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RESEARCH ARTICLE

Sequence Length Polymorphisms Within Primate Amelogenin and Amelogenin-Like Genes: Usefulness in Sex Determination

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Sequence length polymorphisms between the amelogenin (AMELX) and the amelogenin-like (AMELY) genes both within and between several mammalian species have been identified and utilized for sex determination, species identification, and to elucidate evolutionary relationships. Sex determination via polymerase chain reaction (PCR) assays of the AMELX and AMELY genes has been successful in greater apes, prosimians, and two species of old world monkeys. To date, no sex determination PCR assay using AMELX and AMELY has been developed for new world monkeys. In this study, we present partial AMELX and AMELY sequences for five old world monkey species (*Mandrillus sphinx*, *Macaca nemestrina, Macaca fuscata, Macaca mulatta*, and *Macaca fascicularis*) along with primer sets that can be used for sex determination of these five species. In addition, we compare the sequences we generated with other primate AMELX and AMELY sequences available on GenBank and discuss sequence length polymorphisms and their usefulness in sex determination within primates. The mandrill and four species of macaque all share two similar deletion regions with each other, the human, and the chimpanzee in the region sequenced. These two deletion regions are 176–181 and 8 nucleotides in length. In analyzing existing primate sequences on GenBank, we also discovered that a separate six-nucleotide polymorphism located approximately 300 nucleotides upstream of the 177 nucleotide polymorphism in sequences of humans and chimps was also present in two species of new world monkeys (*Saimiri boliviensis* and *Saimiri sciureus*). We designed primers that incorporate this polymorphism, creating the first AMELX and AMELY PCR primer set that has been used successfully to generate two bands in a new world monkey species. Am. J. Primatol. 70:976–985, 2008.

Key words: AMELX; AMELY; *Macaca*; *Mandrillus*; PCR

INTRODUCTION

The human amelogenin gene (AMELX) is located on the short arm of the X-chromosome at Xp22.3-p22.1 [Lau et al., 1989]. This gene is important in mammalian tooth bud development and, specifically, codes for an extracellular matrix protein critical to the formation of tooth enamel [Eastoe, 1979; Fincham et al., 1999; Snead et al., 1984; Termine et al., 1980]. A unique aspect of AMELX is that a highly homologous amelogenin-like gene (AMELY) is located on the Y-chromosome and maps to the pericentric region at Yq11 [Lau et al., 1989]. Comparison of the AMELX and AMELY sequences reveals a large percentage of homology, albeit there are base pair changes and deletions. These polymorphisms, which are present in many mammalian species, have been utilized by researchers to design PCR assays that can be used for sex determination [Ennis & Gallagher, 1994; Ensminger & Hoffman, 2002; Fredsted & Villesen, 2004; Hasegawa et al., 2000; Malaivijitnond et al., 2007; Matsubara et al., 2005; Nakahori et al., 1991; Pajares et al., 2007; Pfeiffer & Brenig, 2005; Sanchez-Morgado et al., 2003; Weikard et al., 2006; Yamamoto et al., 2002; Yamauchi et al., 2000], species identification [Matsubara et al., 2005; Weikard et al., 2006], or to elucidate evolutionary relationships [Delgado et al., 2005; Iwase et al., 2003; Sire et al., 2006]. A summary of published primate AMELX and AMELY sequence data and attempts at sex determination via AMELX and AMELY can be found in Table I. The information obtained from such studies has been helpful in the conservation and captive care of some of these species [DeYoung & Honeycutt, 2005;

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TABLE I. Published Primate AMELX and AMELY Sequences and Sex Determination Attempts Via AMELX and AMELY PCR Assays[a]

Common name	Scientific name	Citation(s)	Sex determination	Sequence
Human	*Homo sapiens*	Nakahori et al. [1991]	Y	XY
Chimpanzee	*Pan troglodytes*	Ensminger and Hoffman [2002];	Y;	N;
		Iwase et al. [2003]	NA	XY
Bonobo	*Pan paniscus*	Ensminger and Hoffman [2002]	Y	N
Lowland gorilla	*Gorilla gorilla*	Ensminger and Hoffman [2002]	Y	N
Baboon	*Papio* spp.	Ensminger and Hoffman [2002];	N;	N;
		Huang et al. [1997]	NA	X
Common squirrel monkey	*Saimiri sciureus*	Ensminger and Hoffman [2002];	N;	N;
		Iwase et al. [2003]	NA	XY
Chamek spider monkey	*Ateles chamek*	Ensminger and Hoffman [2002]	N	N
Black lemur	*Lemur macaco*	Ensminger and Hoffman [2002]	N	N
Cotton-top tamarin	*Saguinus oedipus*	Ensminger and Hoffman [2002]	N	N
Gray mouse lemur	*Microcebus murinus*	Fredsted and Villesen [2004]	Y	N
Berthe's mouse lemur	*Microcebus berthae*	Fredsted and Villesen [2004]	Y	N
Fat-tailed dwarf lemur	*Cheirogaleus medius*	Fredsted and Villesen [2004]	Y	N
Red-tailed sportive lemur	*Lepilemur ruficaudatus*	Fredsted and Villesen [2004]	Y	N
Common brown lemur	*Eulemur fulvus*	Fredsted and Villesen [2004]	Y	N
Giant mouse lemur	*Mirza coquereli*	Fredsted and Villesen [2004]	Y	N
Mandrill	***Mandrillus sphinx***	**This study**	**Y**	**XY**
Pigtail macaque	***Macaca nemestrina***	**This study**	**Y**	**XY**
Japanese macaque	***Macaca fuscata***	**This study**	**Y**	**XY**
Long-tailed macaque	***Macaca fascicularis***	Malaivijitnond et al. [2007];	Y;	N;
		This study	**Y**	**XY**
Rhesus macaque	***Macaca mulatta***	Delgado et al. [2007];	NA;	X;
		This study	**Y**	**XY**
White-tufted-ear marmoset	*Callithrix jacchus*	Sanchez-Morgado et al. [2003];	NA;	X;
		Delgado et al. [2007]	NA	X
Philippine tarsier	*Tarsius syrichta*	Delgado et al. [2007]	NA	X
Orangutan	*Pongo pygmaeus*	Delgado et al. [2008];	NA;	X;
		Hwang et al. [1997]	NA	XY
Small-eared galago	*Otolemur garnettii*	Iwase et al. [2003]	NA	XY
Ring-tailed lemur	*Lamur catta*	Iwase et al. [2003];	NA;	XY;
		Fredsted and Villesen [2004]	Y	N
Bolivian squirrel monkey	*Saimiri boliviensis*	Huang et al. [1997]	NA	XY

Entries in bold were generated in this study.
[a]Sex determination column: Y denotes that successful sex determination for the given species is reported, N denotes that sex determination for the given species was attempted but failed, NA denotes that no attempt was made to determine sex. Sequence column: X denotes that AMELX sequence is reported for the given species, Y denotes that AMELY sequence is reported for the given species, and N denotes that no sequence information is reported.

Ensminger & Hoffman, 2002; Kuhn et al., 2002; Malaivijitnond et al., 2007; Matsubara et al., 2005; Pajares et al., 2007; Pfeiffer & Brenig, 2005; Waits & Paetkau, 2005; Yamauchi et al., 2000].

To date, little information on the AMELX or AMELY genes of old world monkeys has been generated. Two studies [Bailey et al., 1992; Malaivijitnond et al., 2007] reported amelogenin primer sets that were useful for the sex determination of long-tailed and rhesus macaques, whereas another study [Ensminger & Hoffman, 2002] reported that the use of amelogenin primers for the sex determination of baboon (*Papio* spp.) samples was not successful. However, none of these researchers reported any AMELX or AMELY sequence information for any of these species. In fact, the only amelogenin sequence information that has been published to date on any old world monkeys was complete AMELX and AMELY sequences of the rhesus macaque (whole genome shotgun sequence *Macaca mulatta* GenBank accession number AANU00000000) and partial AMELX sequences of the rhesus macaque and yellow baboon (*Papio cynocephalus*) [Delgado et al., 2007; Huang et al., 1997]. Therefore, at the present time the only AMELY sequence that has been published for any old world monkey has been that of the rhesus macaque. In addition, research has yet to detect, via PCR or Southern blot, the AMELY gene in the baboon, green monkey, patas monkey, or talapoin [Bailey et al., 1992; Ensminger & Hoffman, 2002; Huang et al., 1997; Nakahori et al., 1991]. This failure to detect the AMELY gene in these old world monkey species has been hypothesized to be due to the loss of the AMELY gene from their Y-chromo-

somes [Ensminger & Hoffman, 2002; Huang et al., 1997; Nakahori et al., 1991].

For new world monkeys, no PCR amelogenin sex determination assay has been developed. One pair of primers that was shown to work for sex determination in great apes did not work on three new world monkey samples [Ensminger & Hoffman, 2002]. However, in our examination of existing primate sequences on GenBank, we found that the same six-nucleotide polymorphism shown by Ensminger and Hoffman [2002] to be present in four great ape species is also present in two new world species (the common squirrel monkey and the Bolivian squirrel monkey).

In this study, we report partial AMELX and AMELY sequence information for five old world monkey species: the mandrill (*Mandrillus sphinx*), the pigtail macaque (*Macaca nemestrina*), the Japanese macaque (*Macaca fuscata*), the rhesus macaque (*M. mulatta*), and the long-tailed macaque (*Macaca fascicularis*). We also report primer sequences that can be used for the sex determination for these five species and, for the first time, a new world monkey species (*Saimiri boliviensis*). Lastly, we compare these sequences with other primate sequences that have previously been published and discuss the usefulness of the AMELX and AMELY genes in the sex determination of primates.

METHODS

Samples

Mandrill and rhesus macaque blood samples were provided by the Tulsa Zoo, Tulsa, OK. Japanese, long-tailed, and pigtail macaque blood samples were acquired from the Oregon Regional Primate Research Center. Human female DNA was extracted from a female cell line purchased from the American Type Culture Collection (Manassas, VA; ATCC ♯ CRL-10317). Human male DNA was purchased from Sigma (St. Louis, MO; Catalogue ♯ D-7011). Bolivian squirrel monkey DNA was purchased from the Coriell Institute for Medical Research (Camden, NJ; Catalog ID PR00474). DNA extraction of the samples was performed using PUREGENE® DNA Isolation Kits (Gentra Systems, Inc., Minneapolis, MN) following the manufacturer's protocols. All samples used in this study were collected in accordance with the Animal Care and Use Committees of the associated institutions in which they were collected. All research for this study adhered to the legal requirements of the United States of America.

PCR

PCR was performed on each mandrill and macaque sample using primers designed from the human AMELX sequence submitted by Nakahori et al. (GenBank ♯ X14440). Each reaction included approximately 100 ng DNA template, 500 nM forward and reverse primers, and 0.75 U FailSafeTM enzyme (Epicentre® Biotechnologies, Madison, WI) with 1 X PreMix D in a total reaction volume of 25 μL. The primers used for the mandrill DNA were AMXY-1F primer 5′-CTG ATG GTT GGC CTC AAG CCT GTG-3′ and AMXY-2R primer 5′-TAA AGA GAT TCA TTA ACT TGA CTG-3′. The primers used for the macaques were AMXY-8F primer 5′-TGA CCA GCT TGG TTC TA-3′ and AMXY-4R primer 5′-CTT GCT CAT ATT ATA CTT GAC AAA-3′. PCRs were performed in a 9600 thermal cycler (PE Applied Biosystems, Foster City, CA) under the following conditions: initial denaturing at 95°C for 5 min followed by 32 cycles of denaturing at 95°C for 30 sec, annealing at 55°C for 30 sec for mandrills or 53°C for 30 sec for macaques, and extension at 72°C for 1 min. Lastly, there was a final extension cycle at 72°C for 10 min. The PCR products were separated in a 1.6% agarose gel.

Bolivian squirrel monkey and human samples were prepared for PCR as discussed above for mandrill and macaque samples. The primers used for squirrel monkey and human samples were AmelDeg F primer 5′-CCC TGS GCT CTS TAA AGA ATW GTG-3′ and AmelDeg R primer 5′-RTC RGM RCT TAA ACT GGG AAG CTG-3′. PCRs were accomplished in a Bio-Rad iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA) using the above parameters, but having an annealing temperature of 58°C. Samples were loaded onto an 8% polyacrylamide gel and separated using a mini-PROTEAN® 3 (Bio-Rad Laboratories) electrophoresis unit.

Sequencing

Bands to be sequenced were cut from their respective gels and the DNA was extracted using the QIAquick Gel Extraction kit (QIAGEN Inc., Valencia, CA) by following the manufacturer's protocol for use with a microcentrifuge. Samples were sent to Oklahoma State University, Recombinant DNA/Protein Resource Facility, for sequencing. Sequencing was accomplished using an ABI 310 DNA Analyzer (PE Applied Biosystems). Each sample was sequenced in both the forward and reverse directions. Sequence data were manipulated manually as well as by utilizing the BioEdit Sequence Alignment Editor (Carlsbad, CA).

RESULTS

Mandrill

PCR yielded one band in the females and two bands in the males. Approximate band sizes were 950 and 750 bp. The resulting sequences were aligned and comparisons of the sexes were made (partial sequence including major deletion regions is given in

TABLE II. Partial AMELX and AMELY Sequences for Human and Mandrill Samples[a]

```
         438                                 470                                 510
Human    X ACTCTGACTCAGTCTGTCCTCCTAAATATGGCCGTAAGCTTACCCAT-CATGAACCACTACTCAGGGAGGCTCCA
Annie    X ..............................................-..........C................
Pearl    X ..............................................-..........C................
Tammie   X ..............................................-..........C................
Human    Y .T..G..T.G.C...T.................T.....T...TT...T...........G......A...T....
Sabre    Y ....G..T.G.C...C.........---....T.........TT...-...........G......A..AT....
Ed       Y ....G..T.G.C...C.........---....T.........TT...-...........G......A..AT....
         512                                 540                                 580
Human    X TGATAGGGCAAAAAGTAAACTCTGA-4--CCAGCTTGGTTCTAACCCAGCTAGTAAAATGTAAGGATTAGGTAAG
Annie    X ................C.........-4--..............T..............................
Pearl    X ................C.........-4--..............T..............................
Tammie   X ................C.........-4--..............T..............................A
Human    Y ...A............C........CTGA..............T....T.CG...........A..........A
Sabre    Y ...A............C........CTGA..............T....T.TG...........A..........A
Ed       Y ...A............C........CTGA..............T....T.TG...........A..........A
         583                                 620                                 650
Human    X ATGTTATTTAAAACTCTTTCCAGCTCAAAAAACTCCTGATTCTAAGATAGTCACACTCTATGTGTGTCTCTTGCT
Annie    X ............G.....................-.....................................CAT.
Pearl    X ............G.....................-.....................................CAT.
Tammie   X ............G.....................-.....................................CAT.
Human    Y ..-------------------------------------------------------------------------
Sabre    Y ..-------------------------------------------------------------------------
Ed       Y ..-------------------------------------------------------------------------
         658                                 690                                 730
Human    X TGCCTCTGCTGAAATATTAGTGACTAAGTGGTATAGGAGAGACTCCGCAGAACAGCGGAATGCATGAGTTTTGGA
Annie    X ..G.....................................................T.........T..........
Pearl    X ..G.....................................................T.........T..........
Tammie   X ..G.....................................................T.........T..........
Human    Y ----------------------------177-----------------------------------------
Sabre    Y ----------------------------176-----------------------------------------
Ed       Y ----------------------------176-----------------------------------------
         733                                 770                                 800
Human    X CGTCGGGTTTGAGGTTCTCCTCAACCTCTTACTAACTTTGTGATTTTGGGCAAATCATTTCCTCTTTCTGGAACC
Annie    X .A.T.....................................C..............................
Pearl    X .A.T.....................................C..............................
Tammie   X .A.T.....................................C..............................
Human    Y ----------------------------.....---8----C..........A.........C.T.......
Sabre    Y ----------------------------....---8-----..C......G....A.........C.T.......
Ed       Y ----------------------------....---8-----..C......G....A.........C.T.......
         808                                 840                                 880
Human    X CTGGTTTCCTCATCTGGAGAAAGGAAATAATTATAATAACCATATTTCAAAATATTGTTTGGAGAGTAATATAGT
Annie    X ............G.................................A...................A.......CT
Pearl    X ............G.................................A...................A.......CT
Tammie   X ............G.................................A...................A.......CT
Human    Y .......T.....T....C..G......T.C.G.....TT..C.............-4--.....A.........
Sabre    Y .CA..........T....TG.G......T.C.G.....TT..C..........G..-4--.................
Ed       Y .CA..........T....TG.G......T.C.G.....TT..C..........G..-4--.................
         883                                 920
Human    X TAATGAATATGAAAAGTGCTTTGTCAAGTATAATATGAGCAAGGTTACT
Annie    X ...................................................
Pearl    X ...................................................
Tammie   X ...................................................
Human    Y ...CA.T...A....C.............................A...
Sabre    Y ...CAGT..C-....C..........G..................A.T.
Ed       Y ...CAGT..C-....C..........G..................A.T.
```

[a]Numbers above each block designate position on the human AMELX sequence (GenBank accession # X14440), numbers within deletion regions designate sequence polymorphism length between AMELX and AMELY sequences of a given species (associated GenBank accession numbers can be found in Table V).

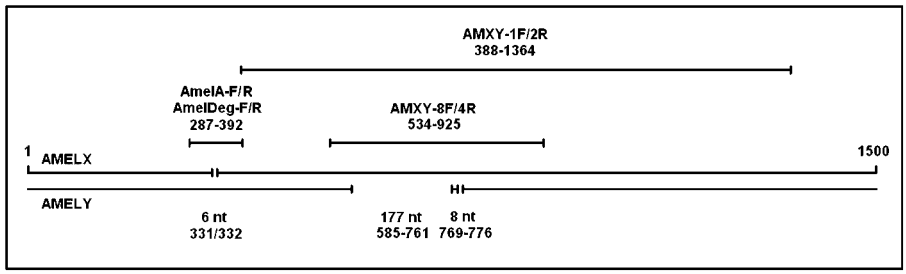

Fig. 1. Positions of amplicons produced by the primer sets used in this study along the human AMELX and AMELY sequences (GenBank accession numbers X14440 and X14439, respectively) along with the diagnostic polymorphisms that exist within them. Notations above the lines representg the gene sequences designate amplicon positions, and those below the lines designate polymorphism regions. All positional numbers relate to nucleotide locations along the human AMELX gene sequence.

Table II). The AMELX sequence had two single base deletions and one four base deletion regions when compared with the AMELY sequence. The AMELY sequence had a total of nine deletion regions when compared with the AMELX sequence; in order, the lengths were 3, 176, 8, 4, 1, 3, 3, 1, and 1 bases. Relative positions of each of the amplicons produced by the primer sets in this study, along with the diagnostic polymorphisms present within them, can be found in Figure 1.

A comparison of human AMELX and AMELY with the mandrill sequences disclosed 51 female to male base pair changes that were identical in the human and mandrills (Table II). A comparison of male and female, human and mandrill, sequences revealed several unique single base pair changes in individual sex and species. The two male mandrill sequences were completely identical, whereas only one of the female mandrills, Tammie, had a single base pair change, relative to the other female mandrills.

Macaque

PCR yielded two bands in the males and one in the females. Approximate band sizes for all species were 400 and 200 bp. The resulting sequences were aligned and comparisons of the sexes were made. Unique differences were found in the AMELY sequences of the macaques. Three deletion regions common to all four macaque species were 176–181, 8, and 4 nucleotides in length. The position of this amplicon along the human AMELX and AMELY genes can be found in Figure 1.

A portion of the amplified sequence that contains these common deletion regions is compared with homologous human AMELX and AMELY sequences (Table III). As expected, the AMELX gene sequences of the rhesus, Japanese, pigtail, and long-tailed macaques were highly similar to human AMELX. The Y-chromosome-specific deletion regions common to each of the macaques mapped to homologous regions within the human AMELY sequence.

Polymorphisms Within Other Primates

In one previous study, an amelogenin PCR assay was used successfully for the sex identification of four great ape species, but was unsuccessful in amplifying a male band from three new world monkey species, one old world species, and one prosimian species [Ensminger & Hoffman, 2002]. We compared the region amplified in this study with the primate AMELX and AMELY sequences that were available on GenBank. From this analysis we discovered that the same six-nucleotide polymorphism between the AMELX and AMELY sequences of humans and chimps in this region was also present in two species of new world monkeys (the common squirrel monkey and the Bolivian squirrel monkey; see Table IV), but was not present in two prosimian species (ring-tailed lemur and small-eared galago, data not shown). The position of this amplicon along the human AMELX and AMELY genes can be found in Figure 1. With redesigned degenerate primers, we were able to amplify two bands from a male Bolivian squirrel monkey sample (Figure 2). In addition, the 176–181 nucleotide polymorphism that was present in human, chimp, mandrill, and macaque samples was not found in either the new world monkey or prosimian sequences. GenBank accession numbers for all primate sequences analyzed can be found in Table V.

DISCUSSION

The sequence length polymorphisms that exist between AMELX and AMELY in the human genome have been utilized for sex determination assays [Nakahori et al., 1991; Sullivan et al., 1993]. In addition, sex determination via PCR assays of the AMELX and AMELY genes have been shown to be successful in greater apes [Bailey et al., 1992; Ensminger & Hoffman, 2002; Matsubara et al., 2005], prosimians [Fredsted & Villesen, 2004], and two species of old world monkeys [Bailey et al., 1992; Malaivijitnond et al., 2007]. Although amelogenin sex determination assays have been successful for the long-tailed macaque and rhesus macaque [Bailey et al., 1992; Malaivijitnond et al., 2007], attempts to

TABLE III. Partial AMELX and AMELY Sequences for Human, Rhesus Macaque, Japanese Macaque, Pig-Tailed Macaque, and Long-Tailed Macaque Samples[a]

```
         548                                 580                                 610
Human    X CTAACCCAGC-TAGTAAAATGTAAGGATT-AGGTAAGATGTTATTTAAAACTCTTTCCAGCTCA--AAAAACTCCTGA
Rhe      X ...T......-..................-.....................................---.............
Jap      X ...T......-................T.....................................CTC.............
Pig      X ...T......-..................-.....................................---.............
Lng      X ...T......C..................-.....................................---.............
Human    Y ...T....T.-CG...........A....-......A..-------------------------------------------
Rhe      Y ...T....T.-.G..G............-......A..-------------------------------------------
Jap      Y ...T....T.-.G..G............-......A..-------------------------------------------
Pig      Y ...C....T.-.G..G........A....-......A..-------------------------------------------
Lng      Y ...T....T.TGGA.G........A....-......A..-------------------------------------------
         622                                 660                                 690
Human    X TTCTAAGATAGTCAC-ACTCTATGTGTGTCTCTTGCTTGC-CTCTGCTGAAATATTAGTGACTAAGTGGTATAGGAGA
Rhe      X ...............-.................CAT....-.....................................
Jap      X ...............-.................CAT....G.....................................
Pig      X ...............-.................CAT....-.....................................
Lng      X ...............C.................CAT....-.....................................
Human    Y ----------------------------------------------------177-------------------------
Rhe      Y ----------------------------------------------------176-------------------------
Jap      Y ----------------------------------------------------180-------------------------
Pig      Y ----------------------------------------------------176-------------------------
Lng      Y ----------------------------------------------------181-------------------------
         698                                 730                                 760
Human    X GACTCCGCAGAACAGCGGAATGCATGAGTTTTGGACGTCGGGTTTGAGGT-TCTCCTC--AACC---TCTTACTAACT
Rhe      X ..................................................-.......--....---...........
Jap      X ..................................................-.......--....---...........
Pig      X ..................................................-.......--....---...........
Lng      X ..............................................A....-......-.....................
Human    Y ------------------------------------------------------------------------------.-
Rhe      Y ------------------------------------------------------------------------------..-
Jap      Y ------------------------------------------------------------------------------..-
Pig      Y ------------------------------------------------------------------------------..-
Lng      Y ------------------------------------------------------------------------------..-
         770                                 800                                 840
Human    X TTGTGATTTTGGGC-AAATCATTTCC-TC-TTTCTGGAACCCTGGTTTCCTCATCT-GGAGAAA-GGAAATAATTATA
Rhe      X .............-...........-..-.........................G.-.......-.............
Jap      X .............-...........-..C.......................G.-.......-.............
Pig      X .............-...........-..-.......................G.-.......-.............
Lng      X .............-...........-..-.......................G.-.......-.............
Human    Y ---8---.......-....A......-..-.C.T.............T.....T.-...C..G-......T.C.GTA
Rhe      Y -8-..........GG....A......-..-.C.T.........CA..........T.-...TG.G-......T.C.GTA
Jap      Y -8-..........G-....A......-..-.C.T.........CA..........T.T...TG.G-......T.C.GTA
Pig      Y -8-..........G-....A......-..-.C.T.........CA..........T.-...TG.G-......T.C.GTA
Lng      Y -8-..........G-....A......-..TAC.T.........CA..........T.-...TG.G-......T.C.GTA
         843                                 880
Human    X ATAACCATATTTCA-AAATA-TTGTTTGGAGAG-TAATATAGTTAATGAA-TATGAAA
Rhe      X ..........A...-.....-...........A-........C.........-.......
Jap      X ..........A...-.....-...........AA.......C.........-.......
Pig      X ..........A...-.....-...........A-........C.........-.......
Lng      X ..........A...-.....-...........AA.......C.........A.......
Human    Y ...TT..C......-.....-....--4-....A-.............CA-.T...A...
Rhe      Y ...TT..C......-....G-....--4-..C..-.............CA-GT..CA...
Jap      Y ...TT..C......-....G-....--4-.CC..-.............CA-GT..CA...
Pig      Y ...TT..C......-....G-....--4-..C..-.............CA-GT..CA...
Lng      Y ...TT..C......T....GG....--4-..C..-.............CATGT..CA...
```

[a]Numbers above each block designate position on the human AMELX sequence (GenBank accession ♯ X14440), numbers within deletion regions designate sequence polymorphism length between AMELX and AMELY sequences of a given species (associated GenBank accession numbers can be found in Table V).

TABLE IV. Six-Nucleotide Polymorphism Used for Sex Determination in Humans Also Present in the Common Squirrel Monkey and the Bolivian Squirrel Monkey[a]

```
          287                                  320                                  350
Human     X CCCTGGGCTCTGTAAAGAATAGTGTGTTGATTCTTTATCCCAGAT---6--GTTTCTCAAGTGGTCCTGATTTTA
Com sq    X ..................................C.........---6--............C..........
Bol sq    X ..................................C.........---6--............C..........
Human     Y ........................G..G.......C......A..AAAGTG.................CA......
Com sq    Y .....C.....C........T...G..-.......C......A..GAAGCG...............T........
Bol sq    Y ..........C........T...G..-.......C......A..GAAGCG...............T........
AmelA     F ........................
AmelDeg   F .....S.....S........W...
          356                                  390
Human     X CAGTTCCTACCACCAGCTTCCCAGTTTAAGCTCT-GAT
Com sq    X .....T.............................T..C
Bol sq    X .....T.............................T..C
Human     Y ............T......................-...
Com sq    Y TG...T......T..................TG.C-...
Bol sq    Y TG...T......T..................TG.C-...
AmelB     R               ....................-...
AmelDeg   R               ..................YK.Y-..Y
```

[a]Numbers above each block designate position on the human AMELX sequence (GenBank accession ♯ X14440), numbers within deletion regions designate sequence polymorphism length between AMELX and AMELY sequences of a given species (associated GenBank accession numbers can be found in Table V). AmelA and AmelB are the primers from Sullivan et al. [1993]. AmelDeg F and R are our novel degenerate primers.

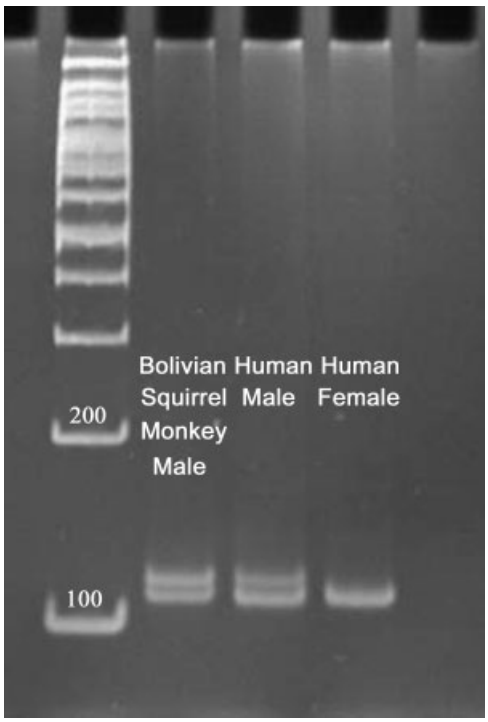

Fig. 2. Polymerase chain reaction products amplified by a novel degenerate primer set on Bolivian squirrel monkey male, human male, and human female samples. PCR products were separated on an 8.0% polyacrylamide gel.

PCR amplify or detect the AMELY gene via Southern blotting in four other old world monkey species (baboon, green monkey, patas monkey, and talapoin) have been unsuccessful [Bailey et al., 1992; Ensminger & Hoffman, 2002; Huang et al., 1997; Nakahori et al., 1991]. To date, no PCR sex determination assays using AMELX and AMELY have been developed for any new world monkeys.

In this study we were able to generate partial AMELX and AMELY sequences for the mandrill and four species of macaque. In each of these species the primers used to amplify the AMELX and AMELY genes proved to be successful in determining gender in all five of these species. In addition, in all five of these species we observed a similar large deletion region (176 nucleotides in mandrills and 176–181 nucleotides in the macaques) to the 177 nucleotide deletion found in the homologous human AMELY sequence.

In analyzing the existing primate AMELX and AMELY sequences that were available on GenBank (Table V), we were not able to see the 176–181 nucleotide deletion in the AMELY gene of either new world monkey samples or prosimian samples. In addition, neither of the prosimian samples contained the homologous six-nucleotide polymorphism that has been utilized for sex determination in greater apes [Ensminger & Hoffman, 2002; Sullivan et al.,

TABLE V. GenBank Accession Numbers for Primate AMELX and AMELY Sequences Analyzed in This Study[a]

Common name	Scientific name	Sequence type	Accession #
Mandrill (Ed)	***Mandrillus sphinx***	**Y**	**EU748887**
Mandrill (Sabre)	***Mandrillus sphinx***	**Y**	**EU748888**
Mandrill (Pearl)	***Mandrillus sphinx***	**X**	**EU748889**
Mandrill (Annie)	***Mandrillus sphinx***	X	EU748890
Mandrill (Tammie)	***Mandrillus sphinx***	**X**	**EU748891**
Rhesus macaque	***Macaca mulatta***	**X**	**EU748892**
Rhesus macaque	***Macaca mulatta***	**Y**	**EU748893**
Japanese macaque	***Macaca fuscata***	**X**	**EU748894**
Japanese macaque	***Macaca fuscata***	**Y**	**EU748895**
Long-tailed macaque	***Macaca fascicularis***	**X**	**EU748896**
Long-tailed macaque	***Macaca fascicularis***	**Y**	**EU748897**
Pig-tailed macaque	***Macaca nemestrina***	**X**	**EU748898**
Pig-tailed macaque	***Macaca nemestrina***	**Y**	**EU748899**
Human	*Homo sapiens*	X	X14440
Human	*Homo sapiens*	Y	X14439
Chimpanzee	*Pan troglodytes*	X	AB091781
Chimpanzee	*Pan troglodytes*	Y	AB091782
Orangutan	*Pongo pygmaeus*	X	U88979
Orangutan	*Pongo pygmaeus*	Y	U88982
Yellow baboon	*Papio cynocephalus*	X	U88980
Rhesus macaque	*Macaca mulatta*	X	EF537871
Common squirrel monkey	*Saimiri sciureus*	X	AB091783
Common squirrel monkey	*Saimiri sciureus*	Y	AB091784
Bolivian squirrel monkey	*Saimiri boliviensis*	X	U88981
Bolivian squirrel monkey	*Saimiri boliviensis*	Y	U88983
White-tufted ear marmoset	*Callithrix jacchus*	X	AY220124
Philippine tarsier	*Tarsius syrichta*	X	EF537873
Ring-tailed lemur	*Lemur catta*	X	AB091785
Ring-tailed lemur	*Lemur catta*	Y	AB091786
Small-eared galago	*Otolemur garnettii*	X	AB091787
Small-eared galago	*Otolemur garnettii*	Y	AB091788

[a]Entries in bold were generated in this study.

1993]. On the other hand, this six-nucleotide polymorphism was detected in both of the new world monkey species. We therefore redesigned the primer set previously used to amplify this region [Ensminger & Hoffman, 2002; Sullivan et al., 1993] by comparing it with the existing new world monkey species and adding degenerate bases where needed in order to match the new world monkey sequences. Using these novel degenerate primers, we were able to amplify two bands of the appropriate size to be AMELX and AMELY amplicons from a male Bolivian squirrel monkey sample (Figure 2). To our knowledge, this was the first use of AMELX and AMELY primers to successfully amplify two bands in a new world monkey species.

In order to be most useful for captive care and conservation efforts, sex determination assays should be designed so that they can be used on noninvasively collected samples. Assays utilizing the amelogenin gene have been successfully carried out on samples that were collected by noninvasive means such as feces, hair roots, or bones [Ensminger & Hoffman, 2002; Faerman et al., 1995; Immel et al., 1999; Kuhn et al., 2002; Matsubara et al., 2005; Pajares et al., 2007; Yamauchi et al., 2000]. Owing to the degraded nature of DNA that exists in such noninvasively collected samples, assays should be designed to produce amplicons that are restricted to lengths of 300 nucleotides or less [Villesen & Fredsted, 2006a]. In addition, multiple primer sets for multiple genes are ideal to ensure correct results. Lastly, variation between multiple bands should be large enough to be easily distinguished on agarose gels.

The existing amelogenin primer set previously used on primate samples that most closely fits the above description is the one designed by Sullivan et al. [1993]. However, this primer set has only been shown to work on great ape samples, and the polymorphism between the AMELX and AMELY amplicons is only six nucleotides long and therefore can be difficult to visualize on agarose gels. For prosimian samples, the only amelogenin primer set that has been shown to successfully determine sex produces amplicon sizes of 1,310 and 1,490 nucleotides [Fredsted & Villesen, 2004]. Such large

amplicons are not ideal for use on noninvasively collected samples. Lastly, although we have shown evidence that redesigned degenerate primers from Sullivan et al. [1993] were able to amplify two bands from a Bolivian squirrel monkey sample, there is no AMELX and AMELY PCR assay presently developed and properly tested for sex determination in new world monkeys. Therefore, further work needs to be done with primate AMELX and AMELY gene sequences to develop rapid PCR assays that are able to consistently determine sex from noninvasively collected samples, or researchers may need to focus on other genes to successfully develop such assays.

Three novel sex determination assays that use other genes to determine the gender in various primate species have recently been published. The first assay has been designed and tested on great ape, old world monkey, and new world monkey samples, producing amplicon sizes of ~180 and 210 nucleotides [Villesen & Fredsted, 2006a]. This primer set amplifies a portion of the DEAD-box polypeptide three gene. The other two assays use multiplex systems to determine the gender in great apes, new world monkeys, old world monkeys, and prosimians, producing 85–200 nucleotide fragments [Di Fiore, 2005; Villesen & Fredsted, 2006b]. Although these assays have proven to be capable of determining sex in various primate species, they have some limitations. The DEAD-box polypeptide three-gene assay is not able to determine sex in prosimians, and the Di Fiore [2005] multiplex assay has shown some reliability issues in consistently amplifying the AMELX gene in some species of prosimians. Keeping in mind the goal of having sex determination primer sets from multiple genes, these primer sets are the only primer sets shown to be able to determine gender in baboons and new world monkeys. Further, only one of the assays has been reliable in determining the gender in prosimian samples without specific alterations [Villesen & Fredsted, 2006b]. Consequently, it would still be advantageous to have an additional primer set for the noninvasive sex determination of baboons and possibly some other old world monkeys, new world monkeys, and prosimians, in order to better ensure correct results.

In this study, we have provided evidence that, with more AMELX and AMELY sequences now available, the design of a noninvasive new world monkey PCR assay based on amelogenin sequences is possible. Additionally, with a novel set of amelogenin primers, we recently obtained two bands from a green monkey sample that corresponded to the sizes of the human AMELX and AMELY bands we obtained with the same primer set (unpublished data). Previous researchers were unable to detect the green monkey AMELY gene via Southern blot [Nakahori et al., 1991]. Therefore, as more primate AMELX and AMELY sequencing occurs, the door may be opened for the design of other noninvasive sex determination assays for primates using the AMELX and AMELY genes.

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REFERENCES

Bailey D, Affara N, Ferguson-Smith M. 1992. The X–Y homologous gene amelogenin maps to the short arms of both the X and Y chromosomes and is highly conserved in primates. Genomics 14:203–205.

Delgado S, Girondot M, Sire J. 2005. Molecular evolution of amelogenin in mammals. J Mol Evol 60:12–30.

Delgado S, Ishiyama M, Sire J. 2007. Validation of amelogenesis imperfecta inferred from amelogenin evolution. J Dent Res 86:326–330.

Delgado S, Vidal N, Veron G, Sire J. 2008. Amelogenin, the major protein of tooth enamel: a new phylogenetic marker for ordinal mammal relationships. Mol Phylogenet Evol 47:865–869.

DeYoung RW, Honeycutt RL. 2005. The molecular toolbox: genetic techniques in wildlife ecology and management. J Wildl Manag 69:1362–1384.

Di Fiore A. 2005. A rapid genetic method for sex assignment in non-human primates. Conservation Genet 6:1053–1058.

Eastoe JE. 1979. Enamel protein chemistry—past, present and future. J Dent Res 58b:753–764.

Ennis S, Gallagher T. 1994. A PCR-based sex-determination assay in cattle based on the bovine amelogenin locus. Anim Genet 25:425–427.

Ensminger AL, Hoffman SMG. 2002. Sex identification assay useful in great apes is not diagnostic in a range of other primate species. Am J Primatol 56:129–134.

Faerman M, Filon D, Kahila G, Greenblatt C, Smith P, Oppenheim A. 1995. Sex identification of archaeological human remains based on amplification of the X and Y amelogenin alleles. Gene 167:327–332.

Fincham AG, Moradian-Oldak J, Simmer JP. 1999. The structural biology of the developing dental enamel matrix. J Struct Biol 126:270–299.

Fredsted T, Villesen P. 2004. Fast and reliable sexing of prosimian and human DNA. Am J Primatol 64:345–350.

Hasegawa T, Sato F, Ishida N, Fukushima Y, Mukoyama H. 2000. Sex determination by simultaneous amplification of equine SRY and amelogenin genes. J Vet Med Sci 62:1109–1110.

Huang W, Chang BHJ, Gu X, Hewett-Emmett D, Li WH. 1997. Sex differences in mutation rate in higher primates estimated from AMG intron sequences. J Mol Evol 44: 463–465.

Immel UD, Hummel S, Herrmann B. 1999. DNA profiling of orangutan (*Pongo pygmaeus*) feces to prove descent and identity in wildlife animals. Electrophoresis 20:1768–1770.

Iwase M, Satta Y, Hirai Y, Hirai H, Imai H, Takahata N. 2003. The amelogenin loci span an ancient pseudoautosomal boundary in diverse mammalian species. Proc Natl Acad Sci USA 100:5258–5263.

Kuhn R, Schwab G, Schroder W, Rottmann O. 2002. Molecular sex diagnosis in Castoridae. Zoo Biol 21:305–308.

Lau EC, Mohandas TK, Shapiro LJ, Slavkin HC, Snead ML. 1989. Human and mouse amelogenin gene loci are on the sex-chromosomes. Genomics 4:162–168.

Malaivijitnond S, Hamada Y, Suryobroto B, Takenaka O. 2007. Female long-tailed macaques with scrotum-like structure. Am J Primatol 69:721–735.

Matsubara M, Basabose AK, Omari I, Kaleme K, Kizungu B, Sikubwabo K, Kahindo M, Yamagiwa J, Takenaka O. 2005. Species and sex identification of western lowland gorillas (*Gorilla gorilla gorilla*), eastern lowland gorillas (*Gorilla beringei graueri*) and humans. Primates 46:199–202.

Nakahori Y, Takenaka O, Nakagome Y. 1991. A human X–Y homologous region encodes amelogenin. Genomics 9: 264–269.

Pajares G, Alvarez I, Fernandez I, Perez-Pardal L, Goyache F, Royo LJ. 2007. A sexing protocol for wild ruminants based on PCR amplification of amelogenin genes AMELX and AMELY (short communication). Archiv Fur Tierzucht—Archives of Animal Breeding 50:442–446.

Pfeiffer I, Brenig B. 2005. X- and Y-chromosome specific variants of the amelogenin gene allow sex determination in sheep (*Ovis aries*) and European red deer (*Cervus elaphus*). BMC Genet 6:16.

Sanchez-Morgado JM, Haworth R, Morris TH. 2003. XY female marmoset (*Callithrix jacchus*). Comp Med 53: 539–544.

Sire J, Delgado S, Girondot M. 2006. The amelogenin story: origin and evolution. Eur J Oral Sci 114:64–77; discussion 93–5, 379–80.

Snead ML, Bringas P, Bessem C, Slavkin HC. 1984. Denovo gene-expression detected by amelogenin gene transcript analysis. Dev Biol 104:255–258.

Sullivan KM, Mannucci A, Kimpton CP, Gill P. 1993. A rapid and quantitative DNA sex test—fluorescence-based PCR analysis of X–Y homologous gene amelogenin. Biotechniques 15:636–641.

Termine JD, Belcourt AB, Christner PJ, Conn KM, Nylen MU. 1980. Properties of dissociatively extracted fetal tooth matrix proteins. I. Principal molecular species in developing bovine enamel. J Biol Chem 255: 9760–9768.

Villesen P, Fredsted T. 2006a. A new sex identification tool: one primer pair can reliably sex ape and monkey DNA samples. Conservation Genet 7:455–459.

Villesen P, Fredsted T. 2006b. Fast and non-invasive PCR sexing of primates: apes, Old World monkeys, New World monkeys and Strepsirrhines. BMC Ecol 6:8.

Waits LP, Paetkau D. 2005. Noninvasive genetic sampling tools for wildlife biologists: a review of applications and recommendations for accurate data collection. J Wildl Manag 69:1419–1433.

Weikard R, Pitra C, Kuhn C. 2006. Amelogenin cross-amplification in the family bovidae and its application for sex determination. Mol Reprod Dev 73:1333–1337.

Yamamoto K, Tsubota T, Komatsu T, Katayama A, Murase T, Kita I, Kudo T. 2002. Sex identification of Japanese black bear, *Ursus thibetanus japonicus*, by PCR based on amelogenin gene. J Vet Med Sci 64:505–508.

Yamauchi K, Hamasaki S, Miyazaki K, Kikusui T, Takeuchi Y, Mori Y. 2000. Sex determination based on fecal DNA analysis of the amelogenin gene in sika deer (*Cervus nippon*). J Vet Med Sci 62:669–671.