})$, we estimated the genetic distance between populations (Rousset 1997). Using IBDWS, we calculated a Mantel test to estimate a correlation between geographic and genetic distances using 30,000 iterations.

Genotype Analysis

We generated multilocus genotypes from a suite of 20 *Indri*-specific microsatellite loci as described in Zaonarivelo *et al.* (2007b). We included only 10 populations in the multilocus genotype analyses as two of the forests, Mananara Nord ($N=4$) and Mangerivola ($N=3$) were underrepresented with respect to statistical thresholds of 10 individuals per population. We checked the genotype file for errors such as typographical, scoring, stutter bands, and allelic dropout with Micro-Checker (van Oosterhout *et al.* 2004) and Microsatellite Analyser (MSA; Dieringer and Schlötterer 2002). We used CERVUS v2.0 (Marshall *et al.* 1998; Slate *et al.* 2000) to identify loci with excessive null allele frequency estimates ($nf > 0.10$) and to estimate relative informative value as polymorphic information content (PIC). Moderate ($0.05 < nf < 0.20$) and high ($0.20 < nf$) null frequencies can have significant effects on population genetics parameter estimates (Chapuis and Estoup 2007). We excluded any loci with moderate null allele frequency ($nf > 0.1$) for the downstream analyses to reduce the bias from misclassification of null heterozygotes as homozygotes (Callen *et al.* 1993; Hoffman and Amos, 2005) and to control the variance of parameter estimates (Chapuis and Estoup 2007). We verified the accepted loci for independence of linkage disequilibrium with Bonferroni-adjusted P -values in FSTAT v2.9.3.2 (Goudet 1995, 2001).

We tested population relationships with two methods to detect levels of structure within and between the populations. Using principal components analysis (PCA) from the multilocus genotype file in PCA-GEN (Goudet 1999), we generated the relationships among the representatives of the pooled populations. The first two axes of greatest inertia were used to generate the PCA output from 10,000 randomizations. We inferred population structure using STRUCTURE v2.1 (Falush *et al.* 2003; Pritchard *et al.* 2000), which uses a Bayesian clustering based method to deduce hidden genetic structure without including population information *a priori*. The program enables the detection of cryptic substructure in populations and defines larger differences between populations. We compared the STRUCTURE results to known phenotypic characteristics (specifically solid and variegated pelage characteristics). STRUCTURE attempts to identify population subsets that minimize HWE and LD from multilocus genotypes (Pritchard *et al.* 2000). We used the ancestry model, correlated allele frequencies, different F_{ST} values assumed for each subpopulation, a uniform prior for α (max: 10, SD for updating: 0.025), constant λ value of 1, prior F_{ST} mean (0.01), and standard deviation (0.05). We estimated 1–13 genetic clusters as Evanno

et al. (2005) suggests testing a range of at least three clusters more than sampling locations. We set the burn-in period at 10^5 repetitions followed by 10^6 MCMC repetitions for 20 iterations of the Gibbs sampler for each K value (1–13). We implemented the Evanno *et al.* (2005) *ad hoc* test statistic to evaluate output files because STRUCTURE occasionally overestimates the optimal K value. We estimated the correlations between log genetic distance (M) and log geographic distance (km) as described earlier. For the nuclear data, we estimated the genetic distance between populations following Rousset's distance, defined as $R = (F_{ST}/1 - F_{ST})$ (Rousset 1997).

Ethical Note

Trained personnel handled all lemurs in this study according to American Society of Mammalogists ethical guidelines (Sikes and Gannon 2011). Immobilization and handling procedures followed Louis *et al.* (2006a). Madagascar's Ministère de l'Environnement et des Forêts authorized all field work in this study and we transported samples in full compliance with the relevant laws of Madagascar and the United States of America. All interactions with the study subjects abided by Omaha's Henry Doorly Zoo and Aquarium's IACUC (97-001, 12-101).

Results

Mitochondrial DNA Analyses

Mitochondrial DNA sequencing was successfully completed for D-loop and the PAST fragment for 114 individuals from 12 forest fragments (ESM Appendix S1).

Table 1 Estimates of gene diversity in the mtDNA D-loop sequences in 12 populations of *Indri*

	N	$\hat{h}$	k	π
pop1	10	0.711 ± 0.118	4.489 ± 2.414	0.008 ± 0.005
pop2	5	0.900 ± 0.161	7.000 ± 3.963	0.012 ± 0.008
pop3	10	0.756 ± 0.130	3.489 ± 1.941	0.006 ± 0.004
pop4	11	0.782 ± 0.107	5.091 ± 2.675	0.009 ± 0.005
pop5	14	0.934 ± 0.045	7.242 ± 3.610	0.0127 ± 0.007
pop6	10	0.511 ± 0.164	4.533 ± 2.435	0.008 ± 0.005
pop7	10	0.644 ± 0.152	2.889 ± 1.656	0.005 ± 0.003
pop8	10	0.844 ± 0.103	7.978 ± 4.053	0.014 ± 0.008
pop9	11	0.618 ± 0.164	2.945 ± 1.669	0.005 ± 0.003
pop10	3	1.000 ± 0.272	6.000 ± 3.928	0.011 ± 0.009
pop11	10	0.933 ± 0.062	4.889 ± 2.602	0.009 ± 0.005
pop12	10	0.911 ± 0.077	5.511 ± 2.895	0.010 ± 0.006
Total		0.971 ± 0.005	9.799 ± 4.520	0.017 ± 0.009

N number of individuals, $\hat{h}$ haplotype diversity, k mean number of pairwise differences, π nucleotide diversity

Table II Pairwise F_{ST} values among different populations of *Indri* based on mitochondrial DNA D-loop haplotype frequencies (below diagonal) and uncorrected pairwise distances (p above diagonal)

1	2	3	4	5	6	7	8	9	10	11	12
1	0.010	0.010	0.012	0.011	0.01	0.009	0.015	0.027	0.011	0.03	0.025
2	0.210**	0.010	0.011	0.013	0.012	0.011	0.016	0.029	0.013	0.033	0.028
3	0.267*	0.130	0.009	0.012	0.012	0.008	0.013	0.026	0.010	0.028	0.024
4	0.253***	0.137	0.163**	0.014	0.013	0.010	0.015	0.027	0.011	0.030	0.025
5	0.159***	0.080*	0.150***	0.139***	0.012	0.011	0.016	0.026	0.013	0.029	0.024
6	0.389***	0.333***	0.367***	0.350***	0.012	0.011	0.016	0.028	0.012	0.032	0.027
7	0.322***	0.250*	0.300***	0.285***	0.422***	0.011	0.012	0.025	0.008	0.029	0.023
8	0.222***	0.132*	0.200**	0.187***	0.322***	0.208*	0.221***	0.018	0.013	0.022	0.017
9	0.336***	0.269***	0.315***	0.300***	0.434***	0.369***	0.221***	0.261	0.023	0.008	0.008
10	0.192	0.058	0.161	0.144	0.338*	0.031	0.069	0.190**	0.042	0.026	0.021
11	0.178***	0.081	0.156**	0.144***	0.278***	0.211***	0.093*	0.186***	0.057	0.029	0.010
12	0.189***	0.094	0.167***	0.155***	0.289**	0.222***	0.035				

1 = Anjanaharibe Sud; 2 = Mananara-Nord; 3 = Marotandrano; 4 = Ambatovaky; 5 = Zaharana; 6 = Betampona; 7 = Anjozorobe; 8 = Mantadia; 9 = Andasibe; 10 = Mangerivola; 11 = Maromizaha; 12 = Anosibe An'ala.

Statistically significant difference from 0 at * $P=0.05$, **0.01, and ***0.001

Fig. 3 Maximum likelihood phylogram generated with D-loop sequences from 114 *Indri indri* and 25 outgroup taxa. Values associated with the branches show proportion of substitutions per site by Phym/ Bayesian support by MrBayes ($-\ln$ likelihood = 9844.99078).

For the 569-bp D-loop sequences, we identified 48 variable sites of which 38 were parsimony informative in 41 haplotypes (ESM Appendix S3). Genetic diversity parameters for and population differentiation measures based on the D-loop are presented in Tables I and II. The mean haplotype diversity (h) was 0.971 ± 0.005 and per site nucleotide diversity (π) was 0.017 ± 0.009 . For the 2367-bp PAST sequences, we identified 93 variable sites of which 54 were parsimony informative. Based on the phylogenetic reconstruction of ML and Bayesian analyses of two sequence alignments, all individuals formed a monophyletic clade (Fig. 3; ESM Appendix S4). The mean absolute genetic distance and uncorrected pairwise distance of mtDNA D-loop sequences among individuals were 9.71 ± 1.71 (from 1 to 22) and 0.017 ± 0.003 (from 0.002 to 0.037), respectively (ESM Appendix S5). The mean absolute genetic distance and uncorrected pairwise distance of mtDNA PAST fragment sequences among individuals were 10.36 ± 1.64 (from 0 to 30) and 0.004 ± 0.001 (from 0.000 to 0.012), respectively (ESM Appendix S6).

Haplotypes from the southern portion of the distribution (Anosibe an'ala, Andasibe, Mantadia, and Maromizaha) formed one haplogroup. Although Mangerivola was geographically proximate to the southern haplogroup it clustered with a second haplogroup that included all other populations. Also, most haplotypes from Anjozorobe populations clustered with the second haplogroup except I21 (Fig. 4). We found no relationship in the isolation-by-distance analyses between log genetic similarity (M) and log geographic distance ($r^2 = 0.01$, $P < 0.31$, $y = 3.06x - 8.07$; ESM Appendix S7).

Microsatellite Testing

After excluding 8 monomorphic loci, 12 *Indri*-specific microsatellite loci met the criteria to screen the 10 populations. The resulting marker suite was highly informative, with an average PIC value of 0.871 ($0.704 < \text{PIC} < 0.937$). None of the loci departed from HWE following a Bonferroni correction for multiple tests. The estimated global locus characterizations are presented in Table III.

The PCA plot of the two most informative axes showed a general mixing of the individuals represented in the data set, suggesting that there is considerable admixture between the individuals sampled, and none of the individuals grouped according to population of origin (Fig. 5). The ΔK estimate identified $K = 4$ as the most likely with $K = 8$ as a secondary possibility to describe the genetic clustering in the 10 population data set (ESM Appendix S8). We identified genetic structure between Anjanaharibe-Sud and Marotandrano plus Ambatovaky populations (with at least 80 % genetic assignment to the two clusters; Fig. 6 and ESM Appendix S9), despite observed pelage differences (Fig. 1a, b). Isolation-by-distance analyses of the 10 populations *Indri* indicated that there was a significant negative correlation between log genetic similarity and log geographic distance ($r^2 = 0.381$, $P < 0.002$, $y = 0.43x + 2.09$; ESM Appendix S7).

Fig. 4 Minimum spanning network of haplotypes of *Indri indri* based on mitochondrial DNA control region sequences. Circles represent haplotypes with size proportional to relative frequencies. Sectors within circles are proportional to the frequency of each haplotype observed in each population. Populations are identified by different colors. The white circle indicates extinct or unsampled haplotypes. When mutation steps were less than 2, they are labeled with dashes; otherwise, they are presented with the number of mutation steps in their connecting lines.

Table III Locus characterizations for 12 *Indri*-specific microsatellite loci showing number of alleles (k), observed (H_O) and expected (H_E) heterozygosities, polymorphic information content (PIC), and Wright's F statistics F_{IS} , F_{ST} , and F_{IT}

Locus	k	H_O	H_E	PIC	F_{IS}	F_{ST}	F_{IT}
67HDZ4	16	0.726	0.900	0.887	0.129	0.081	0.200
67HDZ 16	11	0.821	0.891	0.876	0.045	0.082	0.123
67HDZ 17	9	0.604	0.744	0.704	0.168	0.082	0.237
67HDZ 25	19	0.774	0.870	0.854	0.110	0.054	0.158
67HDZ 39	13	0.755	0.862	0.842	0.084	0.096	0.172
67HDZ 55	18	0.708	0.893	0.879	0.224	0.026	0.244
67HDZ 62	9	0.743	0.854	0.834	0.118	0.067	0.177
67HDZ 76	10	0.745	0.865	0.845	0.114	0.080	0.184
67HDZ	26	0.762	0.944	0.937	0.197	0.041	0.230
67HDZ 161	17	0.876	0.905	0.892	-0.001	0.083	0.083
67HDZ 177	22	0.667	0.815	0.792	0.173	0.062	0.225
67HDZ 210	15	0.755	0.903	0.890	0.173	0.038	0.205
All	15.4	0.745	0.871	0.853	0.128	0.066	0.186

Fig. 5 Plot of the 2 most informative of 29 significant inertia axes detected by principal components analysis for *Indri indri*. Axis 1: 6.06 % total inertia, $F_{ST} = 0.034$ ($P = 0.575$); axis 2: 5.04 % total inertia, $F_{ST} = 0.029$ ($P = 0.681$). Key: Individual ID numbers of individuals sampled at Anjanaharibe-Sud (ANJ, JAR), Marotandrano (TANDRA), Ambatovaky (VAK), Zahamena (ZAH, ZAHA), Betampona (BET), Anjozorobe (ANJZ, JOZO), Mantadia (TAD), Andasibe (DASI), Maromizaha (MIZA), Anosibe An'ala (ANOSIBE).

Discussion

We observed no significant genetic differentiation of populations of *Indri* in our dataset congruent with a two-subspecies classification system. Our dataset supports the monotypic taxonomic designation *Indri indri* for this species. The genus *Indri* was previously split (Groves 2001) into two subspecies (*I. i. indri* and *I. i. variegatus*) based on phenotypic variation, predominantly in the color of the pelage. This pelagic variation appears to be clinal (Louis *et al. unpubl. data*) with the solid dark occurring in the far north and the lighter spotting pattern occurring in the south. Groups found in the middle forests such as Anjozorobe (Mittermeier *et al.* 2010), Mananara Nord (Thalmann *et al.* 1993), Mantadia (6/24/2001–7/6/2001 expedition; this study) and Zahamena (8/29/2004–9/5/2004 expedition, this study) harbor individuals with both pelage variants (Fig. 1f). We observed only solid black individuals comprising the populations in the far northern forests. We also observed only various proportioned black and white patterns in the furthest forest populations to the south across a population and within social groups. However, we observed groups with mixed color representation in many of the mid-range forest populations from Mantadia northward into Mananara Nord.

Mitochondrial DNA sequence analysis of the fastest evolving gene fragment, the control region or D-loop, suggests shared haplotypes in many of the forest populations. While individuals from regional sampling tend to group together, there were also individuals that scattered along the phylogenetic tree demonstrating long-term gene flow within *Indri* that was not observed in other lemur taxa that inhabit the same range (Andriantompohavana *et al.* 2007; Louis *et al.* 2006a, b). We did not detect limited

Fig. 6 Bar graphs of genetic proportions (Q) for 10 populations of *Indri* for $K=2$ (a), $K=4$ (b), and $K=8$ (c) genetic clusters. Population number is on the x -axis with the proportion of genetic contribution on the y -axis. Key: 1 = Anjanaharibe Sud; 2 = Marotandrano; 3 = Ambatovaky; 4 = Zahamena; 5 = Betampona; 6 = Anjozorobe; 7 = Mantadia; 8 = Andasibe; 9 = Maromizaha; 10 = Anosibe An'ala.

genetic variation or substructure using either of the two mtDNA sequence analyses that would have identified the putative subspecific ranges.

As increasing spatial distances infer longer temporal separation since populations exchanged genetic material, population fixation indices should be greater at opposite ends of a large range. Both F_{ST} and DA should be the greatest between Anjanaharibe-Sud of the north and the southern Mantadia, Andasibe, Maromizaha and Anosibe An'ala. That is not the case, as our data demonstrated that the differentiation and genetic distance between Anjanaharibe-Sud and the much closer northern populations, Marotandrano and Ambatovaky, were greater. Isolation by distance analyses based on microsatellite loci demonstrated that there was a significant correlation between population differentiation and the distance separating the populations; however, there was a weak relationship between genetic differentiation and distance based on mtDNA. Historical gene flow or recent habitat fragmentation may lead to this pattern. Survey work around Betampona has shown populations of *Indri* to be susceptible to habitat fragmentation, with populations possibly becoming locally extinct or being driven out of their historical habitats in as little as 2 yr (Glessner and Britt 2005). Data are lacking on dispersal capabilities of *Indri* across fragmented landscapes and about dispersal

differences between sexes [such as those observed in gorillas by Roy *et al.* (2014)], which may also contribute to these genetic patterns.

Microsatellite analyses with *Indri*-specific loci support a single population with no evidence of subspecific structure. From the STRUCTURE global analysis, the greatest likelihood should occur at the K value corresponding to the number of genetic clusters inferred given the populations in the data set. If two distinctive subspecies were represented in the sample collection, then $K = 2$ should give a high likelihood of probability (Fig. 6a). Population 1 (Anjanaharibe-Sud) should absolutely identify one cluster since this forest is considered to harbor the subspecies *I. i. indri* and population 8 (Andasibe) should positively identify the alternative subspecies *I. i. variegatus* (Groves 2001; Thalmann *et al.* 1993). That differentiation is not present in the genetic analyses between these two reference populations. We found that individuals from the two populations at Anjanaharibe-Sud and Anosibe an'ala, the extremes of the species' geographic range, were genetically similar to each other (Table III). This genetic proximity is not expected from individuals that should be, by location and phenotype, representative of the two subspecies.

The STRUCTURE population analyses suggest that excess of homozygotes detected in the three populations (Anjanaharibe-Sud, Betampona and Anosibe an'ala) that deviated from HWE is not likely due to Wahlund effects (Zaonarivelo *et al.* 2010). No significant substructure was detected in any of the 10 populations. The F_{IS} estimates indicated that there was either background inbreeding from bottleneck events or from the mating of pedigree-related individuals, or some other violation of HWE assumptions. Home ranges of *Indri* have been estimated to be 18–37.5 ha with variable densities (6.6–16 individuals/km²; Glessner and Britt 2005) possibly related to habitat quality. If *Indri* have low dispersal capabilities and are at risk to local population declines, it makes sense that rebounding groups would be inbreeding in fragments large enough to support a group(s), and may have poor landscape connectivity, which could affect HWE.

Indri, occupying forests from the lowlands to the highlands and around the headwaters of the major rivers, have maintained more genetic homogeneity over time than many other lemur genera (Andriantompohavana *et al.* 2006, 2007; Louis *et al.* 2006a, b; Mayor *et al.* 2004). Populations of *Indri* exhibit haplotype homogeneity at both the D-loop and PAST mtDNA fragments and no significant population structure using multilocus genotypes. We find no genetic evidence to support the continued use of subspecific nomenclature, and therefore propose suspending the usage of two subspecies for *Indri*. The results of this study support that of other researchers in that all data, including morphometric, qualitative, and molecular, should be considered to reach the most accurate conclusions possible regarding species status (Padial *et al.* 2010).

Acknowledgments We acknowledge the Ministry of Environment and Eaux & Forêts (CAFF/CORE), Madagascar National Parks (MNP), Association FANAMBY, and the Department of Biological Anthropology, University of Antananarivo for their help. This project would not have been possible without the support of the staff of MICET and Omaha's Henry Doorly Zoo & Aquarium (OHDZA) and the Madagascar Biodiversity Partnership (MBP), as well as Parc Botanique et Zoologique de Tsimbazaza, U.S. Fish & Wildlife Service, Bill and Berniece Grewcock, the Ahmanson Foundation, the Theodore F. and Claire M. Hubbard Family Foundation, and the James Family Foundation. We would also like to acknowledge John Callahan, Michael Bosetti, Nikola Miljkovic, Cynthia Frasier, Melissa Hawkins, The Peter Kiewitt Institute, Holland Computing Center, University of Nebraska Foundation, and the University of Nebraska at Omaha for their enthusiastic support for seeing this project through to completion. In addition, we would like to thank the editor and reviewers from the *International Journal of Primatology* for their thoughtful comments and suggestions on this manuscript.

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