

A rapid genetic method for sex assignment in non-human primates

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The field of primate molecular ecology is growing rapidly and holds promise for providing insights into aspects of primate social structure that are difficult to address through observational studies. Recent molecular studies have used samples collected non-invasively from wild animals to shed light on mating systems, dispersal behavior, population structure, and patterns of relatedness in a variety of non-human primates (Di Fiore 2003). Indeed, studies utilizing such samples may be the only tractable way for primate biologists and conservation geneticists to gain critical information about taxa that are highly endangered, elusive, or difficult to habituate. In order to take full advantage of these kinds of samples for investigating a number of features of primate social systems (e.g., population sex ratios, sex biases in dispersal patterns), researchers must be able to confidently and reliably identify the sex of sampled individuals.

Several molecular methods have been developed for sex assignment in humans, most of which rely on fixed polymorphisms between the X- and Y-borne copies of the nuclear gene amelogenin (e.g., Akane et al. 1991; Nakahori et al. 1991; Sullivan et al. 1993; Faerman et al. 1995; Haas-Rochholz and Weiler 1997). Over a decade ago, Sullivan et al. (1993) developed a simple PCR-based human sex test that uses a single primer pair to amplify homologous fragments of amelogenin X and Y that differ in size by 6 bp. Male (XY) samples thus produce two amplicons, while females (XX) produce

just one. Although this basic assay is now the standard used in forensic work (Cotton et al. 2000) and is effective for determining sex in several apes (e.g., gorillas, chimpanzees, gibbons) (Bradley et al. 2001; Ensminger and Hoffman 2002), the procedure, unfortunately, is not broadly applicable across the rest of the primate order. For example, the assay is ineffective for sex-typing orangutans, lemurs, baboons, and many platyrrhines (Ensminger and Hoffman 2002; Steiper and Ruvolo 2003, Di Fiore, unpublished data).

Here, I present a new PCR-based sexing assay that is effective across most primates. The procedure uses a single, multiplex PCR to simultaneously amplify fragments of the amelogenin X gene and the Y-linked sex-determining region (SRY) gene. The amelogenin locus is expected to amplify in all samples containing sufficient nuclear DNA and thus serves as a positive PCR control, while the SRY locus should amplify only if a Y chromosome template is present and thus is used to assign sex. The two amplicons differ in size by ~35 bp and are easily separated and visualized using benchtop procedures. Importantly, the target fragments are short and are expected to amplify reliably even from degraded DNA templates recovered from non-invasive samples.

Primate-specific PCR primers for amelogenin X and SRY were designed based on sequence data available in GenBank. The primers AMEL-F1: 5'-ACCACCAGCTTCCCAGTTTA-3' and AMEL-R1: 5'-GCTGGGWTAGAACCAAGCTG-3' amplify

Table 1. Primate samples typed in this study

Taxonomic group	Genus and species	Source ^a	Provenience of material ^b	Sample type ^c	Sample ID	Reported sex	Assigned sex
Strepsirhini							
Lemuroidea							
	<i>Lemur catta</i>	D Wildman		DNA		Male	Male
	<i>Mirza coquereli</i>	D Wildman		DNA		Unknown	Male
	<i>Otolemur crassicaudatus</i>	D Wildman		DNA		Female	Female
	<i>Daubentonia madagascariensis</i>	D Wildman		DNA		Male	Male
Haplorhini							
Tarsiioidea							
Tarsiidae	<i>Tarsius syrichta</i>	D Wildman		DNA		Female	Female
Platyrrhini							
Cebidae							
	<i>Cebus albifrons</i>	A Di Fiore	Ecuador	Tissue	MF7	Unknown	Male
	<i>Saimiri sciureus</i>	A Di Fiore	Ecuador	Tissue	MF6	Unknown	Female
	<i>Saimiri sciureus</i>	A Di Fiore		Blood		Male	Mal
	<i>Aotus vociferans</i>	A Di Fiore	Ecuador	Tissue	MF20	Unknown	Femle
	<i>Aotus lemurinus</i>	T Disotell		DNA		Female	Female
	<i>Saguinus oedipus</i>	T Tosi		Blood	SAG1	Male	Male
	<i>Leontopithecus rosalia</i>	J Dietz	Brazil	Hair	686H	Male	Male
	<i>Leontopithecus rosalia</i>	J Dietz	Brazil	Hair	601H	Female	Female
	<i>Cebuella pygmaea</i>	A Di Fiore	Ecuador	Tissue	T99	Unknown	Male
Pitheciidae	<i>Pithecia pithecia</i>	R Araya		Tissue	Miles	Male	Male
	<i>Chiropotes satanus</i>	R Araya		Tissue		Unknown	Female
	<i>Callicebus discolor</i>	A Di Fiore	Ecuador	Tissue	CD1	Male	Male
	<i>Callicebus donacophilus</i>	C Lehn		Tissue	961043	Mle	Mle
Atelidae	<i>Lagothrix lagotricha</i>	A Di Fiore	Ecuador	Tissue	T30	Male	Male
	<i>Lagothrix lagotricha</i>	A Di Fiore	Ecuador	Tissue	T13	Female	Female
	<i>Lagothrix lagotricha</i>	A Di Fiore	Ecuador	Tissue	T2	Female	Female
	<i>Lagothrix lagotricha</i>	A Di Fiore	Ecuador	Tissue	LL2000-10	Unknown	Female
	<i>Lagothrix lagotricha</i>	A Di Fiore	Ecuador	Feces	LL2000-F12	Male	Male
	<i>Ateles belzebuth</i>	A Di Fiore	Ecuador	Tissue	Omacal	Female	Female
	<i>Ateles belzebuth</i>	A Di Fiore	Ecuador	Tissue	MF22	Male	Male
	<i>Ateles belzebuth</i>	S Spehar	Ecuador	Feces	Oko	Male	Male
	<i>Ateles belzebuth</i>	S Spehar	Ecuador	Feces	Kuraka	Female	Female
	<i>Ateles belzebuth</i>	S Spehar	Ecuador	Feces	Toma	Female	Female
	<i>Ateles belzebuth</i>	S Spehar	Ecuador	Feces	Oso	Male	Male
	<i>Ateles belzebuth</i>	S Spehar	Ecuador	Feces	Kaya	Female	Female
	<i>Alouatta seniculus</i>	R Rudran	Venezuela	Blood	AS 1	Male	Male
	<i>Alouatta seniculus</i>	R Rudran	Venezuela	Blood	AS2	Female	Female
	<i>Alouatta seniculus</i>	R Rudran	Venezuela	Blood	ASS	Female	Female
	<i>Alouatta seniculus</i>	R Rudran	Venezuela	Blood	AS4	Male	Male
	<i>Alouatta seniculus</i>	A Di Fiore	Ecuador	Tissue	Omaca2	Male	Male
	<i>Alouatta seniculus</i>	A Di Fiore	Ecuador	Tissue	MF21	Male	Male
Cercopithecoidea							
Cercopithecinae							
	<i>Macaca</i> sp	T Disotell		DNA		Male	Male
	<i>Cercopithecus ascanius</i>	T Tosi		DNA	41137B	Male	Male
	<i>Cercopithecus nictitans</i>	R Raaum		DNA	OR1622	Female	Female
	<i>Cercopithecis</i> sp	T Tosi		DNA	100088	Female	Female
	<i>Cercocebus torquatus</i>	R Raaum		DNA	OR538	Male	Male
	<i>Chlorocebus aethiops</i>	T Tosi		DNA	1149	Female	Female
	<i>Chlorocebus aethiops</i>	R Raaum		DNA	VE98007	Male	Male
	<i>Lophocebus aterrimus</i>	R Raaum		DNA		Male	Male

Table 1. Continued.

Taxonomic group	Genus and species	Source ^a	Provenience of material ^b	Sample type ^c	Sample ID	Reported sex	Assigned sex
	<i>Allenopithecus nigroviridis</i>	R Raaum		DNA		Male	Male
	<i>Erythrocebus patas</i>	R Raaum		DNA		Male	Male
	<i>Papio</i> sp	A Burrell		DNA	SWF 18737	Male	Male
	<i>Papio</i> sp	A Burrell		DNA	SWF 18736	Female	Female
	<i>Papio</i> sp	R Palombit	Kenya	Feces		Male	Male
	<i>Papio</i> sp	R Palombit	Kenya	Feces		Female	Female
	<i>Theropithecus gelada</i>	R Raaum		DNA	8910961	Male	Male
	<i>Mandrillus leucophaeus</i>	R Raaum		DNA	OR919	Female	Female
	<i>Mandrillus sphinx</i>	S Clifford	Gabon	DNA		Male	Male
	<i>Mandrillus sphinx</i>	S Clifford	Gabon	DNA		Female	Female
Colobinae	<i>Procolobus badius</i>	T Pope		DNA		Male	Male
	<i>Colobus guereza</i>	N Ting		DNA		Unknown	Female
	<i>Presbytis melalophos</i>	R Raaum		DNA	DJ30	Male	Male
	<i>Nasalis larvatus</i>	R Raaum		DNA		Unknown	Male
	<i>Pygathrix nemaeus</i>	R Raaum		DNA	OR615	Male	Male
Hominoidea	<i>Homo sapiens</i>	T Disotell	Anonymous – US	DNA	HS1	Male	Male
	<i>Homo sapiens</i>	T Disotell	Anonymous – US	DNA	HS2	Female	Female
	<i>Homo sapiens</i>	T Disotell	Anonymous – US	DNA	HS3	Female	Female
	<i>Pan troglodytes</i>	R Raaum		DNA	NA03448A	Unknown	Male
	<i>Pongo pygmaeus</i>	R Raaum		DNA	NA04272	Unknown	Male
	<i>Pongo pygmaeus</i>	T Disotell		DNA	O-1	Unknown	Female
	<i>Pongo pygmaeus</i>	T Disotell		DNA	O-2	Unknown	Male
	<i>Pongo pygmaeus</i>	T Disotell		DNA	O-3	Unknown	Female
	<i>Pongo pygmaeus</i>	T Disotell		DNA	O-4	Unknown	Male
	<i>Gorilla gorilla</i>	J Satkoski		DNA	GG1	Female	Female
	<i>Gorilla gorilla</i>	R Raaum		DNA	NG05251B	Unknown	Female
	<i>Gorilla gorilla</i>	T Disotell		DNA	G-1	Unknown	Female
	<i>Gorilla gorilla</i>	T Disotell		DNA	G-2	Unknown	Female
	<i>Gorilla gorilla</i>	T Disotell		DNA	G-3	Unknown	Male
	<i>Gorilla gorilla</i>	T Disotell		DNA	G-4	Unknown	Female
	<i>Hylobates agilis</i>	R Raaum		DNA	0291	Unknown	Male
	<i>Symphalangus syndactylus</i>	R Raaum		DNA	OR790	Male	Male
	<i>Symphalangus syndactylus</i>	S Lappan		DNA	SL1	Male	Male

^aResearchers providing the source material or DNA used. Contributing organizations for some of these source materials include the Bronx Zoo-Wildlife Conservation Society, the Center for Reproduction of Endangered Species, the Cheyenne Mountain Zoo, the Duke University Primate Center, Harvard University, the Louisiana Purchase Zoo, New York University, the Oakland Zoo, the Southwest Foundation for Primate Research, and the State University of New York at Albany.

^bSource country, if sample is known to be from a wild population.

^cWhere “DNA” is listed as the sample type, high quality genomic DNA extracted from blood, tissue, or cell culture was provided by the researcher listed under “Source”. For all other sample types, genomic DNA was extracted by the author from the material listed using commercially available DNA extraction kits (QIAgen™).

a ~200 bp fragment, while amplification with SRY-F1: 5'-AGTGAAGCGACCCATGAACG-3' and SRY-R1: 5'-TGTGCCTCCTGGAAGAATG G-3' produces a ~165 bp fragment. Multiplex PCR with these four primers was used to screen 77 DNA samples from 38 primate genera (Table 1). The PCR mix contained 2.5 μ l of Mg-free 10 \times

PCR Buffer, 2.0 μ l of 10 mM dNTP mix (2.5 mM each dNTP), 1.5 μ l of 25 mM MgCl₂, 1 μ l of 100 \times BSA (10 mg/ml), 0.8 μ l of each primer at 10 μ M concentration, 1.5 U *Taq* DNA polymerase, ~25–100 ng DNA template, plus ddH₂O to a total volume of 25 μ l. The thermal cycling profile included initial denaturing at 94 °C for 2:00, 40–45

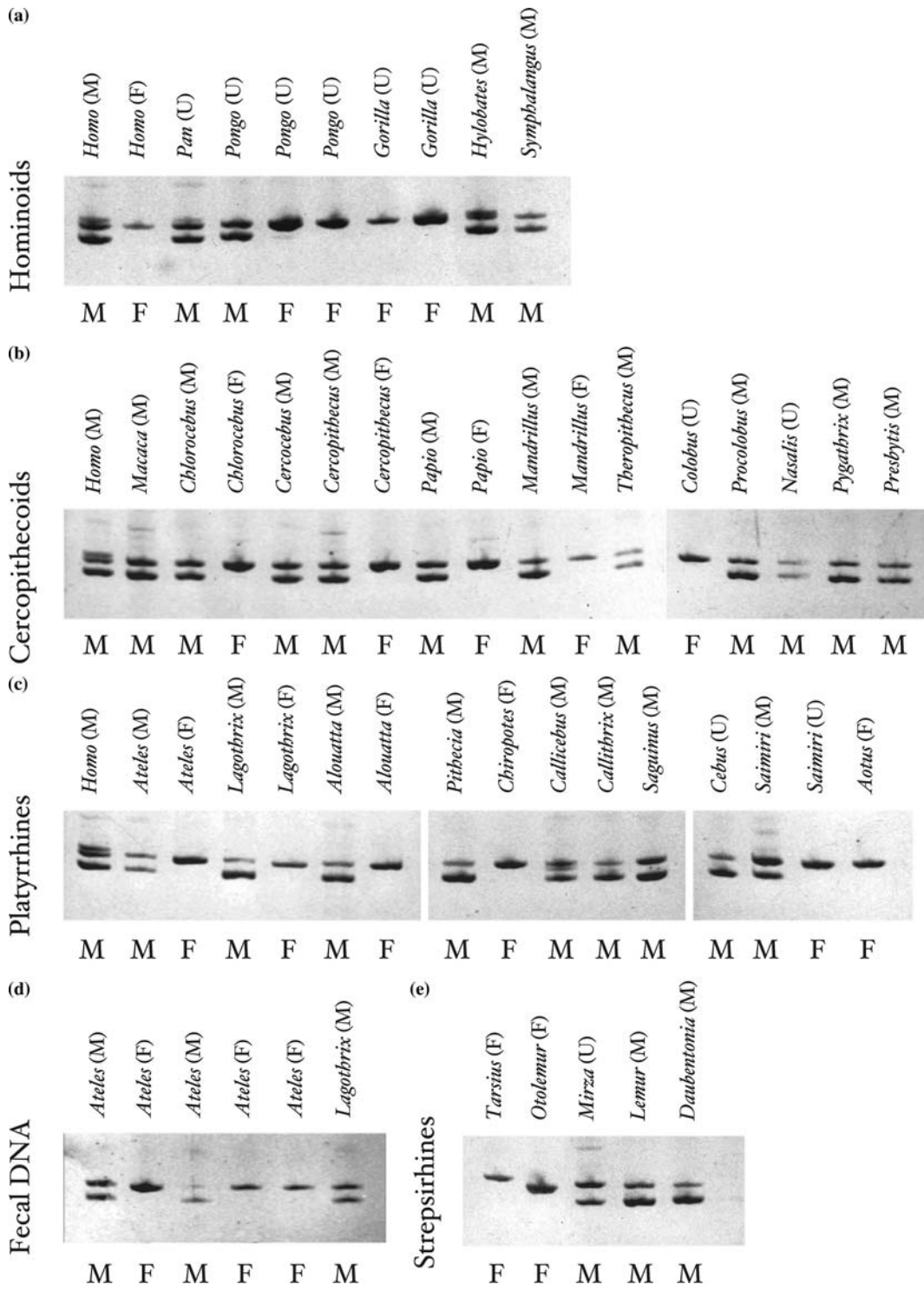


Figure 1. Gel images showing the results of the sexing assay for various taxonomic groups of primates. The genus and reported sex (M = male, F = female, U = unknown) of each primate sample are listed above each lane, and the assigned sex based on the assay is noted below. Only a representative subset of assayed taxa and samples are shown. (a) Hominoids, high quality DNA extracted from tissue or blood. (b) Cercopithecoids, high quality DNA extracted from tissue or blood. (c) Platyrrhines, high quality DNA extracted from tissue or blood. (d) Platyrrhines, DNA extracted from feces. *Ateles* fecal samples were collected and stored in RNAlater® (Ambion) while the *Lagothrix* sample was desiccated in silica gel. The assay was also effective for *Papio* fecal samples stored in RNAlater® (Ambion) and on *Leontopithecus rosalia* hair samples that were collected and stored in plastic envelopes with no desiccating agent or preservative (data not shown). (e) Strepsirrhines, high quality DNA extracted from tissue or blood.

cycles of 94 °C for 0:30, 58°C for 0:30, and 72 °C for 0:30, and a final extension at 72 °C for 5:00. Reactions were set up in a UV-irradiated HEPA-filter equipped PCR workstation using aerosol barrier tips for reagent handling, and negative controls were run for all reactions. PCR products were separated in 8% acrylamide minigels run at ~85 V for 90–120 min and were visualized by UV light following staining with EtBr.

Every anthropoid DNA sample was easily scored as female or male based on the presence of amplification products of appropriate size, and all 52 samples of known-sex individuals from 25 genera were correctly typed (Table 1; Figures 1a–c). Moreover, the assay performed well on DNA extracted from feces or hair of known-sex individuals from several anthropoid genera in which they were tested (Figure 1d). In four strepsirrhine taxa, amplification of the X fragment was either weak or absent, although the SRY band amplified strongly in known males. I thus designed a new set of strepsirrhine-specific X primers (AMEL-F1[strep]: 5'-TGGCCTCAAGCCTGCATT-3' and AMEL-R1[strep]: 5'-AACATCYTACCTAATCCCCAC-A-3') based upon published sequences for *Lemur* and *Otolemur*. When multiplexed with the SRY pair, these primers provided a comparable sex assignment assay (Figure 1e). Finally, for a single female tarsier (Haplorhini: Tarsiidae), the original primer combination produced no PCR product, while the modified set yielded a single amplicon close to the expected X fragment size, suggesting that the strepsirrhine-specific combination may be effective for sexing this genus.

The multiplex strategy described here is analogous to that used by Steiper and Ruvolo (2003) to sex-type orangutans using different amelogenin X (Sullivan et al. 1993) and SRY (Santos et al. 1998) primers originally designed for humans. However, that primer combination is unlikely to be effective across primates because of multiple

sequence mismatches between the primers and most non-hominoid templates. The procedure is also similar that pioneered by Taberlet et al. (1993), except that they used a fragment of mtDNA rather than the X chromosome as a positive control. Given the potentially large difference in mitochondrial and Y chromosome template copy number in a sample, which heightens the chance of falsely concluding that a male sample is female because of Y amplicon “dropout”, the current strategy is preferable. Finally, the only other published sex-typing method effective across a broad range of non-human primates relies on co-amplification of ~700 and ~1150 bp X and Y homologues of the zinc finger protein gene (Wilson and Erlandsson 1998). These fragments are probably too large to amplify reliably from the degraded DNA typically extracted from non-invasive samples. The present method circumvents this problem by targeting much smaller regions for amplification.

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