

Molecular Sex Identification of Five Mustelid Species

Mark J. Statham*, Peter D. Turner, and Catherine O'Reilly

Department of Chemical and Life Sciences, Waterford Institute of Technology, Waterford, Ireland

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Mark J. Statham, Peter D. Turner, and Catherine O'Reilly (2007) Molecular sex identification of five mustelid species. *Zoological Studies* 46(5): 600-608. Determining the sex of animals in natural populations is important for many reasons, such as understanding population dynamics and structure. DNA analysis as a tool for sex determination can be applied to many biological samples of unknown sex, e.g., preserved specimens or samples collected non-invasively. In this study, a number of different DNA analysis techniques for sex determination were tested on the mustelid species; American mink (*Mustela vison*), ermine or stoat (*Mustela erminea*), Eurasian otter (*Lutra lutra*), European pine marten (*Martes martes*), and Eurasian badger (*Meles meles*). These species are representatives of 4 major taxonomic groups within Mustelidae. A region of the ZFX and ZFY genes was amplified from all species. Gender could be determined by directly sequencing the polymerase chain reaction product or by restriction length fragment polymorphism (RFLP) analysis. A new primer was designed to work in combination with a previously published one, which resulted in a smaller product (325 bp). This product was easily analyzed by RFLP to determine the sex of all species examined. This technique is a valuable means of sex identification of genetic samples from the studied species. It is likely to be applicable other closely related species given the conserved nature of this DNA region.
<http://zoolstud.sinica.edu.tw/Journals/46.5/600.pdf>

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Gender determination in natural populations is essential for understanding population dynamics and structure, and also for making associated management decisions (Eggert et al. 2003, Shaw et al. 2003). Knowledge of the sex of remaining individuals in a population (Griffiths and Tiwari 1995) or of the sex ratio is very important when considering the conservation of an endangered or isolated population (Taberlet et al. 1993 1997). Sex identification is necessary when considering ecological aspects of wild populations; for example, daily movement distances, home range sizes, and overlapping ranges of individuals of the same or opposite sex (Zalewski et al. 2004).

Many species are not sexually dimorphic, particularly as juveniles, for example hyenas, beavers, and many bird species (Schwerin and Pitra 1994, Kahn et al. 1998, Dawson et al. 2001, Williams et al. 2004). In the case of rare animals,

it might not be feasible to capture the animal for direct sexing (Taberlet et al. 1993). Observation of the animal, let alone any sexual dimorphisms, can be difficult. It then becomes necessary to work from a biological sample (hair, feather, feces, etc). In addition, museum or other preserved specimens, and samples, as the result of road traffic accidents, might not have the sex recorded.

A number of molecular gender determination methods have been described. The SRY gene is specific to the Y chromosome in mammals and only amplifies a product from male samples. Lack of amplification does not necessarily mean the sample is female as polymerase chain reaction (PCR) failure may occur for many reasons. This is particularly relevant when dealing with DNA from potentially degraded samples, such as feces, hair, or museum samples. The lack of amplification in female samples necessitates the addition of a sec-

*To whom correspondence and reprint requests should be addressed. Division of Biology, Kansas State University, Manhattan, KS 66506, USA. Tel: 1-785-5326413. Fax: 1-785-5326653. E-mail:Statham@ksu.edu

ond set of primers to act as a positive control. This introduces many additional concerns, particularly when applying the system to several different species. Amplification of the SRY gene has been used to sex many species including cetaceans (Palsboll et al. 1992), brown bear (Taberlet et al. 1993), sheep, pig, horse, grey seal, Eurasian badger, pilot whale, rabbit, human (Griffiths and Tiwari 1993), and otters (Dallas et al. 2000).

Another method relies on amplification of homologous sections of the X and Y chromosomes. The amelogenin gene is present on both the X and Y chromosomes but has not proven to be useful in sexing mustelids (Hattori et al. 2003). A gene encoding a zinc-finger protein is also present on both the X and Y chromosomes. Zinc-finger X (ZFX) and zinc-finger Y (ZFY) genes have a high degree of similarity (Palsboll et al. 1992) facilitating amplification by a single primer pair. A number of techniques have been used for gender determination using the ZFX/ZFY genes. Aasen and Medrano (1990) described the use of the primers, P1-5EZ and P2-3EZ, to amplify a 447-base pair (bp) fragment of the ZFX/ZFY genes followed by restriction fragment length polymorphism (RFLP) analysis for sex determination in humans, cattle, sheep, and goats. Direct sequencing of PCR products amplified with the same primers identified males by the presence of double peaks in the chromatograms of 5 species: spotted hyena, Asian elephant, Indian rhinoceros, domestic dog, and Tonkean macaque (Schwerin and Pitra 1994, Fernando and Melnick 2001). These double peaks were identified as the result of the co-amplification of X and Y sequences of a male. Shaw et al. (2003) used size variation in the products of amplification of an intron of a larger region of the ZFX/ZFY genes to sex a wide range of marine and terrestrial mammals.

The zinc finger X and Y are located in sex-specific regions of the X and Y chromosomes, respectively (Slattery et al. 2000). Phylogenetic analysis of the ZFX and ZFY genes in felids found that Xs and Ys predominantly clustered in 2 monophyletic groups, indicating gene divergence prior to the evolution of modern day felids (Slattery et al. 2000). However there was evidence for 2 periods of ancestral gene conversion from X to Y. Analysis of ZFX and ZFY genes in cetaceans also found that Xs and Ys formed 2 distinct clades, while there was no clear evidence for gene conversion (Morin et al. 2005).

Mustelidae is the most specious family in the order Carnivora. Many members require monitor-

ing because they are of conservation concern; for example, the black-footed ferret, *Mustela nigripes* (Wisely et al. 2003), European pine marten, *Martes martes* (Messenger et al. 1997), and Eurasian otter, *Lutra lutra* (Cassens et al. 2000). Monitoring is necessary for other species because of the effect they have on vulnerable species (such as ground nesting birds) as a result of introductions to areas outside their natural distribution, e.g., the ermine or stoat, *Mustela erminea* and American mink, *Mustela vison* (McLennan et al. 1996, Nordstrom and Korpimaki 2004).

No previous sex typing by genetic analysis has been recorded in *Mar. martes*, *Mus. erminea*, or *Mus. vison*. The only previous record for Eurasian badger, (*Meles meles*) genetic sex typing amplified only the male SRY gene and lacked a control sequence for female template DNA (Griffiths and Tiwari 1993).

This paper describes the application of 3 sex typing techniques for 5 mustelid species. These species represent 4 major taxonomic groups within the order Mustelidae. A novel RFLP method for sex determination in these species is described. A new primer pair giving a smaller ZFX/ZFY PCR product was developed which, in combination with RFLP analysis, provides a simple method for gender determination in *Mus. vison*, *Mus. erminea*, *L. lutra*, *Mar. martes*, and *Mel. meles*. Phylogenetic analysis was also carried out on ZFX/ZFY sequences of these species.

MATERIALS AND METHODS

Sample collection and DNA isolation

Tissue was obtained from the Regional Veterinary Laboratory, Kilkenny, Ireland, taxidermists and road traffic accidents. Samples from 2 dried *Mus. erminea* skins were obtained from the Natural History Museum, Dublin, Ireland. Large numbers of sexed specimens were only available for *Mel. meles*. DNA was isolated from tissue using the Qiagen DNeasy® Tissue DNA extraction kit (cat. no. 69504, Germany). DNA was isolated from hair using the Chelex-100 method (Walsh et al. 1991) with some modifications as described by (Goossens et al. 1998).

Sex typing

Tissue samples were sexed by physical examination of the carcass where possible.

Samples for which sex could not be determined by physical examination were sexed using a number of established genetic techniques.

First, a segment (70 bp) of the male-specific SRY gene was amplified using the primers and protocol described for otter (Dallas et al. 2000). This technique has not previously been tested outside Lutrinae.

Second, a portion (~1000 bp) of the ZFX/ZFY genes was amplified with primers LGL331 and LGL335 (Cathey et al. 1998) following the protocol of Shaw et al. (2003). These techniques were first tested on samples of known sex, and when the results concurred, they were used to sex previously unsexed samples.

The 3rd method involved the amplification of a smaller region (447 bp) of the ZFX/ZFY genes using primers P1-5EZ and P2-3EZ (Aasen and Medrano 1990). The PCR amplification protocol used with these primers differed slightly from that published (Aasen and Medrano 1990). The 50 μ l reaction mixture contained ~50 ng DNA, 1.25 units *Taq* DNA polymerase (Promega, Madison, WI, USA) in 50 mM KCL, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.5 μ M of each primer. The program consisted of 5 min at 94°C, then 40 cycles of 94°C for 60 s, 55.5°C for 60 s, and 72°C for 60 s, with a final extension of 72°C for 5 min. A new reverse primer, ZFRev1 (5' TAC TGG CAC TGG TAC GCC TTC 3'), was designed to work in conjunction with P1-5EZ. This primer pair produces a smaller product that should be easier to amplify from potentially degraded DNA samples. The constituents of the reaction were as before except the concentration of $MgCl_2$ was 1.25 mM and the primers used were P1-5EZ and ZFRev1. The PCR program consisted of 94°C for 5 min, then 40 cycles of 94°C for 60 s, 58°C for 30 s, and 73°C for 30 s, with a final extension of 72°C for 5 min. All PCR reactions were run with a negative control containing no template DNA.

Restriction enzyme digestion of the PCR products

PCR products generated with the primers sets, P1-5EZ/P2-3EZ and P1-5EZ/ZFRev1, were analyzed by restriction analysis. *Mar. martes*, *Mel. meles*, and *Mus. vison* samples were incubated with 5 units *Sac* 1, 2 μ l of 10x SuRE/Cut buffer A (Roche, Ireland), ~200 ng DNA, and water to a total volume of 20 μ l. Samples were digested for 3 h at 37°C. *Lutra lutra*, *Mus. erminea*, and *Mus.*

vison samples were restricted for 3 h at 65°C with 5 units of *Bsm* 1 and 2 μ l of 10x SuRE/Cut buffer H (Roche), ~200 ng DNA, and water to a total volume of 20 μ l.

Electrophoresis, product visualization, and sequence analysis

DNA extractions were analyzed on 0.8% agarose gels. PCR products and restriction enzyme digests were analyzed using 2% agarose gels. Gels were made using 1x TAE buffer (40 mM Tris acetate and 1 mM EDTA; pH 8) containing 0.25 μ g/ml ethidium bromide. Products were visualized under UV light and photographed with a Gene Genius Bioimaging System from Syngene (UK). PCR products were purified using Montage™ PCR Centrifugal Filter Devices from Millipore (Ireland). DNA was sequenced commercially by Qiagen and MWG Biotech (Germany). The EMBL accession numbers for the sequences are AM039476-AM039485. EditSeq™, MegAlign™, and MapDraw™ from DNASTAR (USA) were used for DNA analysis.

Phylogenetic analysis

Phylogenetic analysis was carried out on the ZFX and ZFY sequences obtained following amplification with P1-5EZ and P2-3EZ. Phylogenetic trees were constructed using an exhaustive search based on maximum parsimony with PAUP* 4.0 Beta vers. 10 (Swofford 1998).

RESULTS

Sex identification using established techniques

SRY amplification with primers Lut-SRY F and Lut-SRY R yielded a ~70 bp product from known male samples ($n = 7$), and no fragment was obtained from known female samples tested ($n = 5$) (Fig. 1A). This method was used to identify 22 tissues of animals of unknown sex as being male. Six females were tentatively identified by the absence of a product in multiple reactions in samples that amplified well with other primer sets. A summary of the results for each of the different sex typing methods is given in table 1.

Shaw et al. (2003) used the primers, LGL331 and LGL335, for sex identification in a range of mammals. These primers yielded a single product

in females and 2 products in males of a variety of mammal species. When this method was applied to *Mar. martes*, *Mus. erminea*, and *L. lutra*, a single product of ~900 bp was found in all known females tested ($n = 5$) and 2 fragments of ~900 and 1000 bp in all known males tested ($n = 5$) (Fig. 1B). One available known male *Mus. vison* tissue sample also yielded 2 fragments of ~900 and 1000

bp. When this technique was applied to 26 samples of unknown sex, 6 females and 20 males were identified. All sex identifications agreed with those obtained using SRY. The badger, *Mel. meles*, yielded 1 product of 900 bp from known male ($n = 3$) and female ($n = 2$) tissue samples and therefore the unknown *Mel. meles* samples could not be sexed using this method.

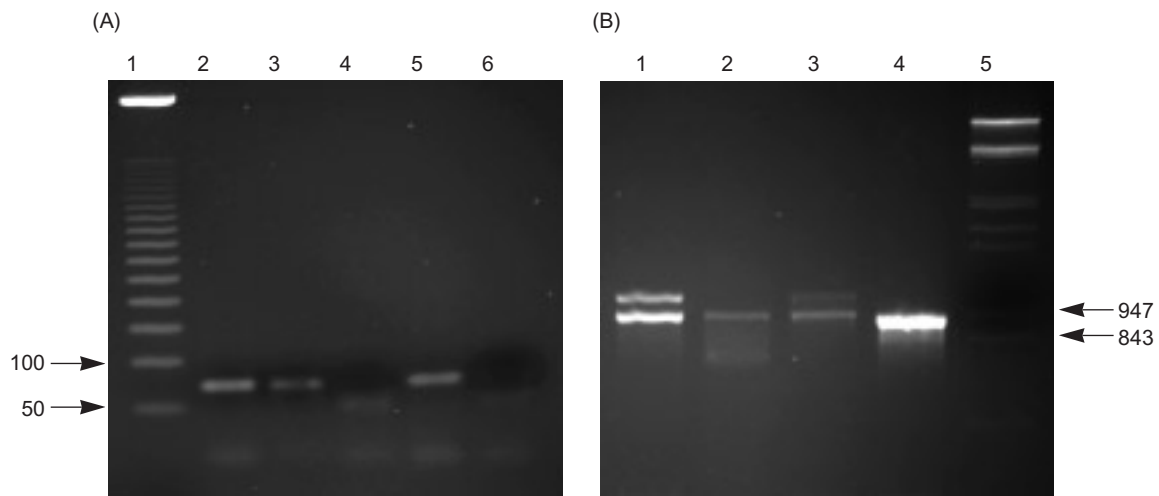


Fig. 1. Examples of sex identification using the SRY gene and zinc finger X and Y genes amplified with the primers, LGL331 and LGL335. (A) Amplification of SRY PCR products. The PCR products were amplified with *Mustela erminea* DNA as the template. Lane 1 contains a 50 bp size standard; lanes 2 and 3 were from animals of unknown sex; lane 4 contains female DNA; lane 5 contains male DNA; lane 6 is the no-template control. (B) ZFX/ZFY products amplified with the primers, LGL331 and LGL335. Lane 1 and 3 are male *Martes martes*; lanes 2 and 4 are female *Mar. martes*; lane 5 λ DNA restricted with *Eco* RI and *Hind* III.

Table 1. Summary of results from different sex determination methods. SRY indicates the number of samples that were sexed by amplification of a Y-specific product. ZFX/ZFY LGL331/335, indicates the number of samples that were sexed by the number of bands amplified. ZFX/ZFY restriction indicates the number of samples that were sexed by restriction analysis

Species		Known sex	SRY ^a	ZFX/ZFY LGL331/335	ZFX/ZFY restriction
<i>Lutra lutra</i> ($n = 8$)	♀	2	1	2	1
	♂	4	6	4	1
<i>Martes martes</i> ($n = 13$)	♀	2	4	4	4
	♂	2	8	9	5
<i>Meles meles</i> ($n = 20$)	♀	6	1	ND	2
	♂	11	1	ND	2
<i>Mustela erminea</i> ($n = 12$)	♀	1	4	3	3
	♂	1	8	7	6
<i>Mus. vison</i> ($n = 9$)	♀	0	1	1	1
	♂	1	6	5	2

^aFemales tentatively identified by the absence of product. ND, no difference in the product size seen.

Analysis of ZFX/ZFY using primers P1-5EZ and P2-3EZ

Aasen and Medrano (1990) described the use of primers P1-5EZ and P2-3EZ to amplify a 447 bp fragment of the ZFX/ZFY genes followed by RFLP analysis for sex determination in humans, cattle, sheep, and goats. Direct sequencing of PCR products identified double peaks in the chromatograms from males that could be used for sex determination or the selection of restriction enzymes for RFLP analysis (Fernando and Melnick 2001). In this study, primers P1-5EZ and

P2-3EZ amplified fragments of 447 bp from male and female *L. lutra*, *Mel. meles*, *Mar. martes*, *Mus. erminea*, and *Mus. vison*. Sequencing of the amplified products revealed double peaks in the chromatograms of male samples (Fig 2). Double peaks were not seen in female samples. The female sequence only differed from the male sequence at these sites. The double peaks were due to the co-amplification of X and Y products. The female template only gave ZFX products. By removing the base seen in the ZFX sequence from the double peak in the ZFX/ZFY mixed sequence it was possible to deduce the ZFY sequence as

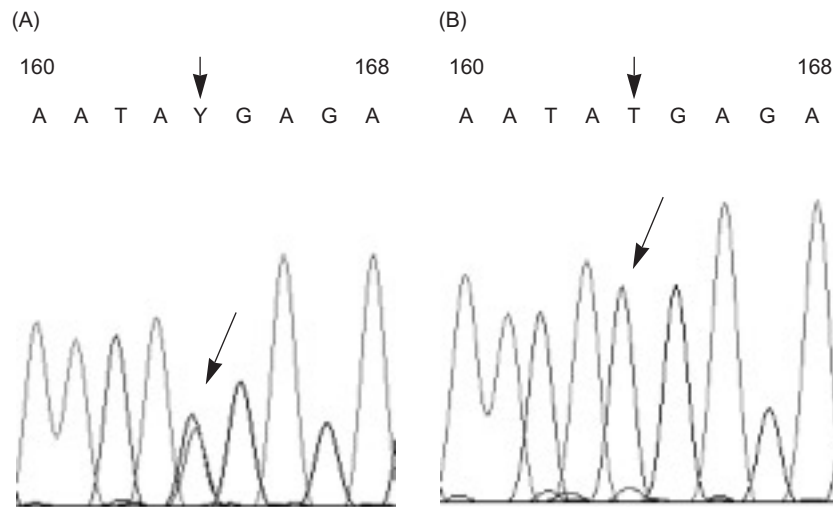


Fig. 2. DNA sequencing chromatograms of zinc finger X and Y (ZFX and ZFY) gene PCR products amplified with P1-5EZ and P2-3EZ. (A) Male *Mustela erminea* and (B) female *Mus. erminea*. The arrows highlight the double peak in the male and the corresponding single peak in the female. Y indicates the presence of both a C and T in the nucleotide sequence.

Table 2. Variable sites of the zinc finger X (ZFX) and ZFY genes in 5 species of mustelids. Identity with the consensus is denoted by a dash (-)

Nucleotide	0	0	0	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	3	3	3	3	3	3	3	3	3	3	3
Position	1	2	3	3	3	4	5	5	7	8	0	0	2	2	3	4	4	5	7	7	9	0	1	1	1	2	2	4	7	7	9
	3	8	1	0	9	8	1	7	2	1	5	8	0	3	8	1	4	0	4	7	7	4	1	6	9	2	8	8	3	6	1
Consensus	A	A	C	C	C	T	A	G	C	G	T	T	C	G	C	G	A	C	C	G	A	C	A	C	A	C	T	A	A	C	C
<i>L. lutra</i> X	-	-	-	-	T	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>L. lutra</i> Y	-	-	T	-	T	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-
<i>Mar. martes</i> X	-	G	-	-	-	C	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Mar. martes</i> Y	-	-	T	-	-	-	-	-	-	-	-	C	-	-	-	-	-	T	-	-	-	-	-	-	-	T	-	-	-	-	-
<i>Mel. meles</i> X	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	T
<i>Mel. meles</i> Y	-	-	-	-	-	-	-	-	T	A	-	-	-	-	A	-	T	-	-	G	-	-	-	-	-	-	-	T	-	G	T
<i>Mus. erminea</i> X	-	-	-	-	T	-	-	C	-	-	C	-	-	T	A	C	-	-	C	-	-	C	T	-	-	-	-	-	G	-	-
<i>Mus. erminea</i> Y	-	-	T	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	G	-	-
<i>Mus. vison</i> X	G	-	-	-	-	-	G	C	-	-	C	-	-	-	-	-	-	-	-	-	C	-	-	C	-	G	-	C	-	G	-
<i>Mus. vison</i> Y	-	-	T	T	-	-	-	-	-	-	-	-	-	-	A	-	T	-	-	-	-	-	C	-	G	-	C	-	G	-	-

Individuals sequenced: *Lutra lutra*, ♀ = 4, ♂ = 2; *Martes martes*, ♀ = 3, ♂ = 3; *Meles meles*, ♀ = 3, ♂ = 4; *Mustela erminea*, ♀ = 3, ♂ = 3; *Mustela vison*, ♀ = 1, ♂ = 5.

described previously by Fernando and Melnick (2001). The positioning of the variable sites is shown in table 2. The sequences were analyzed to identify restriction enzyme sites that would allow differentiation between ZFX and ZFY for each species under study. A *Bsm* 1 (GAATGCN) recognition sequence was present in the ZFX sequence of *L. lutra*, *Mus. erminea*, and *Mus. vison* but not in ZFY, while a *Sac* 1 restriction site (GAGCTC) was identified in the ZFX sequence of *Mar. martes*, *Mus. vison*, and *Mel. meles*, which was not present on the ZFY sequence. Importantly, the restriction enzyme sites were present in the ZFX genes and absent from the ZFY genes in all cases, ensuring that digestion was always seen. The sizes of the expected fragments are shown in table 3. Digestion of the PCR products yielded the expected products indicating that these enzymes could

be used for RFLP sex identification (Fig. 3A) of the mustelid species under study. All restriction digests gave the expected RFLP patterns for samples of known sex and those sexed using the established techniques of Shaw et al. (2003) and Dallas et al. (2000).

Where incomplete digestion was evident, this was dealt with either by adding less PCR product to the mixture, adding more restriction enzyme, or allowing further time for digestion.

Amplification and RFLP analysis of the short ZFX/ZFY products

The amplification of shorter fragments is generally considered to be more efficient than that of longer fragments, particularly from degraded samples (e.g., museum samples, hair, and feces).

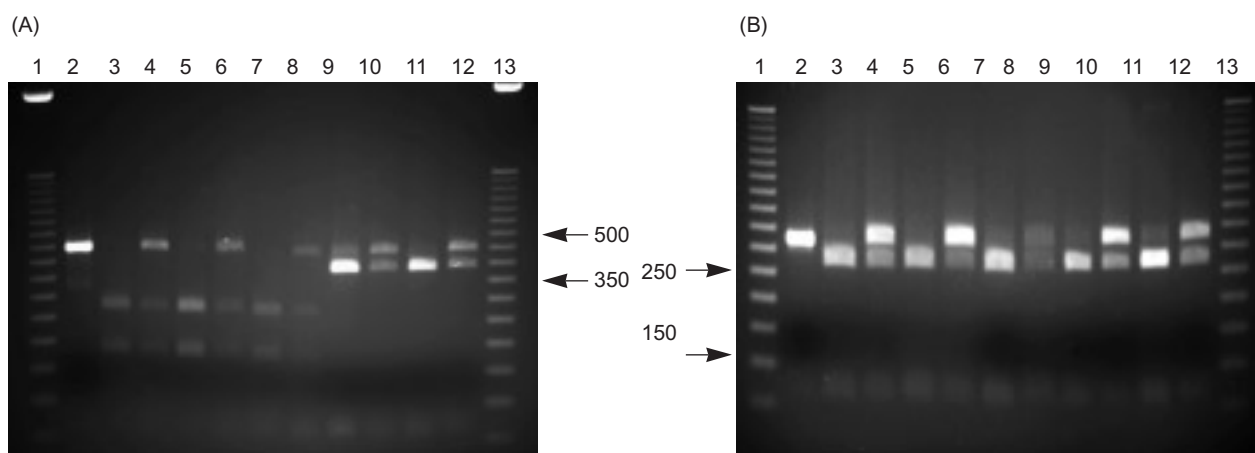


Fig. 3. RFLP analysis of zinc finger PCR products restricted with *Sac* 1 and *Bsm* 1. (A) Amplified with P1-5EZ and P2-3EZ. (B) Amplified with P1-5EZ and ZFRev1. Lanes 1 and 13 contain a 50-bp size standard; lane 2, *Mustela erminea* ♀ unrestricted; lanes 3 and 4, *Martes martes* ♀ + ♂; lanes 5 and 6, *Mus. vison* ♀ + ♂; lanes 7 and 8, *Meles meles* ♀ + ♂; lanes 3-8 restricted with *Sac* 1; lanes 9 and 10, *Mus. erminea* ♀ + ♂; lanes 11 and 12, *Lutra lutra* ♀ + ♂; lanes 9-12 restricted with *Bsm* 1.

Table 3. Sizes of the restriction fragments from ZFX/ZFY products obtained with the primer sets, P1-5EZ/P2-3EZ and P1-5EZ/ZFRev1

Species	Restriction enzyme	Sex	Primer sets	
			P1-5EZ and P2-3EZ Fragments sizes (bp)	P1-5EZ and ZFRev1 Fragments sizes (bp)
<i>Martes martes</i> , <i>Meles meles</i> , <i>Mustela vison</i>	<i>Sac</i> 1	Female	270, 177	270, 55
		Male	447, 270, 177	325, 270, 55
<i>Lutra lutra</i> , <i>Mustela erminea</i> , <i>Mustela vison</i>	<i>Bsm</i> 1	Female	391, 56	269, 56
		Male	447, 391, 56	325, 269, 56

Therefore a new primer, ZFRev1, was designed to amplify a shorter region of the ZFX/ZFY region in combination with P1-5EZ. The new primer pair reliably amplified 325 bp PCR products from each of the studied species. RFLP analysis results

agreed with those previously obtained with SRY and ZFX/ZFY using primers P1-5EZ and P2-3EZ. The sizes of the expected restriction fragments are shown in table 3, and the results of the RFLP analysis with *Sac* 1 and *Bsm* 1 are in figure 3B.

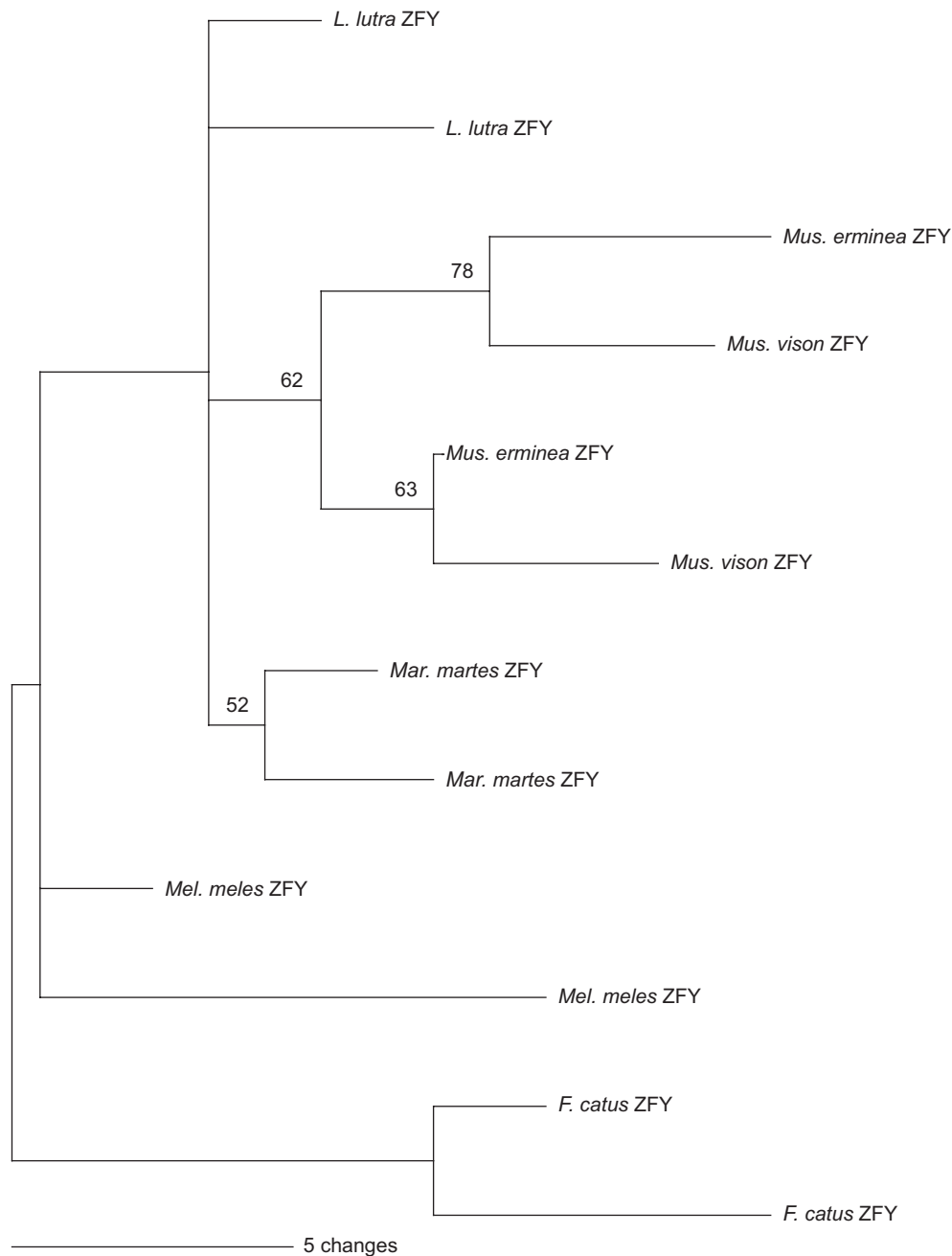


Fig. 4. Maximum parsimony tree of zinc finger X and Y (ZFX and ZFY) genes for 5 species of mustelids. The tree shown is one of 2 most parsimonious trees. All branches and nodes shown were retained in the consensus. The analysis was based on 397 total characters, 24 of which were parsimony informative. *Felis catus* X (AF252989) and Y (AF253014) sequences (Slattery *et al.* 2000) are included as an outgroup. Bootstrap values ≥ 50 in support of the adjacent node are given as a percentage derived from 1000 replicates. The consistency index was 0.7966, and the retention index was 0.6842.

Phylogenetic analysis of the ZFX and ZFY sequences

The phylogenetic relationship of the ZFX and ZFY sequences gained following amplification with primers P1-5EZ and P2-3EZ was examined (Fig. 4). The positioning of *Mel. meles* sequences (Fig. 4) is consistent with the basal relationship relative to other members of Mustelidae based on *cytochrome b* and complex repetitive flanking region sequences (Marmi et al. 2004). Xs and Ys of the 2 members of the genus *Mustela*, *Mus. erminea* and *Mus. vison*, formed monophyletic groups with moderate levels of bootstrap support (78 and 63, respectively). This likely indicates the divergence of ZFX and ZFY in *Mustela* prior to speciation. Similar results were seen in Felidae (Slattery et al. 2000) and cetaceans (Morin et al. 2005), where ZFX and ZFY form 2 distinct clades, although this occurred at higher taxonomic levels than genus. When members of different Mustelid genera (*Martes*, *Meles*, and *Lutra*) were taken into account, this pattern did not continue. The lack of formation of X and Y clades for all mustelid species in this study may have been the result of X-to-Y gene conversions, as seen in Slattery et al. (2000), prior to separation of mustelid genera.

DISCUSSION

In this study, a variety of methods were tested for sex typing *Mar. martes*, *Mus. vison*, *Mus. erminea*, *L. lutra*, and *Mel. meles*. Amplification of a small section of the male-specific SRY gene was successful in all species. The small size of the product (70 bp) makes it ideal for degraded samples; but this method, when used on its own, requires a 2nd amplification of a control nuclear sequence to confirm that non-amplification from a sample is not due to PCR inhibition or poor DNA quality/quantity. In this study, all samples sexed with this method were tested with a number of methods, and therefore it was established that they amplified well. The small size of the product can lead to some difficulty in visualization as primer dimers may be confused with a positive product (Fig. 1A). A positive control of a known male animal should be included in all experiments.

The 2nd method used primers LGL331 and LGL335 (Cathey et al. 1998) to amplify an intron from the ZFX/ZFY gene. A single band of ~900 bp was amplified from females and 2 bands of ~900 and ~1000 bp from males for all species except

Mel. meles. *Meles meles* gave only 1 product in both sexes, and therefore this method was uninformative in this species. Shaw et al. (2003) amplified 2 products from male animals of all species in their study, which included a number of whale species, harbour seal, franciscana dolphin, canids, black bear, and moose. This method is well suited for sex determination from non-degraded DNA, but the size (~1 kb) of the products makes it prohibitively difficult to apply to more-degraded samples.

The 3rd method for gender determination used primers P1-5EZ and P2-3EZ (Aasen and Medrano 1990) to amplify a 447 bp region of the ZFX/ZFY genes. Sequencing of the amplified products from known males and females of each species allowed the determination of the ZFX and ZFY sequences in all species. While males can be identified from the sequencing chromatogram by the presence of double peaks, this method is dependent on the quality of the sequence data. RFLP analysis of the amplified product with *Bsm* 1 and *Sac* 1 is a simple and reliable method for gender identification. Amplification with primers P1-5EZ and ZFRev1 followed by RFLP analysis is particularly useful. A smaller, more-easily amplified product is produced, and the same sex-specific RFLP pattern was seen across all 5 studied species. This method works well with many sample types and should be readily adaptable to closely related species.

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