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MOLECULAR DIAGNOSTICS AND DNA TAXONOMY

A primer set to determine sex in the small Indian mongoose, *Herpestes auropunctatus*

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Abstract

To enable the accurate sexing of individuals of introduced populations of the small Indian mongoose, *Herpestes auropunctatus*, we designed a primer set for the amplification of the sex-specific fragments *EIF2S3Y* and *EIF2S3X*. Using this primer set, the expected amplification products were obtained for all samples of genomic DNA tested: males yielded two bands and females a single band. Sequencing of each PCR product confirmed that the 769-bp fragment amplified from DNA samples of both sexes was derived from *EIF2S3X*, whereas the 546-bp fragment amplified only from male DNA samples was derived from *EIF2S3Y*. The results indicated that this primer set is useful for sex identification in this species.

Keywords: *EIF2S3X*, *EIF2S3Y*, invasive species, sex identification

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The small Indian mongoose, *Herpestes auropunctatus* (Family Herpestidae, Order Carnivora, Class Mammalia, synonym: *Herpestes javanicus*), has been introduced into many different areas of the world, mainly islands (Simberloff *et al*. 2000). However, the mammal's introduction has had unwelcome consequences and there is now an urgent need to eradicate it. In Japan in 1910, 13–17 mongooses were released on Okinawa Island and four on Tonaki Island for the control of poisonous snakes and rats. Furthermore, 30 individuals were released on Amami-Oshima Island in 1979. Since then, the range of these mongooses has increased (Yamada & Sugimura 2004).

Molecular sexing by means of PCR amplification is a rapid and convenient, and hence effective, way to study introduced populations. This system can be applied to various samples, which include tissue from animals that have been captured alive, for example, blood, skin and hair; tissue obtained from decomposing corpses that are found in a trap; hair that has been shed; and tissue that has been frozen for a long period. Molecular sex identification normally involves PCR amplification of regions that differ in length between sexes, and the fragments of different sizes are separated by gel electrophoresis and then visualized. The constitution of sex chromosomes in female mammals is homogametic (XX), whereas that of males is heterogametic (XY). As a consequence, primer sets for sex determination are generally designed to amplify homologous regions of the X and Y chromosomes that differ in length. However, the small Indian mongoose has a unique constitution of sex chromosomes among mammals. No separate Y chromosome can be identified, and the male has an odd number of diploid chromosomes, $2n = 35$, whereas the female has an even number, $2n = 36$. The odd number of chromosomes in males is thought to be caused by the translocation of the Y chromosome to an autosome (Fredga 1964; Raman & Nanda 1982). We performed Southern blot analysis using mouse cDNA clones as probes to identify the mammalian Y-linked genes, *EIF2S3Y*, *ZFY* and *KDM5D*, which are present in the genome of males, but not females, of the small Indian mongoose (C. Murata, G. Ogura, A. Kuroiwa, unpublished data). Male-specific bands were clearly detected for *EIF2S3Y* (data not shown); therefore, we selected this gene as a molecular marker for sexing. We designed a degenerate PCR primer set by comparing the *EIF2S3Y* sequences of *Mus musculus* (NM_012011) and *Xenopus tropicalis* (NM_001011456) using ClustalW (http://clustalw.ddbj.nig.ac.jp/top-j.html). The primer set consisted of the forward primer 5′- CAA GCC ACA ATT AAY ATA GGC AC -3′ and the reverse primer 5′- CTC CTA CTT CTG TGC AYA CTG G -3′. Amplification was carried out in a 50 μl mixture that contained 25–50 ng of template cDNA, which was synthesized from mRNA from the brain of male *H. auropunctatus*, 2 mM

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$MgCl_2$, each primer at 0.5 μM, each of the four deoxynucleotide triphosphates (dNTPs) at 0.2 mM, 1.25 U of *ExTaq* (TaKaRa), and a one-tenth volume of 10× *ExTaq* buffer. PCR was performed under the following conditions: denaturation for 5 min at 94 °C, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C, extension for 35 s at 72 °C, and finally 5 min at 72 °C using a GeneAmp PCR System 9700 (Applied Biosystems). Fragments of 1156 and 1087 bp (excluding the primer sequences), which corresponded to a male-specific fragment and a fragment from the X chromosome, were amplified by degenerate PCR from the cDNA from the brain, and both fragments were cloned using the pGEM-T Easy System (Promega). The cDNA clones were labelled with a BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems).The following primers were used for sequencing: SP6, 5′- ATT TAG GTG ACA CTA TAG AA -3′; T7, 5′- TAA TAC GAC TCA CTA TAG GG -3′. The fragments were sequenced in both directions after insertion into the vector pGEM-T Easy (Promega). The sequencing was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). We registered the sequences in the genetic sequence database Genbank, with the following accession numbers : *EIF2S3Y*, AB550793; *EIF2S3X*, AB550794. The sequences of the two fragments were compared to identify homologous regions, and the boundaries between exons and introns were estimated on the basis of the genomic sequences of these genes in the domestic cat (UCSC Genome Bioinformatics, http://genome.ucsc. edu/). Using Primer Express (Applied Biosystems), we designed four sets of primers that bound to the homologous regions and selected a set that showed no non-specific bands: forward primer, 5′- TGA TGT CAA CAA ACC TGG CTG -3′; reverse primer, 5′- CTG GAG CAG CAT ACT GAA GAT CA -3′. To test the efficacy of this primer set for sexing, tissue samples were obtained from individuals captured on Okinawa Island using traps. Genomic DNA was extracted from various types of tissue to confirm that this sexing system could be applied to samples from various sources and held under various conditions, which included fresh muscle obtained from euthanized individuals and kept at −80 °C, muscle obtained from euthanized individuals and kept in 100% ethanol, muscle obtained from decomposing corpses and kept in 100% ethanol, hair from living individuals and hair from decomposing corpses. We used DNA extracted from 30 males and 29 females. DNA was extracted from muscle

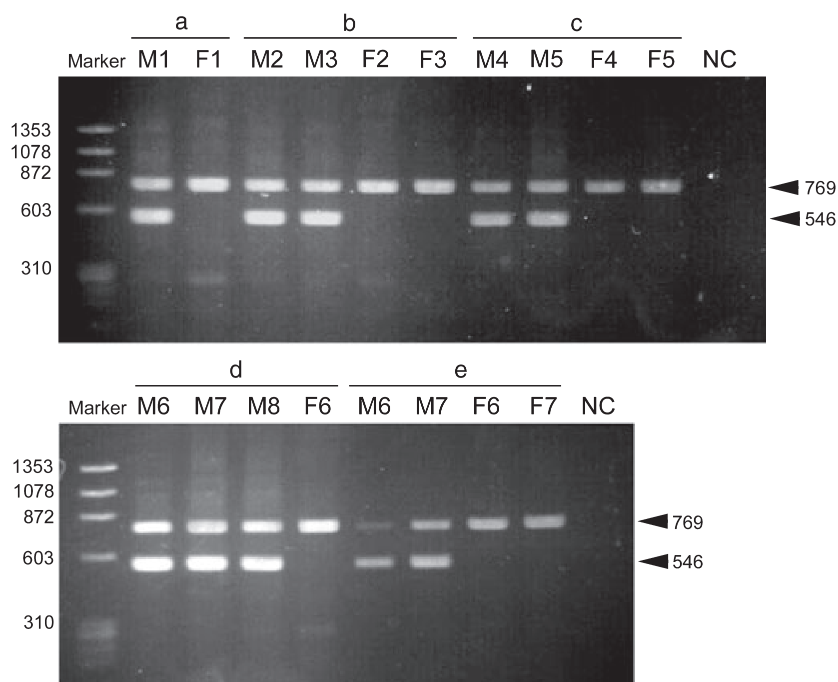

Fig. 1 Molecular sex identification in the small Indian mongoose using a primer set for PCR. PCR amplification was performed using genomic DNA samples extracted from eight males and seven females. The size of bands is indicated in bp on both sides of the lanes. The X174/*Hae*III digest (Toyobo) was used as a marker of molecular size. Numbers indicate the individual. M: male; F: female. (a) DNA extracted from fresh muscle obtained from euthanized individuals and kept at −80 °C; (b) DNA extracted from muscle obtained from euthanized individuals and kept in 100% ethanol; (c) DNA extracted from the hair of living individuals; (d) DNA extracted from muscle obtained from decomposing corpses and kept in 100% ethanol; (e) DNA extracted from the hair of decomposing corpses, used as a template. NC, negative control.

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using a standard technique (Sambrook & Russell 2001). DNA was extracted from hair using Isohair (Nippon Gene) in accordance with the manufacturer's protocol. The fragments were amplified using Ampdirect Plus PCR buffer (Shimadzu) and Nova *Taq*™ Hot Start DNA Polymerase (EMD Biosciences). Amplification was carried out in a 10 μl mixture that contained 25–50 ng of template DNA, each primer at 0.5 μM, 0.25 U of Nova *Taq*™ Hot Start DNA Polymerase (EMD Biosciences), and one-half volume of 2× Ampdirect Plus (Shimadzu). The PCR was performed using a GeneAmp PCR System 9700 (Applied Biosystems) and the following reaction conditions: 94 °C for 2 min, then 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 35 s, and finally 72 °C for 5 min. Amplification was successful in all DNA samples tested. After 2% agarose gel electrophoresis, two bands (546 and 769 bp) could be visualized for male DNA samples and a single band (769 bp) for female samples. The sex determined from the genotype was consistent with the sex determined from the phenotype for all samples. The results for eight males and seven females are shown in Fig. 1. Each DNA band was eluted from the gel using a QIAquick Gel Extraction kit (Qiagen), ligated into the pGEM-T Easy Vector (Promega), and the resulting construct was used to transform *Escherichia coli* JM109 competent cells (Takara Bio). The inserted fragments were sequenced as described earlier. The results of the sequencing confirmed that the 769-bp fragment that was amplified from DNA of both sexes, was derived from *EIF2S3X*, whereas the 546-bp fragment that was amplified only from DNA samples from males was derived from *EIF2S3Y*. The results of our study showed that this primer set is useful for sexing the small Indian mongoose and that this method of molecular sex determination could be an effective tool for research and the control of introduced populations.

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