Noninvasive Molecular Sexing: An Evaluation and Validation of the SRY- and Amelogenin-Based Method in Three New Lemur Species

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ABSTRACT Many lemur species are arboreal, elusive, and/or nocturnal and are consequently difficult to approach, observe and catch. In addition, most of them are endangered. For these reasons, non-invasive sampling is especially useful in primates including lemurs. A key issue in conservation and ecological studies is to identify the sex of the sampled individuals to investigate sex-biased dispersal, parentage, social organization and population sex ratio. Several molecular tests of sex are available in apes and monkeys, but only a handful of them work in the lemuriform clade. Among these tests, the coamplification of the SRY gene with the amelogenin X gene using strepsirhine-specific X primers seems particularly promising, but the reliability and validity of this sexing test have not been properly assessed yet. In this study, we (i) show that this molecular sexing test works on three additional lemur species (*Microcebus tavaratra*, *Propithecus coronatus* and *P. verreauxi*) from two previously untested genera and one previously untested family, suggesting that these markers are likely to be universal among lemurs and other strepsirrhines; (ii) provide the first evidence that this PCR-based sexing test works on degraded DNA obtained from noninvasive samples; (iii) validate the approach using a large number of known-sex individuals and a multiple-tubes approach, and show that mismatches between the field sex and the final molecular consensus sex occur in less than 10% of all the samples and that most of these mismatches were likely linked to incorrect sex determinations in the field rather than genotyping errors. Am J Phys Anthropol 000:000–000, 2013.

Noninvasive samples, such as feces and hairs, are particularly valuable in primates because most species are arboreal, highly mobile, and elusive, which makes them difficult to catch in the wild for the purpose of collecting blood or other tissue samples (e.g., Goossens et al., 2000, 2002). In addition, nearly half of the 634 recognized primate species and subspecies face extinction (Mittermeier et al., 2009) which makes noninvasive sampling techniques desirable to avoid the negative impact of animal captures and excessive handling (Waits and Paetkau, 2005).

Since the first successful attempts of DNA extraction and amplification from noninvasive samples on free-ranging mammals (Höss et al., 1992; Taberlet and Bouvet, 1992; Taberlet et al., 1993, 1996; Woodruff, 1993; Constable et al., 1995; Gerloff et al., 1995; Gagneux et al., 1997; Goossens et al., 1998), noninvasive molecular techniques have been increasingly used in primates for investigating issues as diverse as parentage, dispersal, and kinship (e.g., Morin et al., 1994; Gerloff et al., 1999; Constable et al., 2001; Oka and Takenaka, 2001), genetic structure and diversity (e.g., Reinartz et al., 2000; Eriksson et al., 2004; Quéméré et al., 2010), phylogeography (e.g., van der Kuyl and Dekker, 1996; Jensen-Seaman and Kidd, 2001) or population censuses (e.g., Bergl and Vigilant, 2007; Guschanski et al., 2009).

For such noninvasive studies, identifying the sex of the sampled individuals can be a key issue to infer and quantify sex-biased dispersal (e.g., Bradley et al., 2004; Eriksson et al., 2006), assign parentage, build pedigrees and study the mating system and social structure (e.g., Vigilant et al., 2001; Bradley et al., 2005; Boesch et al., 2006)

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or determine the population sex ratio (e.g., McGrew et al., 2004). Incorporating sex-related information into ongoing analyses of fecal or hair samples (Bradley et al., 2001) can also be important to determine, for instance, whether there are sex-related biases in parasite load (Landsoud-Soukate et al., 1995) or diet (Marriott et al., 1996). Even when animals can be observed or handled in the field, sex identification can be difficult because of the limited sexual dimorphism between males and females (especially at the juvenile stage) in some species (Ensminger and Hoffman, 2002). Molecular sexing may be necessary to confirm sex determination in the field (Griffiths and Tiwari, 1993).

The general method used to molecularly identify the sex of sampled individuals is based on the PCR amplification of sex-specific regions, followed by the visualization of the PCR products using a standard electrophoresis (Villesen and Fredsted, 2006a). Basically, two main approaches have been developed in mammals (Fernando and Melnick, 2001): 1) the amplification of a homolog region on the X and Y chromosomes with known length polymorphism between sexes, such as the amelogenin gene "AMEL" (e.g., Bradley et al., 2001; Ensminger and Hoffman, 2002; Fredsted and Villesen, 2004), the zinc finger protein gene (e.g., Wilson and Erlandsson, 1998; Fernando and Melnick, 2001), the ubiquitously transcribed tetratricopeptide repeat protein gene "UTX/UTY" (Villesen and Fredsted, 2006b) and the Dead-Box gene (Villesen and Fredsted, 2006a) in primates; 2) the coamplification of a Y-specific region (i.e. the sex determination region Y gene "SRY" in primates) with an autosomal or an X-linked marker (e.g., Amelogenin X gene, Di Fiore, 2005).

Until recently, no test was specifically available for the molecular sexing of lemurs (see Table 1 for more details). Indeed, because the lemuriform clade diverged from other primates more than 60 million years ago (Nowak, 1999), most primers designed for humans, great and lesser ape or Old and New World monkey species could not amplify lemur DNA successfully because of the mutation accumulation since the divergence time (Fredsted and Villesen, 2004).

By co-amplifying the SRY gene with the amelogenin X gene using strepsirhine-specific X primers and using high quality DNA extracted from tissue and blood, Di Fiore (2005) managed to determine the sex of three sampled individuals from three different lemur species: *Lemur catta*, *Daubentonia madagascariensis* and *Mirza coquereli*. While this method potentially provides a promising protocol, it still requires further testing and validation. First, lemurs are subdivided into five major families, two of which (Lepilemuridae and Indriidae) were not represented in the original study, and extending the tests to other species from the same and from new families is thus necessary. Second, all the samples used were from tissue and the performance of the sexing test with degraded DNA obtained from hair or feces is unknown. Third, only the samples of the first two species were from known-sex individuals making the sexing of the third species difficult to validate. Fourth, the assessment of the sex-specificity nature of the sexing test was based on only one male individual for each of the two species with known-sex sample (no female was tested). Finally, the accuracy of the test was not quantified by molecularly sexing a reasonably large number of known-sex individuals for each species (see Robertson and Gemmell, 2006 for a discussion on this issue).

Our study therefore aims at further assessing the reliability and validity of Di Fiore's approach to determine the sex of lemurs, especially when dealing with noninvasive samples, which are likely to become increasingly available for many endangered species due to the fact that technological advances now allow to use fecal samples for metagenomic studies (Pompanon et al., 2012; Sharma et al., 2012). In particular, we: (i) tested this approach on three new lemur species (*Microcebus tavaratra*, *Propithecus coronatus* and *P. verreauxi*), from two new genera and one new family, (ii) tested whether this method works with noninvasive samples, (iii) validated the approach using known-sex individuals (from field observation and captures) and estimated the rate of mismatching between field and molecular sexing results, and (iv) evaluated the number of replicates necessary for reliable molecular sex assignments by repeating amplifications of individual samples via a multiple-tubes approach (Taberlet et al., 1996) that is commonly used for genotyping but not for sexing.

MATERIALS AND METHODS

Sampling

We studied three lemur species from Madagascar, one mouse lemur (*Microcebus tavaratra*) considered as endangered, and two sifakas (*Propithecus coronatus* and *P. verreauxi*) considered respectively as endangered and as vulnerable by The IUCN Red List of Threatened Species, 2012.1 (http://www.iucnredlist.org/).

The mouse lemur samples were obtained in July-August 2010 in the Daraina region (Northern Madagascar, Meyler et al., 2012) using Sherman traps (H.B. Sherman Traps®) to capture individuals. For each individual captured, skin tissue samples (ca. 2 mm^2) were taken using a specific 1-3 systematic ear biopsy code (following Rakotondravony et al., 2009) for later individual identification. The biopsies were stored in Queens Lysis Buffer (Seutin et al., 1991; Dawson et al., 1998) until extraction, first in Madagascar at room temperature and then in Lisbon at 4°C (see Table 2 for more details). Morphometric measures were taken and the sex was recorded for all individuals from visual inspection of the genitalia. In this study, 75 samples (49 identified in the field as females and 26 as males) were used for the sex identification and validation procedure.

For the two sifaka species, fecal samples were obtained non-invasively just after defecation from known individuals belonging to social groups that are being followed for behavioral, ecological and evolutionary studies (Pichon et al., 2010; Lewis and Rakotondranaivo, 2011). For *P. coronatus*, the sex was known from distant but repeated observation of the genitalia and 65 (35 identified in the field as females and 30 as males) individuals were sampled in July–August 2010 in Antrema (Northwestern Madagascar). Note, however, that there were differences in the intensity and frequency of observations among *P. coronatus* individuals. For instance, eight samples from individuals identified in the field as females and six identified as males were the object of a more intensive behavioral study and were observed longer and more often in the field than the other 51 individuals. Their sex could therefore be identified in the field with more confidence than for individuals from less intensively observed social groups. The *P. verreauxi* individuals were easily identified by nylon collars and numbered tags or radio collars worn as a part of a

TABLE 1. Summary of five molecular sex determination assays

Sexing assay ID	Sexing marker	Forward primer (5′-3′)	Reverse primer (5′-3′)	Band size for X (bp)	Band size for Y (bp)	Lemur sp. for which the sexing assay was diagnostic	Lemur sp. for which the sexing assay was not diagnostic	Does it work on non-invasive samples?	References[a]
1	Sullivan Amelogenin gene	AmelA: CCCTGG GCTCT-GT AAAGAATAGTG	AmelB: ATCAGA GCTTA-AACTG GGAAGCTG	106	112		*Lemur macaco*	NA	1
2	Amelogenin X gene with Strepsirhine-specific primers	AmelF1(strep): TGG CCTCA-AGCCTGCATT	AmelR1(strep): AACATCYTACC-TAATCCCCACA	≈ 200		*Lemur catta, Mirza coquereli, Daubentonia madagascariensis*		yes?	2
	Sex determination region Y gene	SRY-F1: AGTGA AGCG-ACCCAT GAACG	SRY-F2:, TGTGCC TCCT-GGAAG AATGG		≈ 165				
3	Zinc finger protein gene	ZFSex_F: AAGTG CCCT-CTTGCAC ATAGAT	ZFSex_R: CCT TTTTCCTT-ATGCACCATTT	≈ 1560	≈ 1137		*Microcebus murinus, Microcebus berthae, Cheirogaleus medius, Mirza coquereli, Eulemur fulvus rufus, Propithecus verreauxi, Lepilemur ruficaudatus, Lemur catta*	NA	3
4	Amelogenin gene with degenerate primers	Amel2_F: CTCAT CCTGGG-CACCC TGSTTATATC	AMEL2_R: GGTACC ACTTCAR-AGGGGTRAGCAC	≈ 1490	≈ 1310	*Microcebus murinus, Microcebus berthae, Cheirogaleus medius, Mirza coquereli, Eulemur fulvus rufus, Lepilemur ruficaudatus, Lemur catta*	*Propithecus verreauxi*	no?	3
5	Ubiquitously transcribed tetratricopeptide repeat protein gene	UTXY: TGCTA CCTCAG-GTGGACAAC AAGG	UTY: TGCTTGTTTCA GGC-ACCAAGGRT CTATK		86	*Eulemur fulvus, Mirza coquereli, Microcebus murinus, Microcebus berthae, Cheirogaleus medius, Lemur catta*		yes	4
			UTX: CTCGACACTGG-CAGTGCTGTTAGG	127					

The assays presented in this table have all been tested in lemurs by different authors (see last column for the reference). NA, not applicable.
[a] References: 1. Ensminger and Hoffman (2002), 2. Di Fiore (2005), 3. Fredsted and Villesen (2004), 4. Villesen and Fredsted (2006b).

TABLE 2. Storage methods and time and extracted DNA quality

	M. tavaratra	*P. coronatus*	*P. verreauxi*
Sample type	ear biopsies	feces	feces
Storage method	in Queens lysis buffer	dry with silica gel	dry with silica gel
Period of collection in the field	July-August 2010	July-August 2010	June 2011
Date of arrival in the lab in Lisbon	October 2010	October 2010	July 2011
Period of DNA extraction	between January and June 2011	between April 2011 and January 2012	October 2011
Time between collection and arrival in the lab	2-3 months	2-3 months	1 month
Time between arrival in the lab and extraction	3-8 months	6-15 months	3 months
Time between collection and extraction	5–11 months	8–18 months	4 months
Number of extracts	82	36	74
Mean DNA concentration (ng/μL)	59 (range = 4–440)	524 (range = 20–991)	609 (range = 327–1249)
Mean $A_{260/280}$ ratio	1.89 (range = 1.38–2.72)	1.98 (range = 1.63–2.17)	1.97 (range = 1.89–2.02)

DNA concentration and $A_{260/280}$ were measured with a Nanodrop (Thermo Scientific Nanodrop 1000 spectrophotometer). Note that for fecal samples, the estimated template DNA includes that of any organism (e.g., fungi, plants, bacteria) present in the feces and is therefore not necessarily a reliable measure of lemur DNA.

long-term study of the population. Thus, sex was based upon previous capture and handling of individuals. Eighteen (10 identified in the field as females and eight as males) samples were obtained in June 2011 in the Ankoatsifaka field station of the Kirindi Mitea National Park (Central-Western Madagascar).

All field handling and sampling procedures adhered to the legal requirements of Madagascar and were approved by the *Ministère de l'Environnement et des Forêts* of Madagascar and the Malagasy government permitting committee CAFF/CORE (permit ID: 175/10). The research was conducted with the approval of the USA, French, Portuguese governments and was approved by the University of Texas at Austin's Institutional Animal Care and Use Committee (permit ID: 08110301).

DNA extraction and amplification

For the fecal material, DNA extraction was performed following the 2CTAB/PCI protocol adapted from Vallet et al. (2008), according to Quéméré et al. (2010). For the *Microcebus* ear biopsies, total genomic DNA was extracted using a standard mammalian DNA isolation protocol adapted from Laird et al. (1991). Each sample was incubated overnight at 37°C in 300 μl digestion buffer (100 mM EDTA, 100 mM NaCl, 50 mM Tris pH8, and 1% SDS) and 30 μl of Proteinase K at 10 mg/ml (Promega #V3021). The extractions were performed in a DNA free Hood and each set of samples included a negative control to ensure that no cross-contamination occurred and a positive control (i.e., one sample from the same species that had already amplified successfully) to validate the genotypes. We then quantified for each sample the extracted DNA using a Nanodrop (Thermo Scientific Nanodrop 1000 spectrophotometer) (Table 2). Note that for fecal samples, the estimated template DNA includes that of any organism (e.g., fungi, plants, bacteria) present in the feces and is therefore not necessarily a reliable measure of lemur DNA.

Molecular sexing was performed using the two primer pairs published by Di Fiore (2005) (strepsirhine-specific X primers AMEL-F1[strep]: 5′-TGGCCTCAAGCCTGCATT-3′ and AMEL-R1[strep]: 5′-AACATCYTACCTAATCCCCA CA-3′; SRY primers SRY-F1: 5′-AGTGAAGCGACCCA-TGAACG-3′ and SRY-R1: 5′-TGTGCCTCCTGGAAGAA TGG-3′). A single multiplex PCR was performed to simultaneously amplify fragments of the amelogenin X gene and the Y-linked sex-determining region (SRY) gene. While the SRY locus is used to assign sex (amplifying only if a Y chromosome template is present) and should yield a ~165 bp fragment (this varies between species), the amelogenin locus serves as a positive PCR control and should be present in all samples with sufficient DNA, producing a ~200 bp fragment. As a result, males are expected to produce two bands, whereas females are expected to produce only one band.

For fecal samples, PCR amplification was carried out in a total volume of 10 μl consisting of 5 μl of 2x MyTaq HS Mix from Bioline, 0.1 μl of each primer (for a final concentration of 10 μM) and 1 μl of total template DNA. For ear biopsy samples, we added only half of the total volume of the above mix (i.e., 4.5 μl) to 1 μl of total template DNA of each sample.

PCR reactions were conducted in a BIO-RAD MyCycler™ Thermal Cycle under the following conditions: for fecal samples, initial denaturation of 15 min at 94°C, followed by 40 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min, and a final extension step for 10 min at 72°C; for ear biopsies, initial denaturation of 15 min at 95°C, followed by 30 cycles of 95°C for 30 s, 58.5°C for 90 s, and 72°C for 1 min, and a final extension step for 30 min at 72°C. The PCR templates finally staid at 4°C until the gels were run. Electrophoresis was carried out on a 3% agarose gel for 35–40 min at 90V, and the bands were visualized under a UV light using a RedSafe staining (RedSafeTM Nucleic Acid Staining Solution (20,000x) iNtRON Biotechnology, Inc.) and a 100 bp Step ladder (PROMG6951-SC) (see Fig. 1 for an example of gel image). We defined a positive PCR according to Goossens et al. (2000), i.e., when a PCR product was obtained and alleles were identified.

Multiple-tubes procedure and definition of the "true" sex

A key issue when dealing with both field sex identification and molecular sexing determination is to decide what we consider as the "true" sex. Indeed, both sexing approaches can result in incorrect sex assignment. The misidentification of an individual's sex in the field can be caused by various factors such as bad conditions of observation (due for instance to animal distance and/or hiding in the foliage), ambiguous or subtle morphological sexing cues (especially in juveniles), or the widespread belief that only females carry infants, which is not true in all species (Ensminger and Hoffman, 2002). Molecular

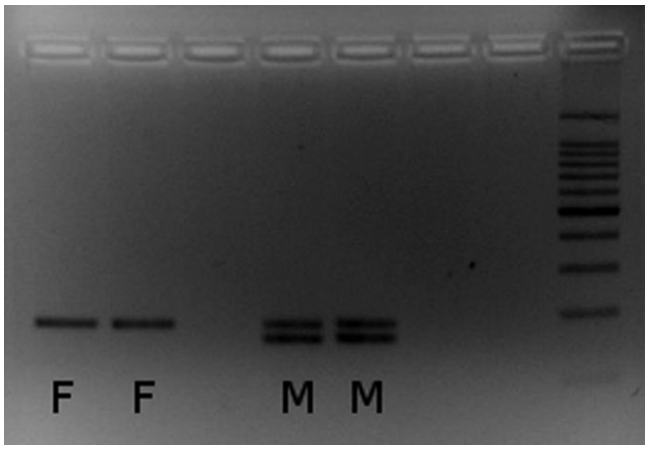

Fig. 1. Gel image of the molecular sexing assay. This figure shows the result for four fecal samples of *Propithecus verreauxi*. The assigned sex (M, male; F, female) based on the assay of each sample is noted below each lane. The two females (Lanes 1 and 2, starting from the left) are identified by a single band (the X fragment) whereas the two males (Lanes 4 and 5) are identified by two bands (the X and Y fragments). The size standard (100 bp Step ladder PROMG6951-SC) is shown on the rightmost lane (Lane 8).

sexing can also provide incorrect sexing results for other reasons such as amplification failure due to technical (allelic dropout, null alleles, or preferential amplification; Hoffman and Amos, 2005; Robertson and Gemmell, 2006) or nontechnical (primer region mutations or laboratory bookkeeping errors; Robertson and Gemmell, 2006; Villesen and Fredsted, 2006b) problems, false alleles (when amplification artifacts can be misinterpreted as true alleles), sporadic contaminations by human male manipulators or cross-sample contaminations (Taberlet et al., 1999; Goossens et al., 2000). A multiple-tubes procedure independently repeating amplifications of individual samples should allow for the detection of most of these problems and avoid incorrect sex assignment (Taberlet et al., 1996, 1999). Finally, both approaches may provide correct but apparently contradictory results. This situation occurs when field sexing is correct but there is missampling of an individual's feces due to the simultaneity in several individuals' defecations leading to the correct molecular sexing of the wrong individual. This problem is crucial when one wants to estimate error rates and validate methods as we did in this study.

Our aim was therefore to follow the general multiple-tubes procedures suggested to validate microsatellite markers genotyped from feces (e.g., Taberlet et al., 1996; Goossens et al., 2005; Quéméré et al., 2010) and perform at least three independent replicates of the sexing result (i.e., three positive PCRs) for each sample. Because of the degraded nature of the DNA present in *Propithecus* fecal samples, several samples failed to amplify in some or all three first PCR replicates. We therefore carried out additional PCRs and in some cases additional DNA extractions (with a limit of 5–8 independent amplifications and three independent extractions for a given sample) so as to achieve a minimum of three successful amplifications (positive PCRs) per sample.

We used the following rules to score the final consensus molecular sex. The sex bands had to appear at least three times over the different replicates to be considered as the final consensus sex. Hence, whenever the sexing results of the three first positive PCRs were consistent, we stopped doing additional PCRs and scored the final consensus sex. Whenever an inconsistency in the sexing results occurred between the three first replicates, we considered that the sexing results were ambiguous and we performed additional PCRs until we obtained three similar sexing results out of four or five PCR repetitions. We must note here that this approach may be problematic if the rate of sexing error is high. Indeed, after five replicates a 3/5 ratio favoring one sexing result is only marginally better than 50%. In our study where sexing error rates were very low this is not an issue.

Estimation of the different error rates

We compared the sex determined in the field with the molecular sex as determined after three consistent results of the positive PCRs and estimated the rate of mismatching between both sexing results.

Different genotyping errors could be identified by comparing individual PCRs and the final molecular sex: (i) Y fragment dropout, when the Y fragment was not amplified in males due to allelic dropout, (ii) Y spurious amplification, when the Y fragment amplified in females due to a contamination or an amplification artifact misinterpreted as a true allele. The Y fragment dropout and spurious amplification rates were thus calculated as the number of times the Y fragment dropped out in males and spuriously amplified in females, respectively, times 100, divided by the total number of successful amplifications in males and females, respectively.

To evaluate the number of replicates necessary for reliable molecular sex assignments, we first estimated the percentage of positive PCRs among all replicates providing a different sexing result than the final molecular consensus sex (i.e., sex determined after at least three consistent results of the positive PCRs). We then considered two independent positive PCRs per sample and estimated the rate of sexing result mismatches between the two replicates (when the two replicates provided ambiguous sexing results). This rate was estimated by comparing the sexing results of the first with the second positive PCRs, as well as of the first with the third positive PCRs, but not of the second with the third positive PCRs since adding this last comparison would introduce a problem of nonindependency between data. Finally, we estimated the percentage of samples with inconsistent molecular sexing results between the three first positive PCRs, and which therefore necessitated additional PCRs to obtain the final consensus sex.

RESULTS

Amplification success rate

The proportion of positive PCRs out of the 508 PCR amplifications carried out overall for the study was 92%, but the amplification success rate varied between species. For *M. tavaratra* ear biopsies, all extracted DNA amplified successfully at the first attempt (amplification success rate = 100% for a total of 228 PCRs performed). For *P. verreauxi* fecal samples, only one extract failed to amplify twice, but the second extract from the same sample amplified successfully for the three replicates (amplification success rate = 96.4% for a total of 56 PCRs performed). For *P. coronatus* fecal samples, the amplification success rate was much lower (about 83%): 37 of 224 PCRs failed and these failures concerned 14 samples (22% of the 65 *P. coronatus* samples). Note

TABLE 3. Amplification success of the amelogenin X gene

Number of successful PCRs	*M. tavaratra*	*P. coronatus*	*P. verreauxi*	Total
0	0	2	0	2
1	0	0	0	0
2	0	2	0	2
3	75	61	18	154
Total	75	65	18	158

This tables shows, for each species and when all the three species were pooled, the number of individuals for which 0, 1, 2 and $\geq$3 successful PCR amplification could be obtained (with a limit of 5-8 independent amplifications and 3 independent extractions for a given sample).

that for these 14 samples, the amplifications were often tested on several independent extracts (1.6 extracts/sample on average, with a maximum of 3 extracts).

We obtained a minimum of three independent molecular sexing results (i.e., three positive PCRs) for a total of 154 out of the 158 individuals sampled among the three species, that is for 97.5% of all individuals (100% for *M. tavaratra* and *P. verreauxi*, 94% for *P. coronatus*, Table 3). Two *P. coronatus* fecal samples could never give any specific PCR product despite five independent amplification attempts using three different DNA extracts. The other two *P. coronatus* fecal samples were only successfully amplified twice (Table 3) despite the use of five or six independent PCRs.

Rate of mismatching between the field and molecular sexing results

The rate of mismatching between the field and molecular sexing results was 9% over the three species (mismatchings occurred in 14 of the 154 individuals with three independent molecular sexing results), with important disparities between species (Table 4). While for *P. verreauxi* we observed no difference between the field and molecular sexing results, we detected differences for five *M. tavaratra* (6.7%) and nine *P. coronatus* (14.8%) individuals (Table 4). We also noticed that the mismatching rate was much higher in individuals assigned to the male sex than to the female sex by the molecular sexing test in *M. tavaratra* (13.8% vs. 2.2%) and *P. coronatus* (22.9% vs. 3.9%, Table 4). This result means that individuals finally identified as males on the basis of the PCRs were more often identified as females in the field than the opposite (identified males in the field that were genetically identified as females).

Genotyping error rates for the Y chromosome

Assuming that the sex determined after three consistent sexing results of the positive sexing PCRs was the "true" sex, we found that genotyping errors associated with nonamplification of the Y allele were infrequent, and males were properly identified in more than 99% of the 216 PCRs over the three species (Table 4). The Y fragment dropped out only twice over 87 amplifications in *M. tavaratra* (dropout rate = 2.3%), and never in *P. coronatus* and *P. verreauxi* (Table 4). Spurious amplification of the Y allele in DNA from a female occurred in only one of 246 PCRs over the three species (in a *M. tavaratra* sample), giving a very low global spurious amplification error rate of 0.4% (Table 4).

TABLE 4. Molecular sexing results

Species	Consensus molecular sex	# of samples with mismatching between field and molecular sexing results	Total # of samples	Mismatching rate (%)	# of Y fragment dropouts	# of Y fragment spurious amplifications	Total # of successful amplifications	Y fragment dropout rate (%)	Y fragment spurious amplification rate (%)
M. tavaratra	Female	1	46	2.17		1	138		0.72
	Male	4	29	13.79	2		87	2.30	
	Total	5	75	6.67					
P. coronatus	Female	1	26	3.85		0	78		0.00
	Male	8	35	22.86	0		105	0.00	
	Total	9	61	14.75					
P. verreauxi	Female	0	10	0.00		0	30		0.00
	Male	0	8	0.00	0		24	0.00	
	Total	0	18	0.00					
The three species pooled	Female	2	82	2.44		1	246		0.41
	Male	12	72	16.67	2		216	0.93	
	Total	14	154	9.09					

Here only the samples with at least three positive PCRs were considered.The consensus molecular sex represents the sex identified after three consistent results of the positive PCRs.

Molecular sexing error rates

Among the 154 individuals with at least three independent positive PCRs over the three species, only three positive PCRs, all from *M. tavaratra* samples, provided a different sexing result than the final consensus sex (0.6% out of 493 positive PCRs over the three species or 1.3% out of 228 positive PCRs in *M. tavaratra*). When considering two positive PCRs per sample, the rate of sexing result mismatches between the two replicates was about 1.0% over the three species, 2.0% in *M. tavaratra* and still 0.0% in *P. coronatus* and *P. verreauxi*.

Finally, the molecular sexing results were consistent over the three first replicates in 151 out of 154 individuals over the three species (98%). For these individuals, the final consensus sex could be determined unambiguously based on these three replicates: 70 individuals were assigned to the male sex and 81 individuals were assigned to the female sex. The three remaining *M. tavaratra* individuals showed inconsistent molecular sexing results between the three first positive PCRs and required additional PCRs to obtain the final consensus sex. With a single additional positive PCR, we managed to obtain three consistent molecular sexing results and determined the final sexing consensus. Note that these three individuals were not among the five individuals that had different sex assignments in the lab and field.

DISCUSSION

In this study, we applied Di Fiore's (2005) approach to new lemur species and tested it with both noninvasive and tissue samples. We also assessed its reliability by comparing field sex identifications to the molecular sexing results using several independent PCRs. The results presented here are therefore of great importance to field biologists working on lemurs. We note that most of the issues discussed here are actually valid across all vertebrates and thus to most field biologists interested in determining the sex of unknown individuals which cannot be reliably observed for long periods or for which only noninvasive samples are available.

Our study showed that Di Fiore's approach worked on *M. tavaratra*, *P. coronatus*, and *P. verreauxi* and thus provides for the first time a way to molecularly determine the sex for these three lemur species. In his original study, Di Fiore had applied his approach on *Lemur catta* (Lemuridae), *Daubentonia madagascariensis* (Daubentonidae), and *Mirza coquereli* (Cheirogaleidae). Our study, by adding three species from two new genera and one new family (Indriidae), thus confirms that Di Fiore's molecular sex determination assay has been validated in at least one species of four out of the five families recognized in Madagascar (Mittermeier et al., 2008). This finding suggests that this approach may work on most if not all lemur species. It is noteworthy, though, that Fredsted and Villesen (2004) tried to sex *P. verreauxi* individuals using the Zinc finger protein system and the amelogenin gene system with degenerate primers but failed to obtain positive results on this species even though it worked on seven other lemur species (see Table 1). The fact that this other protocol was unsuccessful suggests that more tests should be performed across species, including the Lepilemuridae family not yet tested, and using different protocols.

Importantly, we also provided a validation of Di Fiore's lemur sexing protocol on reasonably large samples of known-sex individuals (from field observations and from captured animals) and the first evidence that it worked on degraded DNA obtained from noninvasive samples (here fecal material), at least in the two *Propithecus* species studied. A high rate of amplification success was observed using a large number of independent PCR reactions from *M. tavaratra* ear biopsy extracts as well as from *P. verreauxi* fecal extracts, with PCR products obtained in 100 and 96% of PCRs, respectively. In contrast, we had more amplification failure problems for *P. coronatus* fecal samples (amplification success rate = 83%). In comparison, Vigilant (2002) demonstrated that the success rate of typing the amelogenin locus from chimpanzee (*Pan troglodytes*) fecal samples dried with silica was on average >85%. Bradley et al. (2001) reported some amplification success rates of the amelogenin locus in chimpanzees (94%) and gorillas *Gorilla gorilla* (97%) fecal samples very similar to what we found for *P. verreauxi* fecal extracts. The complete amplification success that we found for *M. tavaratra* was expected because of the high quality DNA that can be extracted from ear biopsies.

The differences in amplification success between the fecal samples of the two *Propithecus* species are more striking. One hypothesis to explain this result is that sample degradation has probably been more important in *P. coronatus* than in *P. verreauxi* fecal samples. Indeed, while fecal samples from both species were collected fresh just after defecation and then preserved dry in small tubes containing silica gel beads, *P. coronatus* samples spent much more time in the field with important variations of temperature and humidity and then in the lab at constant room temperature and humidity before DNA extraction (total time between collection and extraction = 8–18 months) than *P. verreauxi* ones (total time = 4 months, see Table 2 for more details). This hypothesis is also supported by the lower DNA concentration measured with the Nanodrop in *P. coronatus* extracts than in *P. verreauxi*, as well as the larger deviation of the $A_{260/280}$ ratio from the optimal value (i.e., between 1.8 and 2.0) observed in *P. coronatus* than in *P. verreauxi*, although the average $A_{260/280}$ values were similar in the two species (Table 2). Note, however, as mentioned above, that for fecal samples, the estimated template DNA includes that of any organism (e.g., fungi, plants, bacteria) present in the feces and is therefore not necessarily a reliable measure of lemur DNA. The figures above should therefore not be taken as measures of lemur DNA quantity but rather as proxies for total DNA quality (less degraded), including that of lemurs.

We validated and assessed the accuracy of Di Fiore's lemur PCR-based sex test by comparing field sex identifications to the molecular sexing results using a rather large number of known-sex individuals (>60 for *M. tavaratra*, *P. coronatus* and 18 for *P. verreauxi*) and by independently repeating amplifications of individual samples at least three times (as recommended by Robertson and Gemmell, 2006). The results were in general very consistent: over the three species, field sex identifications and molecular sexing results (based on the final sexing consensus) were identical in more than 90% of all the samples. However, we found large differences across species in the mismatch rate between field and molecular sexing results (*M. tavaratra*: about 7%, *P. coronatus*: 15%, *P. verreauxi*: 0%).

For *P. verreauxi* fecal samples, we found that the field and molecular sexing results were all consistent,

probably because all subjects had been captured and handled at some point prior to fecal sample collection. The *P. verreauxi* results are notable for demonstrating that Di Fiore's lemur PCR-based sex test is highly reliable even with low quality DNA extracted from noninvasive samples. Given that the time spent between collection and sexing seemed crucial, sexing (and most probably genotyping) should be conducted as soon as possible.

For *M. tavaratra* ear biopsies, four individuals molecularly sexed as males and one individual molecularly sexed as a female were assigned the opposite sex in the field. This mismatch is likely due to sex misassignments in the field, because we followed a multiple-tubes procedure to assign the final molecular consensus sex, allowing for the detection of most genotyping errors. Although the sex of *Microcebus* individuals has been identified in the field after capture and handling of the animals, morphological sexing cues are sometimes subtle. In particular, juvenile and subadult males with small testes can easily be misidentified as females, likely explaining why these four males, with pretty low body mass (between 36 and 47 g) compared with the average male body mass for this species (50 $\pm$ 5.6 g, n = 12, Radespiel et al., 2012; 49.7 $\pm$ 7.4 g, n = 85, Salmona et al. unpubl. data) and no swollen testes, were misidentified as females in the field. The case of the female misidentified as a male in the field is more difficult to explain. This could be due to a misspelling or the wrong recording of the data.

Finally, for *P. coronatus* fecal samples, six individuals molecularly sexed as males and one individual molecularly sexed as a female were assigned the opposite sex in the field. Most of these sex mismatches are probably due to incorrect sex identification in the field because a multiple-tubes approach was used. Indeed, all these seven individuals were part of the less intensively followed social groups in the field for which the sex was identified with less confidence than the most intensively followed social groups (see Materials and Methods). We noticed that the mismatching rate was 18.0% for the less intensively observed individuals whereas it was zero for the most intensively observed individuals. We found that the mismatching rate between the field and molecular sexing results was much higher in individuals assigned by the molecular sexing test to the male sex than to the female sex, which could be explained by the fact that sifaka males are known to have particularly small testes size for their body size (Lewis, 2009) and so can easily be misidentified as females in the field, especially when they are juveniles. Interestingly, one of the six individuals identified in the field as a female but as a male when using the molecular sexing test was observed providing infant care. Paternal care in this species has never been reported but it has been observed in several other *Propithecus* species (e.g., in *P. coquereli*: Bastian and Brockman, 2007, *P. verreauxi*: Lewis, 2004, *P. tattersalli*: Meyers, 1993, *P. candidus*: Patel, 2007). Hence, in that case, the widespread misconception that only females provide infant care and can carry infants in most lemur species (Tecot et al., in press) might be the underlying cause for incorrect sex assignment in the field. However, another potential explanation could be that we sampled the wrong individual: the feces of the targeted female could have been mis-sampled due to the simultaneity in defecation with a male situated close by.

The low rates of Y fragment dropout in males (0.9%) and spurious amplification in females (0.4%) are encouraging. We found only one case of spurious amplification of the Y allele in a female of *M. tavaratra*. This case is probably due to a sporadic lab contamination, since two additional PCRs from the same extracted DNA amplified only the X allele. Even though allelic dropout is frequently a problem with DNA extracted from noninvasive samples (Ensminger and Hoffman, 2002), we detected a very low dropout rate across our three species. The Y fragment dropped out only twice in *M. tavaratra*. Thanks to the replicate approach used in our molecular sexing test, we correctly identified these samples as males (additional PCRs from the same extracted DNA amplified properly the Y fragment). Our study, therefore, provides good examples of the interest and importance of doing replicates of the PCR reactions in order to decrease the probability of sex misassignments (see also Ensminger and Hoffman, 2002). Similarly, Robertson and Gemmell (2006) strongly recommended repeating amplifications of individual samples via a multiple-tubes approach, in order to check for genotyping errors and contaminations, and be able to distinguish the true absence of the sex dependent fragment from its amplification failure, especially when dealing with noninvasive samples. Yet, very few studies developing molecular sexing assays in nonhuman primate taxa (see the references in Table 5) appear to have applied any validation test. Only one study (Bradley et al., 2001) quantified the error rate associated with the nonamplification of the Y allele in males based on the Sullivan amelogenin gene system and using noninvasive samples of chimpanzees and gorillas. They found very similar values (2 and 3%, respectively) to what we found. But we note that they only amplified samples with >25 pg of genomic DNA, whereas in our study, we tried to amplify all samples whatever the amount of extracted DNA. In addition, some of the validation procedures of the authors remained unclear (in particular, the number of replicates carried out per DNA extraction). Ensminger and Hoffman (2002) also mentioned that they did amplify each extract from invasive sample at least twice and each fecal extracts in triplicate, but they did not provide clear values of their Y fragment dropout rates for the three great ape species (*Pan paniscus*, *P. troglodytes* and *Gorilla gorilla*) that reliably amplified the Amel-A/B primers. Finally, Robertson and Gemmell (2006) investigated the occurrence of sexing errors in studies using PCR-based tests of sex. Unlike the recent interest in microsatellite genotyping errors, they found that very little attention has been paid to molecular sexing errors. Interestingly, among the 16 species for which sexing errors were reported (all from mammalia, aves or Osteichthyes taxa), the lowest error rates were found in the tree swallow (*Tachycineta bicolor*) (0.5%, B. Robertson unpubl. data), the chimpanzee and the gorilla (see values mentioned above, Bradley et al., 2001).

Robertson and Gemmell (2006) also recommended, especially when dealing with noninvasive samples, to repeat amplifications of individual samples via a multiple-tubes approach, in order to determine the number of independent amplifications necessary to have a high confidence in the results of the sexing test. From our results, we found that the percentage of positive PCRs providing a sexing result different from the final consensus sex was 0.6% over the three species. This finding means that if a single positive PCR is used to

TABLE 5. Advantages and drawbacks of the eight different sexing markers tested so far in non-human primates

		Be diagnostic in a range of primate species					Work on non- invasive samples	Products difier substantially in length (15–30 bp)	One amplification step only	Internal positive control	References[a]
		Great apes	Lesser apes	Old World monkeys	New World monkeys	Prosimians					
Amelogenin gene systems	Sullivan amelogenin gene system	YES[b]	YES	NO	NO	NO	YES	NO	YES	YES	1, 2, 3
	Fredsted & Villesen amelogenin gene system	?	?	?	?	YES[c]	NO	YES	YES	YES	4
	Primate specific amelogenin X system	YES	YES	YES	YES	NO	YES	NA	YES	NO	5
	Strepsirhine-specific amelogenin X system	?	?	?	?	YES	?	NA	YES	NO	5
Zinc finger protein system		YES	YES	YES	YES	NO	NO	YES[d]	YES	YES	4, 6, 7
Dead-box gene		YES	YES	YES	YES	NO	YES	YES	YES	YES[e]	8
Ubiquitously transcribed tetratricopeptide repeat protein gene		YES	YES	YES	YES	YES	YES	YES	YES	YES[f]	9
Sex determination region Y gene		YES	YES	YES	YES	YES	YES	YES	YES	NO	5, 10, 11, 12

We used the criteria identified by Villesen and Fredsted (2006a) for the "optimal primate sexing marker" (see main text) and applied them to all the different sexing markers tested so far in nonhuman primates.

[a] References: 1. Bradley et al. (2001), 2. Ensminger and Hoffman (2002), 3. Steiper and Ruvolo (2003), 4. Fredsted and Villesen (2004), 5. Di Fiore (2005), 6. Wilson and Erlandsson (1998), 7. Fernando and Melnick (2001), 8. Villesen and Fredsted (2006a), 9. Villesen and Fredsted (2006b), 10. Steiper and Ruvolo (2003), 11. Malaivijitnond et al. (2007), 12. He et al. (2010).

[b] But *Pongo pygmaeus*.

[c] But *Propithecus verreauxi*.

[d] Except prosimians.

[e] But primer region mutations may be an issue in untested primate species.

[f] But primer region mutations may result in nonidentification of males due to PCR failure. NA, not applicable.

molecularly sex lemur samples, sexing errors will be obtained in slightly more than one sample out of 200 on average. But this value is misleading as it can vary widely according to the species considered. In our study, the sexing error was 1.3% in *M. tavaratra*, but 0.0% in *P. coronatus* and *P. verreauxi*. Adding a single PCR replicate can allow to detect these sexing errors. In our study, only two *M. tavaratra* individuals out of 75 were assigned an incorrect sex in the first positive PCR. Repeating the amplification for all individuals enabled us to identify these individuals as having potential sex identification problems, because inconsistent molecular sexing results were found between the first and second positive PCRs. Our results showed that inconsistent sexing results between the first and second positive PCRs are expected in 1% of the samples over the three species. With two additional sexing replicates, we managed to obtain, for these two *M. tavaratra* individuals, a final consensus sex, which was identical to the field sex. Interestingly, we also showed that less than 2% of the total samples showed inconsistent molecular sexing results between the three first positive PCRs and required additional PCRs to obtain the final consensus sex.

As a final test, because it was the species with the highest mismatch between field and molecular sexes, we randomly chose 11 individuals among the 61 *P. coronatus* samples (for which we could obtain at least three independent positive PCRs for the first molecular sexing test) and molecularly sexed them a second time using independent PCRs from a different sample collection in the field and extraction in the lab. The final consensus sexing results were identical to the previous ones in all these 11 cases.

In the light of these results, we suggest the following strategy in order to minimize molecular sexing errors and costs (in time and money) when using Di Fiore's lemur sexing test, especially with non-invasive samples. We recommend amplifying twice each individual sample. Each amplification should be done independently to avoid contamination or bias in the sex identification. Whenever the two replicates provide consistent sexing results, the final consensus sex should be based on these two replicates. Whenever the two replicates provide ambiguous sexing results, two additional amplifications should be performed in order to identify the sex with the highest support, which will be retained as the final consensus sex.

In summary, Di Fiore's sexing test based on the co-amplification of the SRY gene with the amelogenin X gene using strepsirhine-specific X primers appears to be an interesting and reliable molecular sexing test for lemurs. First, it has been shown to work thus far on six different lemur species from five different genera and four different families and we may therefore expect that these markers will be universal among lemurs and other primates. Second, we showed that Di Fiore's sexing test works well on degraded DNA obtained from noninvasive samples (at least in *P. coronatus* and *P. verreauxi*). In particular, the results from *P. verreauxi* were extremely good with 100% success amplification and the markers did not seem to suffer much from technical problems such as allelic dropouts, null alleles, or preferential amplification. However, the amount of time between sampling and genotyping/sexing seems to be an important factor. Third, the sexing test can be conducted with a single multiplex PCR, so that it is fast, inexpensive, and requires only small amounts of sample. Hence, the sexing protocol could in principle be performed in Madagascar provided access to a lab where PCRs can be performed is available. Provided that the time between sampling and sexing is short, our results suggest that the success rate would be very high, which stresses again the need to develop local laboratories and train local field biologists to these techniques. Fourth, Di Fiore's lemur sexing test includes an internal positive control (amelogenin X gene) which amplifies in both sexes. Finally, bands are easily visualized on the agarose gel thanks to the large difference of size between bands (~35 bp). Hence, overall, Di Fiore's sexing protocol fits well the five criteria listed by Villesen and Fredsted (2006a) to define the optimal primate sexing marker. The only minor difference with the "optimality criteria" is that it is not a single marker but the co-amplification of two markers (i.e. the SRY gene and the amelogenin X gene). Among the five markers that have been tested so far for molecular sex identification of non-human primates (i.e., AMEL, zinc finger protein, UTX/UTY gene, Dead-Box and SRY), only one, the UTX/UTY gene, could be considered as an optimal primate sexing marker, as defined by Villesen and Fredsted, 2006b (Table 5). Our study shows that Di Fiore's sexing test based on the co-amplification of the SRY gene with the amelogenin X gene using strepsirhine-specific X primers constitutes an interesting alternative. It would be interesting in the future to compare these two methods (UTX/UTY and SRY/amelogenin X).

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