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RESEARCH ARTICLES

Genetic Assessment of a White-Collared × Red-Fronted Lemur Hybrid Zone at Andringitra, Madagascar

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We examined a purported lemur (*Eulemur fulvus rufus* × *E. albocollaris*) hybrid zone at Andringitra, Madagascar, using sequences from five genes (one mitochondrial gene (d-loop) and four nuclear introns (hemopexin, malic enzyme, ceruloplasmin, and microsatellite 26 flanking region)), from 60 individuals (*E. albocollaris* (n = 16), *E.f. rufus* (n = 14), *E. collaris* (n = 9), and purported hybrids from Andringitra (n = 21)). Diagnostic (d-loop and microsatellite 26) and private sites (all other genes) were found in all gene regions for *E. albocollaris* and *E.f. rufus*. Also, private sites were found for the purported hybrid population in two gene regions (d-loop and ceruloplasmin). When the putative hybrids were examined for diagnostic and private markers, 18 of 21 were found to contain markers from both *E. albocollaris* and *E.f. rufus* populations. The remaining three individuals were found to contain only markers for *E. albocollaris*. These results indicate that the population at Andringitra is a hybrid population between *E. albocollaris* and *E.f. rufus*. Am. J. Primatol. 57:51–66, 2002. © 2002 Wiley-Liss, Inc.

Key words: *Eulemur fulvus*; species units; hybridization; primates

INTRODUCTION

Hybrid Zones

Many hybrid zones have been documented and studied in primates, including cercopithecines (baboons [Phillips-Conroy & Jolly, 1986; Nagel, 1973]; guenons

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[Struhsaker et al., 1988]; Sulawesi macaques [Bynum et al., 1997; Froehlich & Supriatna, 1996], gibbons [Brockelman & Gittins, 1984], and New World monkeys (spider monkeys [Rossan & Baerg, 1977]; capuchins [Torres de Caballero et al., 1976]; and saddleback tamarins [Cheverud et al., 1993; Peres et al., 1996]). In Madagascar, hybrid zones have been observed between black lemur subspecies [Meyers et al., 1989] and among brown lemur taxa [Lehman & Wright, 2000; Sterling & Ramarason, 1996]. The discovery of hybrid zones between lemur populations should not be surprising given the high species richness and close proximity of many related taxa. An examination of lemur hybrid zones can shed light on the role of hybridization in lemur speciation.

Hybrid zones have been the focus of intense research about the speciation process [Harrison, 1990]. Because such zones represent areas of secondary contact after speciation, how genes of individuals in a hybrid zone interact is of interest in the study of speciation. Once secondary contact is established as a part of the history of individuals in the putative hybrid zone, the introgression and integration of genetic differences can be assessed using classical population genetic approaches [Harrison, 1990] or phylogenetic methods [Davis & Nixon, 1992; Goldstein et al., 2000].

Brown Lemurs

A hybrid zone between separate brown lemur (*Eulemur fulvus*) populations has been recorded at Andringitra National Park in the southeast of Madagascar [Sterling & Ramarason, 1996]. Until recently, the *E. fulvus* complex was considered to be comprised of six subspecies (*E.f. sanfordi*, *E.f. albifrons*, *E.f. fulvus*, *E.f. rufus*, *E.f. albocollaris*, and *E.f. collaris*) distributed parapatrically around coastal areas of Madagascar. Despite this distribution, *E. fulvus* does not follow a classic ''ring species'' model; extant populations of *E.f. rufus* and *E.f. fulvus* are found in both the east and west, suggesting previously continuous populations across the central high plateau [Tattersall, 1982]. Typically, coat color pattern, range, and chromosome number differentiate the subspecies from one another [Mittermeier et al., 1994; Buettner-Janusch & Hamilton, 1979; Hamilton et al., 1980]. *E.f. albocollaris* and *E.f. collaris* maintain very different karyotypes (2N = 48 and 50–52, respectively, vs. 2N = 60 in all other *E. fulvus* subspecies [Buettner-Janusch & Hamilton, 1979; Hamilton et al., 1980]). Despite the greater similarity in karyotype, captive breeding experiments have shown that *E.f. albocollaris* and *E.f. collaris* do not produce fertile offspring when crossed with one another [Dutrillaux & Rumpler, 1977]. Recently the addition of diagnostic mtDNA data and the reanalysis of chromosomal data have led to the suggestion that *E.f. albocollaris* and *E.f. collaris* should be classified as distinct phylogenetic species from each other and from the four other *E. fulvus* subspecies [Djlelati et al., 1997; Wyner et al., 1999]. Their names have been changed accordingly to *E. albocollaris* and *E. collaris* [Djlelati et al., 1997; Wyner, 2000]. This nomenclature will be used for the rest of the discussion.

Brown Lemur Contact Zone at Andringitra

Based on pelage, the lemurs found at Andringitra National Park appear to be a mixture of three phylogenetic species: *E.f. rufus*, *E. albocollaris*, and *E. collaris* [Sterling & Ramarason, 1996]. This putative contact zone thus provides an opportunity to examine processes in the origin and maintenance of very closely related species. A full understanding of this system requires information on

habitat structure, ecology, demography, dispersal patterns, social structure, mate recognition and preference, and population genetics. As a first step in this endeavor, we examine the genetic structure of the local brown lemur populations to provide a baseline for understanding the Andringitra hybrid zone and its role in brown lemur speciation. Mitochondrial (d-loop) and nuclear intron (hemopexin, microsatellite 26, malic enzyme, and ceruloplasmin) markers were developed to characterize the three suspected source species (*E.f. rufus*, *E. albocollaris*, and *E. collaris*). These markers were then used to examine the contribution of these species to the putative hybrid zone, the age and stability of the zone, and whether there is any gene flow across the zone.

METHODS

Study Site

Andringitra National Park is located in southeastern Madagascar. The reserve centers on high mountains (2600+ m) that divide the arid central high plateau and the humid eastern escarpment. Consequently, the park maintains tremendous floristic diversity, including three distinct zones: lowland rain forest, mid-altitude rain forest, and dwarf montane habitats [Goodman & Lewis, 1996]. This area contains the headwaters of two major eastern river systems. Tributaries that ultimately drain into the Mananara River originate in the dry southwestern portion of the reserve; this river system separates *E. albocollaris* and *E. collaris* farther south in the eastern corridor. There are no suitable forest habitats near the Andringitra Mananara headwaters region. Therefore, we do not expect *E. collaris* to be able to migrate into the area or contribute to the putative contact zone (though this may have been possible prior to deforestation in the region). The Manampatrana River (known locally as the Iantara) divides a population of *E.f. rufus* located southwest of Andringitra from *E. albocollaris* populations. Farther south, this river bisects the eastern corridor and the center of *E. albocollaris*'s range (Fig. 1).

The Iantara River also appears to serve as an important boundary in the Andringitra hybrid zone. It is thought that source populations of *E.f. rufus* enter into the purported hybrid zone from two separate corridors (from the southwest and northeast) and that *E. albocollaris* accesses the forest from a southeastern rain forest corridor [Johnson & Wyner, 2000] (Fig. 1). The putative hybrid zone appears to be confined to the eastern rain forest slope of the park; brown lemurs in the western portion closely resemble *E.f. rufus* and are isolated by the high mountain range. The center of the zone is thought to be where the smaller Korokoto River drains into the Iantara. Above this point, the two rivers are likely narrow enough that *E.f. rufus* and *E. albocollaris* can cross and hybridize. During dry months, these rivers are less than 10 m wide, with numerous stepping-stones. Downriver, the Iantara River is too wide (20+ m) for extensive dispersal. Social groups bordering the river in this area were observed crossing only once (using a large fallen tree as a bridge) in over 12 months of observation (Johnson, personal observation).

Based on the river structure and corridor limits, we predict that brown lemur samples collected near the point where the Korokoto drains into the Iantara will demonstrate the greatest evidence of hybridization. Accordingly, lemurs captured downstream and east of the Iantara River should appear more like *E. albocollaris* than *E.f. rufus*, while those captured on the western bank should appear more like *E.f. rufus*. In accordance with these predictions, we collected in three sites in Andringitra: 1) Korokoto, near the confluence of the Korokoto and Iantara (S 22

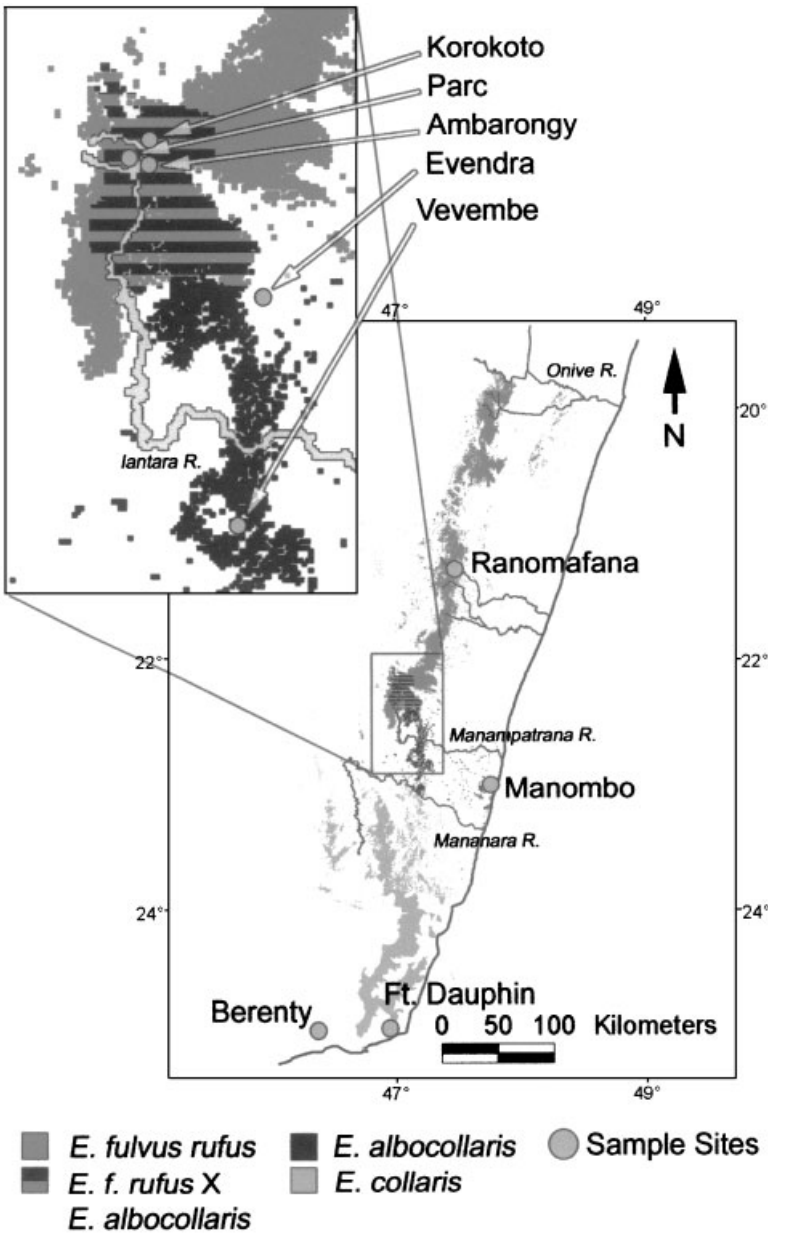

Fig. 1. Distribution of southeastern brown lemur species. This figure indicates the distribution of brown lemurs within the narrow strip of forested habitats of southeastern Madagascar. The forest area (shaded areas on the map) was calculated from 1999–2000 Landsat 7 multispectral satellite images (Irwin et al., unpublished data). A separate, noncontiguous population of *E.f. rufus* is located in western Madagascar (where some samples were collected); the western range of *E.f. rufus* in not shown on this map. The eastern range of this taxon extends from the Andringitra region in the south to the Onive River in the north. The extent of the *E.f. rufus* × *E. albocollaris* hybrid zone is estimated based on the location of source populations (from observations of phenotypes) outside the area, including *E.f. rufus* populations to the west, southwest, and northeast of the Andringitra hybrid zone, as well as *E. albocollaris* populations to the southeast. The range of *E. albocollaris* also appears to be limited by the Andringitra region, although most populations are found between the Manampatrana and Mananara rivers. The Mananara River divides *E. albocollaris* from *E. collaris*. *E. collaris* ranges to the extreme south at the transition from forest to more arid habitats. All known eastern sampling localities for all three taxa (and hybrids) are also indicated.

11 44.2″ E 47 1 55.6″); 2) Ambarongy (S 22 13′ 21.4″ E 47 1 15.9″) 3.2 km SSW of Korokoto, on the eastern bank of the Iantara; and 3) Parc (S 22 13′ 24.2″ E 47 1′ 6.0″), across the river from Ambarongy (Fig. 1). The latter two sites are less than 0.5 km apart; however, as the river is normally impossible to cross up to the Korokoto site, they are effectively 6.5 km apart.

Study Animals

To determine the genetic structure of the hybrid zone at Andringitra, we collected blood samples at the three study sites. At Korokoto, five individuals were captured from one social group. At the Parc site, four individuals from one group were sampled. At Ambarongy, we captured 12 individuals from two groups with adjacent home ranges. The animals were darted with a CO_2-powered rifle using disposable nonbarbed 9-mm darts [Glander, 1993; Glander et al., 1992]. The anesthetic/tranquilizer Telazol™ (A. H. Robbins Co., Richmond, VA) was used to immobilize the animals. Blood samples were stored in Queens lysis buffer (recipe: 10 mM Tris, 10 mM EDTA, 1% N-laurlsarcosine, 10 mM NaCl). Each animal was photographed in uniform lighting positions and measured according to standard techniques [Glander et al., 1992]; morphometric analyses will be presented elsewhere.

As noted, the study animals may be hybrids from up to three distinct populations: *E.f. rufus*, *E. albocollaris*, and *E. collaris*. These taxa are distinguished by chromosome number (*E.f. rufus*: 2N = 60; *E. collaris*: 2N = 50, 51, or 52; and *E. albocollaris*: 2N = 48 [Buettner-Janusch & Hamilton, 1979; Hamilton et al., 1980]), diagnostic DNA positions [Wyner et al., 1999], and pelage characteristics. All three parental taxa and the putative hybrids are sexually dichromatic. The following pelage descriptions of parental taxa are adapted from Mittermeier et al. [1994]. *E.f. rufus* females are reddish brown. Males are gray or gray-brown with a lighter ventrum. They have thick white facial fur (but no beard) and a variable rufous crown. Both males and females have distinctive white patches over the eyes, with a black vertical stripe extending from the dark snout to the crown. *E. albocollaris* females are somewhat more rufous than *E.f. rufus* females, with solid gray faces. Males are a darker brown-gray than *E.f. rufus* males, with black tails and a variable black stripe on the dorsum. Their faces are dark, with thick white beards; somewhat lighter eye patches may occur but are not nearly as striking as in *E.f. rufus*. *E. collaris* females are nearly indistinguishable from *E. albocollaris* females (their coats may be somewhat darker). *E. collaris* males are also similar to *E. albocollaris*, but with rufous beards.

The putative hybrids at Andringitra show a blend of these pelage characteristics. Females tend to closely resemble *E. albocollaris*, but with occasional faint dark stripes between the eyes and somewhat browner coats, particularly at the Parc site. Males are more distinctive. They tend to have some variation of white eye patches (more prominent in *E.f. rufus*) with the full white beard of *E. albocollaris*. *E.f. rufus*-like reddish crowns are also occasionally present, especially on the western bank. Rarely, males with reddish, *E. collaris*-like beards are also sighted.

The parental populations and hybrids may also differ ecologically. All well-studied brown lemurs are primarily frugivores, with a lesser reliance on leaves and flowers [Overdorff, 1993; Sussman, 1977; Freed, 1996] (Johnson, unpublished data). However, *E.f. rufus* may be more adaptable; their range includes a wide range of environments from the humid eastern region to western dry forests. *E.*

albocollaris is restricted to a very small range in the eastern rain forest. *E. collaris* is also largely limited to humid forests, though this species is also found in the transition to the arid south at Andohahela [Mittermeier et al., 1994]. Brown lemurs have multi-male, multi-female social groups [Overdorff et al., 1999] (Johnson, unpublished data). *E. albocollaris* groups also practice frequent fission-fusion (Johnson, unpublished data). Females are philopatric, while males leave their natal groups at sexual maturity [Overdorff et al., 1999].

Regions Sequenced

We sequenced 543 base pairs (bp) from the d-loop region, 396 bp from the hemopexin intron, 358 bp from the malic enzyme intron, 299 bp from the ceruloplasmin intron, and 225 bp from microsatellite 26 for 16 *E. albocollaris* individuals from outside the hybrid zone, 14 *E.f. rufus* individuals from outside the hybrid zone, and the 21 *Eulemur* individuals from three sites at Andringitra National Park. Nine *E. collaris* individuals from outside the hybrid zone were sequenced for d-loop, and fewer *E. collaris* individuals were sequenced for the nuclear introns (Table I). The GenBank accession numbers are AF257943-AF258002 for d-loop sequences, AF258070-AF258132 for ceruloplasmin intron 16 sequences, AF258020-AF258069 for hemopexin intron 5 sequences, AF258133-AF258182 for malic enzyme intron 8 sequences, and AF258183-AF258240 for microsatellite 26 sequences.

DNA Isolation and Manipulation

DNA was isolated according to the protocol discussed in DeSalle et al. [1993]. Polymerase chain reaction (PCR) was performed to amplify the mitochondrial and nuclear gene regions (Table II). The samples were cleaned (Bio101) and sequenced on the ABI 373 and ABI377 sequencer. For all samples, both strands of the PCR product were sequenced. For some samples internal primers were used to sequence the PCR products. Since the nuclear samples were directly sequenced, two peaks appeared on the chromatogram at polymorphic sites. Direct sequencing of allelic DNA has been shown to be effective in determining the sequences from both haplotypes [Hare & Palumbi, 1999].

Chromosomal Location of Markers

In humans, the hemopexin gene, the malic enzyme gene, and the ceruloplasmin gene are found on chromosomes 6, 11, and 3, respectively [Law et al., 1988; Tracey, 1999; Daimon et al., 1995]. Chromosome painting experiments in humans and lemurs show that chromosome 6 and 11 are conserved across taxa [Vezuli et al., 1997; Apiou et al., 1996; Muller et al., 1997]. Chromosome 3 does not appear to be conserved in lemurs. However, since the other two chromosomes are conserved in lemurs, it is likely that the ceruloplasmin gene is found on a different chromosome in lemurs than the other two genes, and that therefore all the gene regions examined are unlinked.

Diagnostic and Private Site Identification

There were few differences among the observed sequences that indicate insertions or deletions (d-loop: 8 gaps; ceruloplasmin intron: 9 gaps, including a 6-bp insertional deletion event; hemopexin intron: 0 gaps; malic enzyme intron: 0 gaps; microsatellite 26: 2 gaps). Therefore, alignment of the various sequences

TABLE I. List of Samples Containing Catalogue ID #, Species Name, Area collected, and Gene Region Sequenced.

AMNH 1D#	Species name[a]	Location of origin[a]	Genes sequenced
Eac 145	*E. albocollaris*	Vevembe	D, H, ME, C, M26
Eac 146	*E. albocollaris*	Vevembe	D, H, ME, C, M26
Eac 147	*E. albocollaris*	Vevembe	D, H, ME, C, M26
Eac 148	*E. albocollaris*	Vevembe	D, H, ME, C, M26
Eac 149	*E. albocollaris*	Vevembe	D, H, ME, C, M26
Eac 169	*E. albocollaris*	Vevembe	D, H, ME, C, M26
Eac 170	*E. albocollaris*	Evendra	D, H, ME, C, M26
Eac 171	*E. albocollaris*	Vevembe	D, H, ME, C, M26
Eac 172	*E. albocollaris*	Vevembe	D, H, ME, C, M26
Eac 174	*E. albocollaris*	Vevembe	D, H, ME, C, M26
Eac 175	*E. albocollaris*	Vevembe	D, H, ME, C, M26
Eac 176	*E. albocollaris*	Evendra	D, H, ME, M26
Eac 178	*E. albocollaris*	Vevembe	D, H, ME, C, M26
Eac 179	*E. albocollaris*	Evendra	D, H, ME, C, M26
Eac 180	*E. albocollaris*	Manombo	D, H, C, M26
Eac 181	*E. albocollaris*	Evendra	D, H, C, M26
Efr048	*E.f. rufus*	Ranomafana-east	D, H, ME, C, M26
Efr049	*E.f. rufus*	Ranomafana-east	D, H, ME, C, M26
Efr050	*E.f. rufus*	Befasy-West	D, H, ME, C, M26
Efr051	*E.f. rufus*	Ranomafana-east	D, H, ME, C, M26
Efr052	*E.f. rufus*	Ranomafana-east	D, H, ME, C, M26
Efr212	*E.f. rufus*	Morondava-west	D, H, ME, C, M26
Efr213	*E.f. rufus*	Maintirano-west	D, H, ME, C, M26
Efr214	*E.f. rufus*	West	D, H, ME, C, M26
Efr215	*E.f. rufus*	West	D, H, ME, C, M26
Efr218	*E.f. rufus*	Southeast	D, H, ME, C, M26
Efr219	*E.f. rufus*	Southeast	D, H, ME, C, M26
Efr220	*E.f. rufus*	Southeast	D, H, ME, C, M26
Efr221	*E.f. rufus*	Southeast	D, H, ME, C, M26
Efr222	*E.f. rufus*	Southeast	D, H, ME, C, M26
Ec001	*E. collaris*	Fort Dauphin	D, C
Ec002	*E. collaris*	Fort Dauphin	D, C
Ec004	*E. collaris*	Fort Dauphin	D, C
Ec055	*E. collaris*	Fort Dauphin	D, C
Ec057	*E. collaris*	Fort Dauphin	D, C
Ec058	*E. collaris*	Near Berenty	D
Ec059	*E. collaris*	Near Berenty	D, C
Ec060	*E. collaris*	?	D, C
Ec187	*E. collaris*	?	D, C
Hybrid173	Hybrid	Andringitra-Korokoto	D, H, ME, C, M26
Hybrid177	Hybrid	Andringitra-Korokoto	D, H, ME, M26
Hybrid182	Hybrid	Andringitra-Korokoto	D, H, ME, C, M26
Hybrid185	Hybrid	Andringitra-Korokoto	D, H, ME, C, M26
Hybrid186	Hybrid	Andringitra-Korokoto	D, H, ME, C, M26
Hybrid231	Hybrid	Andringitra-Ambarongy	D, H, ME, C, M26
Hybrid232	Hybrid	Andringitra-Ambarongy	D, H, ME, C, M26
Hybrid233	Hybrid	Andringitra-Ambarongy	D, H, ME, C, M26
Hybrid234	Hybrid	Andringitra-Ambarongy	D, H, ME, M26
Hybrid235	Hybrid	Andringitra-Ambarongy	D, H, ME, C, M26
Hybrid236	Hybrid	Andringitra-Ambarongy	D, H, ME, C, M26
Hybrid237	Hybrid	Andringitra-Ambarongy	D, H, ME, C, M26
Hybrid238	Hybrid	Andringitra-Ambarongy	D, H, ME, C, M26

TABLE I. Continued.

AMNH 1D#	Species name[a]	Location of origin[a]	Genes sequenced
Hybrid239	Hybrid	Andringitra-Ambarongy	D, H, ME, C, M26
Hybrid265	Hybrid	Andringitra-Ambarongy	D, H, ME, C, M26
Hybrid266	Hybrid	Andringitra-Ambarongy	D, H, ME, C, M26
Hybrid267	Hybrid	Andringitra-Ambarongy	D, H, ME, C, M26
Hybrid268	Hybrid	Andringitra-Parc	D, H, ME, C, M26
Hybrid269	Hybrid	Andringitra-Parc	D, H, ME, C, M26
Hybrid270	Hybrid	Andringitra-Parc	D, H, ME, C, M26
Hybrid271	Hybrid	Andringitra-Parc	D, H, ME, C, M26

[a]D, D-loop; H, hemopexin intron 5; ME, malic enzyme intron 8; C, ceruloplasmin intron 16; M26, microsatellite 26. [a]Andringitra and *E. albocollaris* samples were collected by authors. *E.f.rufus* and *E. collaris* samples are from Duke University Primate Center and Y. Rumpler.

TABLE II. Primers in Study

Primer	Fragment size
dlp5 (Dloop) [Baker et al., 1993][a]	~540bp
dlp1.5 (Dloop) GCA-CCC-AAA-GCT-GAR-RTT-CTA[a]	~540bp
hmpxn5f (hemopexin intron 5) GAA-TGY-CAC-CGT-GGA-GAA-TG[b]	~400bp
hmpxn6r (hemopexin intron 5) CCC-YGG-AAG-CAR-TAG-TAG-CG[b]	~400bp
Malic8f (malic enzyme intron 8) ATI-ACC-AAI-AAC-AAG-CTY-TC[c]	~350bp
Malic9r (malic enzyme intron 8) TTC-YTT-YTC-YAI-GGC-CAT-RAC[c]	~350bp
Cplsmn16f (ceruloplasmin intron 16) TAG-AAA-GCA-ATA-AAA-TGC[c]	~300bp
Cplsmn17r (ceruloplasmin intron 16) GTC-TAT-TTC-ATT-GCC-CAT[c]	~300bp
efr26r (microsatellite 26) [Jekeliek and Strobeck, 1999][c]	~220
efr26r (microsatellite 26) [Jekeliek and Strobeck, 1999][c]	~220

[a]Reaction conditions of 94°C 1′, 47°C 1′, 72°C 1′30″ for 35 cycles on PE486.
[b]Reaction conditions of 94°C 1′, 48°C 1′, 72°C 1′ 30″ for 40 cycles on PE9600.
[c]Reaction conditions of 94°C 30″, 51°C 10″, 72°C 35″ for 38 cycles on PE9600.

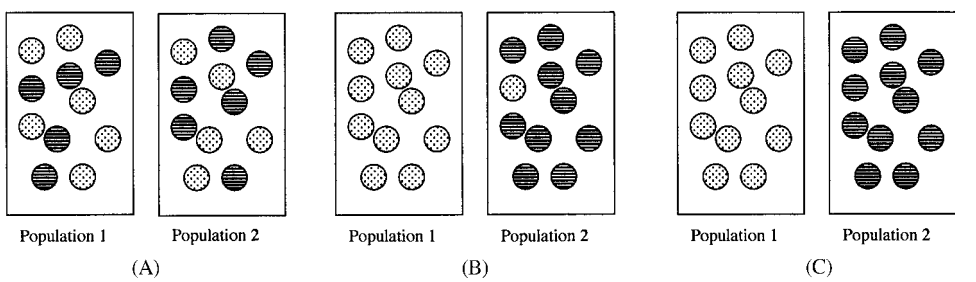

Fig. 2. **A:** The patterned circles are traits found in populations 1 and 2. **B:** The lined circle is a trait private to population 2. **C:** The dotted circle is a fixed character of population 1, and the lined circle is a fixed diagnostic character of population 2. (Figure modified from Davis and Nixon [1992].)

could be done confidently by eye, with subsequent direct importation into MacClade version 3.01 [Maddison & Maddison, 1992]. The match first option was used to identify diagnostic and private sites. Diagnostic sites were determined

through population aggregation analysis (PAA) [Davis & Nixon, 1992] (Fig. 2). PAA was used to differentiate between attributes that are characters and attributes that are traits. An attribute was considered a trait when its state was found to be variable and polymorphic between populations (Fig. 2A). It was considered a character when its state was found to be fixed and different between populations (Fig. 2C). Only characters were used to assess diagnosis because they provide evidence that gene flow does not occur between populations [Davis & Nixon, 1992]. Diagnosis can only be achieved with characters, but traits sometimes can be described as private to a population. A trait is private if it exists in one population, but not in the other (Fig. 2B). A private trait is not a character because it is not fixed. However, since it is found only in one population and not the other, it is still useful as a hybrid indicator.

Hybrid Assessment

Once diagnostic and private sites were determined in the species (*E. albocollaris*, *E.f. rufus*, and *E. collaris*), the purported hybrids from Andringitra were examined for the presence or absence of diagnostic and private sites. Diagnostic and private mitochondrial and nuclear sites were used to determine whether Andringitra individuals contain markers from more than one predicted source species.

RESULTS

Diagnostic and Private Sites

Sequencing and analysis of the four nuclear introns and the d-loop mtDNA revealed diagnostic and private sites for the *Eulemur* species (*E. albocollaris*, *E.f. rufus*, and *E. collaris*). Both the d-loop mtDNA and the microsatellite 26 nuclear gene region contain diagnostic characters for *E. albocollaris*, *E. collaris* (d-loop data set only), and *E.f. rufus*. All of the other gene regions–the ceruloplasmin intron, the hemopexin intron, and the malic enzyme intron–were shown to contain private sites for the purported source species. Additionally, the d-loop region and the ceruloplasmin intron contain private sites for the putative hybrid population as well (Table III).

Analysis of the d-loop gene region shows that the Andringitra population contains a mixture of haplotypes characteristic of *E. albocollaris* and *E.f. rufus*, and that none of these individuals contains sites characteristic of *E. collaris* (Table III). The ceruloplasmin intron data set appears to corroborate the mitochondrial results. A relatively common private site from the ceruloplasmin intron of *E. collaris* was not found in the purported hybrids (Table III).

The hemopexin intron contains private sites for both *E. albocollaris* and *E.f. rufus* that were also found in the Andringitra individuals (Table IV). The malic enzyme intron contains a private site for *E.f. rufus* that was found in the Andringitra animals (Table IV). Finally, microsatellite 26 contains diagnostic characters for *E.f. rufus* and *E. albocollaris* that were found in the purported hybrids (Table IV). Some of the Andringitra lemurs were homozygous for the *E. albocollaris* diagnostic sites (5/21; Table IV), some were homozygous for the *E.f. rufus* diagnostic sites (8/21; Table IV), and some were heterozygous for *E. albocollaris* and *E.f. rufus* diagnostic sites (8/21; Table IV). Sample size was too small to calculate whether Hardy-Weinberg expectations were met.

TABLE III. Diagnostic and Private Sites

	D-loop		Ceruloplasmin,	Hemopexin,	Malic enzyme,	Microsatellite 26	
	Diagnostic	Private	Private	Private	Private	Diagnostic	Private
E. albocollaris	205, 206, 216, 487	–	–	270	–	36, 37, 50, 54, 107	–
E. collaris	202, 212	–	254	–	–	–	–
E.f.rufus	3, 26, 35, 166, 171, 213	20, 219, 230, 302	154	58, 115	13	36, 37, 50, 54, 107	142
Putative hybrids	–	34	40, 292, 67, 79, 240–245	–	–	–	–

Table of diagnostic and private sites for each gene region sequenced. Each gene sequence was numbered sequentially from #1 corresponding to the first base position of the amplified region. The numbers in each row indicate which base position is private or diagnostic for the corresponding taxa and gene region. A dash (–) indicates that no diagnostic or private sites were found in the corresponding gene region for the corresponding taxa. See http://research.amnh.org/molecular/sequence.html for tables of the diagnostic and private sites for each gene region presented here.

TABLE IV. List of Hybrid Indicators Based Upon Private and Diagnostic Sites

Sample	Dloop		Hemopexin		Malic enzyme		Microsatellite 26		Samples w/albocollaris markers	Samples w/rufus markers
	Albocollaris	Rufus	Albocollaris	Rufus	Albocollaris	Rufus	Albocollaris	Rufus		
Hybrid173	X	–	X	*	*	*	X	X	X	X
Hybrid177	–	X	*	X	*	*	X	–	X	X
Hybrid182	–	X	*	*	*	*	X	X	X	X
Hybrid185	–	X	X	X	*	X	X	X	X	X
Hybrid186	–	X	*	X	*	X	X	–	X	X
Hybrid231	X	–	X	*	*	*	X	X	X	X
Hybrid232	–	X	X	*	*	*	–	X	X	X
Hybrid233	X	–	*	*	*	*	–	X	X	X
Hybrid234	X	–	X	*	*	*	X	–	X	–
Hybrid235	X	–	*	X	*	*	–	X	X	X
Hybrid236	X	–	X	*	*	*	X	–	X	–
Hybrid237	X	X	*	*	*	*	X	X	X	X
Hybrid238	X	–	*	*	*	*	X	X	X	X
Hybrid239	X	–	X	*	*	*	–	X	X	X
Hybrid265	X	–	X	*	*	*	X	–	X	–
Hybrid266	X	–	X	*	*	X	X	X	X	X
Hybrid267	X	–	X	*	*	*	–	X	X	X
Hybrid268	X	–	X	*	*	*	–	X	X	X
Hybrid269	X	–	X	*	*	*	–	X	X	X
Hybrid270	–	X	X	*	*	*	–	X	X	X
Hybrid271	–	X	X	*	*	*	X	X	X	X
Total	13	8	14	4	0	3	13	16	21	18

Hybrid indicates *E. albocollaris X E.f. rufus* hybrid. Numbers after species name correspond to AMNH ID #. Table indicates whether individual contains markers for specified gene region for *E. albocollaris* and *E.f.rufus*. An ex (X) indicates that a marker is present. A dash (–) indicates that the marker is absent. An asterisk (*) indicates that a marker is ambiguous. An individual may contain a haplotype from the species; however, since the site is private and not diagnostic, it is not possible to conclude definitively whether a haplotype from the labeled species is present. The last two columns total whether an individual contains private or diagnostic sites from the species heading the column. If an individual contains a marker for the species then the row is marked with an ex (X). If an individual does not contain a marker for that species, then the row is marked with a dash (–).

DISCUSSION

E.f. rufus and *E. albocollaris*, but no *E. collaris*, Are Present in the Putative Hybrid Zone

The diagnostic and private nuclear and mitochondrial data lead to the assertion that the *E. fulvus* population at Andringitra is a hybrid population between *E.f. rufus* and *E. albocollaris* individuals. The hybrid population contains markers for both *E.f. rufus* and *E. albocollaris*, but does not contain markers for *E. collaris* (Tables III and IV). Since neither *E. collaris*'s d-loop diagnostics nor *E. collaris*'s ceruloplasmin private site were found in the hybrid population, it is reasonable to assert that *E. collaris* is not part of this hybrid system, although testing more hybrids may indicate otherwise. Hybrids with phenotypes reminiscent of the reddish beards of *E. collaris* in some males may be the result of *E.f. rufus* (reddish face) × *E. albocollaris* (bearded males) hybridization events, rather than the result of actual *E. collaris* gene introgression. Distribution patterns also support the result that there is no *E. collaris* gene introgression into the hybrid zone.

Diagnostic and Private Site Evidence for Hybridization

Most Andringitra hybrids have both *E.f. rufus* and *E. albocollaris* markers. Eighteen of 21 individuals sampled (Table IV) contain both *E.f. rufus* and *E. albocollaris* markers. Only three individuals (hybrids 234, 236, and 265) do not contain *E.f. rufus* markers (Table IV). These individuals are either *E. albocollaris* individuals or hybrids. If they are *E. albocollaris* individuals, then no matter how many more genes are sampled, no *E.f. rufus* markers will be found in these individuals. However, if they are hybrids, sampling more gene regions should uncover *E.f. rufus* markers.

It is useful to discuss the phenotypes of these three individuals. Hybrid 234, like the other females at the site, is difficult to type by species. Hybrid 236 is a male whose reddish beard most closely resembles beards found in *E. collaris* males. Lastly, hybrid 265 is a male with *E.f. rufus*'s characteristic white eye patches and *E. albocollaris*'s characteristic white beard. These three individuals appear to be phenotypically more similar to the other individuals in the hybrid zone than they are to *E. albocollaris*. It is important to note that the three hybrid samples with only *E. albocollaris* markers were collected from Ambarongy, the collection site east of the Iantara River. This result fits with the prediction that lemurs from this site should most closely resemble *E. albocollaris* (this area is closest to the source population of *E. albocollaris*; Fig. 1). However, since most samples were collected from this site (n = 12/21), it is more likely to contain outlier samples. Also, 83% of the individuals from Ambarongy carry the *E. albocollaris* mitochondrial maternal marker as compared to 20% and 50% of the individuals from the Korokoto and Parc sample sites, respectively. Since males disperse more freely than females, it is logical that Ambarongy, the site closest to *E. albocollaris*'s range, should contain the greatest percentage of *E. albocollaris* maternal markers. However, since the sample size is limited, more sampling is needed to validate this conclusion.

Hybridization as a Source of Variation

Inspection of the data (Table III, Appendices A and B; http://research.amnh.org/molecular/sequence.html) shows that the hybrid population contains private sites not found in either source species. Ceruloplasmin intron 16 is the

source of most of these private sites. Hybrids 236, 237, and 239 share two private sites not found in either *E. albocollaris* or *E.f. rufus*. Hybrids 271 and 266 share three private sites, including a 6-bp deletion event, that are found only in the hybrid population. The d-loop region in the mtDNA contains a private site shared by hybrids 177, 185, and 186. This site is found only in the hybrids and is not found in any of the other sampled groups.

These private sites may be the result of sequence evolution unique to the hybrid zone. In that case, the contact zone must have been stable for many generations. It also indicates that gene flow has not occurred from the hybrid zone to the source species. More sampling needs to be performed in order to validate these conclusions. However, if after more sampling it is found that these sites are still unique to the hybrid zone, we may consider the hybrid zone as a source for new variation or as a reservoir for variation that originated but no longer exists in the source populations.

Stability of the Hybrid Zone

This analysis suggests that a hybrid zone exists between *E. albocollaris* and *E.f. rufus* at Andringitra. In the following we address the stability of the zone. Is this a first-generation hybrid zone that results from heterozygote advantage? Or have the animals at Andringitra been hybridizing and backcrossing for generations? An assessment of the diagnostic sites (microsatellite 26; Table IV) and private sites (hemopexin; Table IV) helps to elucidate this problem. If all the hybrid individuals were found to be heterozygous for the diagnostic and private sites, it can be concluded that this is a first-generation hybrid system resulting from breeding incompatibility between the component species. However, it was found the hybrid population contains a mixture of both homozygous and heterozygous individuals, indicating that this hybrid zone is stable.

Nevertheless, undetected substructure in the hybrids may mean that the zone is less stable than it appears. Sample sizes from the three regions inside the hybrid zone were too small to analyze separately, possibly masking subregion structure. Some of that structure is suggested by the result that the individuals with only *E. albocollaris* markers were found at Ambarongy and the greatest percentage of *E. albocollaris* maternal markers were found at Ambarongy, the area predicted to contain the most *E. albocollaris*-like samples.

The future stability of the hybrid zone may depend on external factors as well. If there is still migration into the zone, the impact of ongoing deforestation in southeastern Madagascar may be significant. While Andringitra National Park is relatively well protected, forest corridors linked to the contact zone from the northeast, south, and southwest are subject to persistent human encroachment. Maintenance of the National Park may be enough to keep the hybrid zone vital. However, if the stability of the zone depends on regional migration, the zone may eventually be eliminated even if the Andringitra forest stays intact.

In summary, examination of the brown lemur population at Andringitra indicates that this population is comprised of hybrids between *E. albocollaris* and *E.f. rufus*. A combination of diagnostic and private mitochondrial and nuclear markers from the three potential parental species (*E. albocollaris*, *E. collaris*, and *E.f. rufus*) was used to detect and characterize hybridization in the study population. Eighteen of 21 study individuals showed highly variable mixtures of both *E. albocollaris* and *E. rufus* haplotypes, and no two individuals appeared to have the same genetic makeup with respect to the nuclear and mitochondrial markers used. Also, although no fixed diagnostic sites were located, several

private sites were found in the hybrids. Based on the genetic data, the hybrid zone at Andringitra appears to be stable. Moreover, with its unique private sites, the hybrid population may be a source of genetic diversity.

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