Original Article

Evaluation of Amelogenin and Zinc-finger Loci for Sex Identification in Captive Felids

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Abstract

The amelogenin and zinc-finger regions were used for sex determination in six species of felids: *Felis catus* (Persian cat), *Felis chaus* (jungle cat), *Prionailurus bengalensis* (leopard cat), *Pardofelis temminckii* (Asiatic golden cat), *Panthera tigris* (tiger) and *Prionailurus viverrinus* (fishing cat). The amelogenin region in all six felids could be successfully amplified and gave a heterozygous genotype in males and a homozygous genotype in females, except for leopard cats which showed a heterozygous genotype in both sexes. The PCR products from the zinc-finger region could be amplified in Persian cats, Asiatic golden cats, tigers and fishing cats. In the Persian cat, Asiatic golden cat and tiger, the heterozygous genotype of PCR products was amplified in males and the homozygous genotype was amplified in females, whereas the heterozygous genotypes of PCR products in both males and females were amplified from the fishing cat. Thus, the amelogenin region can be used for sex identification in the fishing cat, Asiatic golden cat, jungle cat, Persian cat and tiger. However, the zinc-finger region can be used to determine the gender in the Persian cat, tiger and Asiatic golden cat. In conclusion, both amelogenin and zinc-finger regions can be used to determine sex in certain felid species in Thailand.

Keywords: amelogenin region, Felidae, sex determination, zinc-finger region

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Introduction

Sex identification in populations is important to understand population dynamics and structure (Statham et al., 2007). This is necessary when determining the ecological manners of a wildlife population including daily movement distances, home range sizes and behavior in the population and the mating system (Shaw et al., 2003). In addition, gender determination is a tool for monitoring and investigating forensic cases (Shaw et al., 2003). Molecular gender determination is essential in situations when the animal is presented with an incomplete carcass and sex-specific characters are either absent or difficult to identify (Shaw et al., 2003). Knowledge of sex ratio is an important basis for the management and conservation of endangered animals (Barbosa et al., 2009).

A typical gene used to identify sex in mammals is the SRY gene (the testes determining factor), which is present only on the male Y chromosome. By amplification with SRY-specific primers, one band of the PCR products is amplified for males but there is no PCR product band for females. A negative result can mean either that the sample originated from a female or that it was of low quality and did not contain an adequate amount of DNA (Goossens and Salgado-Lynn, 2013). Thus, the SRY tests can be problematic when used on poor-quality DNA samples (Pilgrim et al., 2005). Identification of the zinc-finger region (*Zfx* and *Zfy*) is one of the several methods for identifying sex that can avoid a false female result because it is present on both X and Y-chromosomes. These genes have been tested to be useful as a molecular method for determining gender in several mammals such as canids, sea otters, rodents, pinnipeds, ungulates, ursids, prosimians, human, cetaceans and felids (Curtis et al., 2007). When determining the sex in a carnivore using the zinc-finger region, the male shows two bands of PCR products, whereas the female shows only one band of PCR products (Goossens and Salgado-Lynn, 2013). Amelogenin genes were also identified first on the sex chromosomes in both mouse and man (Lau et al., 1989). The amelogenin (AMEL) locus encodes a matrix protein forming mammalian tooth enamel. Mutations in the AMEL locus can lead to an enamel defect known as amelogenesis imperfecta, which results in the abnormal formation of tooth enamel (Butler and Li, 2014). The AMEL marker is one of the forensic markers for human gender identification. Moreover, the amelogenin gene primers are useful in sex identification of sheep and red deer (Pfeiffer and Brenig, 2005), cattle (Grzybowski et al., 2006) and domestic cat (Pilgrim et al., 2005). In *Bos taurus*, the most striking difference between the X- and Y-chromosome *AMEL* genes is in the 63 bp deletion in the exon 6 of the Y-linked copy. In domestic cat, the Y-chromosome copy (*AMELY*) has 20 bp deletions when compared to the X-chromosome copy (*AMELX*) and the PCR products present a heterozygous genotype in the male cat and a homozygous genotype in the female cat. The objective of this study was to evaluate whether the amelogenin gene and zinc-finger regions can be used to develop a sex determination tool for captive felids in Thailand.

Materials and Methods

Animals and sample collections: One-milliliter EDTA-blood samples were collected from 31 Persian cats (*Felis catus*) from a private Persian cat farm; 39 jungle cats (*Felis chaus*) from Songkhla Zoo, Nakornratchasima Zoo and Khao Kheow Open Zoo; 42 leopard cats (*Prionailurus bengalensis*) from Nakornratchasima Zoo, Khao Kheow Open Zoo and the Khaozon Wildlife Breeding Center; 16 Asiatic golden cats (*Pardofelis temminckii*) from Songkhla Zoo, Khao Kheow Open Zoo, Chiang Mai Zoo and the Khao Pratub Chang Wildlife Breeding Center; 35 tigers (*Panthera tigris*) from Chiang Mai Zoo, Nakornrachasima Zoo and the Khaozon Wildlife Breeding Center; and 23 fishing cats (*Prionailurus viverrinus*) from the Chiang Mai Night Safari. The EDTA-blood samples were kept at -20°C until used.

DNA extraction: The EDTA-blood samples were subjected to DNA extraction using the phenol-chloroform extraction method (Sambrook et al., 1989). Concentration and purity of the extracted DNA was measured using a spectrophotometer (SmartSpec™ Plus Spectophotometer, BIO-RAD, USA).

DNA amplification: Two sets of specific primers (Pilgrim et al., 2005) were used for amplification of the zinc-finger region and the amelogenin gene, Fz (5′-AAG-TTT-ACA-CAA-CCA-CCT-GG-3′) and Rz (5′-CAC-AGA-ATT-TAC-ACT-TGT-GCA-3′), and Fa (5′-CGA-GGT-AAT-TTT-TCT-GTT-TAC-T-3′) and Ra (5′-GAA-ACT-GAG-TCA-GAG-AGG-C-3′), respectively. Twenty microliters of PCR mixture (Invitrogen®, Brazil) was composed of 50 ng of DNA template, 2 µl of PCR buffer (10xbuffer), 1 µl of 50 mM $MgCl_2$, 0.4 µl of 10 mM dNTPs, 0.1 µl of each forward and reverse primers (100 µM), 0.1 µl of Taq DNA polymerase and DNase-free water. PCR conditions were modified from those described earlier (Menotti-Raymond et al., 1999). After an initial denaturation at 93°C for 3 min; the amplification was performed by 10 cycles of 94°C for 15s, 55°C for 15s, 72°C for 30s; followed by 20 cycles of 89°C for 15s, 55°C for 15s, and 72°C for 30s and a final extension at 72°C for 30 min. The PCR products were electrophoresed using 1.5% agarose gel (ADVANCE, Japan) and visualized under ultraviolet illumination. DNA fragment analysis was performed using automated capillary electrophoresis (QIAGEN®, Germany).

Results

The amplified products of male fishing cats, Asiatic golden cats, jungle cats, Persian cats and tigers were 206 and 228 bp, 208 and 224 bp, 208 and 224 bp, 216 and 236 bp, and 216 and 236 bp, respectively (Fig 1 and Table 1). The amplified products of the female fishing cats, Asiatic golden cats, jungle cats, Persian cats and tigers were 228 bp, 224 bp, 224 bp, 236 bp, and 236 bp, respectively. The PCR products produced by the zinc-finger region specific primers were 182 and

188 bp for the males and 188 bp for the females of Asiatic golden cats, Persian cats and tigers; whereas there were two bands (198 and 202 bp) for both male and female fishing cats (Fig 2 and Table 2). There were not enough samples for analysis of the jungle cats and leopard cats.

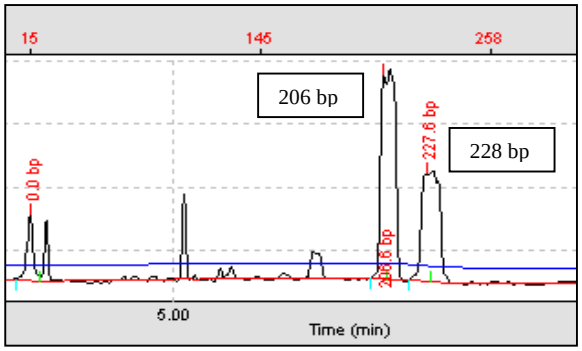
Table 1 Allele sizes of amelogenin region according to gender

Species	Number	DNA product (bp)	
		Male	Female
Fishing cat	23 (M = 13, F = 10)	206, 228	228
Asiatic golden cat	16 (M = 15, F = 1)	208, 224	224
Jungle cat	39 (M = 17, F = 22)	208, 224	224
Persian cat	31 (M = 15, F = 16)	216, 236	236
Tiger	35 (M = 20 , F = 15)	216, 236	236
Leopard cat	42 (M = 33 , F = 9)	196, 216	196, 216

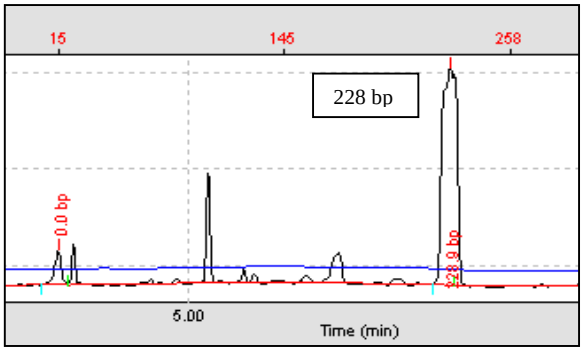
Table 2 Allele sizes of zinc-finger region according to gender

Species	Number	DNA product (bp)	
		Male	Female
Fishing cat	23 (M = 13, F = 10)	198,202	198,202
Asiatic golden cat	16 (M = 15, F = 1)	182,188	188
Persian cat	31 (M = 15, F = 16)	182,188	188
Tiger	35 (M = 20, F = 15)	182,188	188
Jungle cat		ND*	
Leopard cat		ND*	

ND* = There were insufficient samples for analysis.



A



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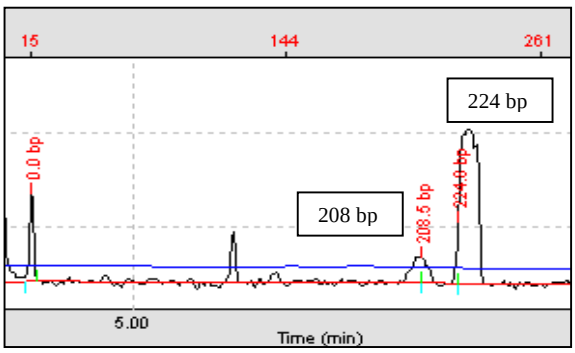

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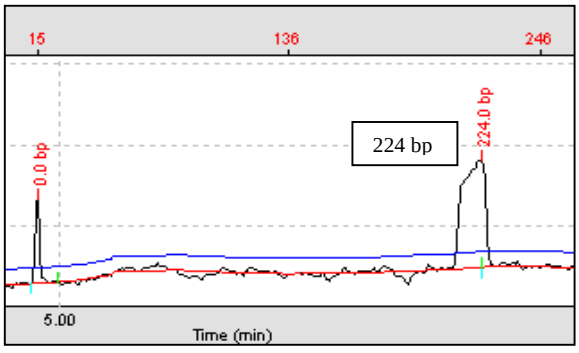

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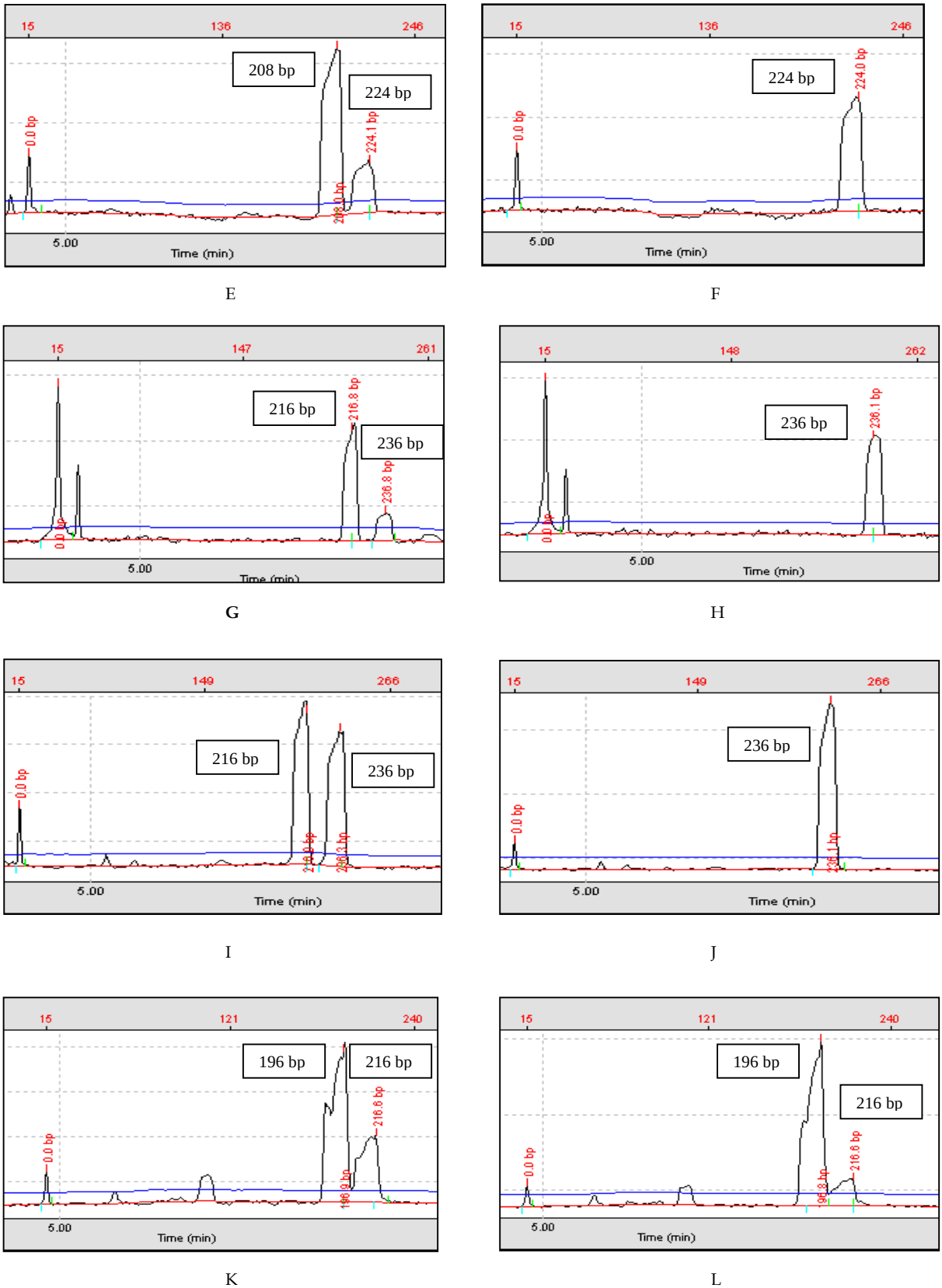

Figure 1 Photographs of peaks of amplified products of amelogenin gene in male (A, C, E, G, I and K) and female (B, D, F, H, J and L) fishing cats (A and B), Asiatic golden cats (C and D), jungle cats (E and F), Persian cats (G and H), tigers (I and J) and leopard cats (K and L)

Discussion

The amelogenin region was successfully amplified and showed a heterozygous genotype in the males and a homozygous genotype in the females of all felids in this study except for the leopard cats, which showed a heterozygous genotype of 196 and 216 bp, in both sexes. The sizes of the amplified products of the fishing cats (206 and 228 bp), Asiatic golden cats (208 and 224 bp), jungle cats (208 and 224 bp), Persian cats

(216 and 236 bp) and tigers (216 and 236 bp) were different from those of the previous study by Pilgrim et al. (2005), in which the amplified products of the wild cat (*Felis silverstris*), bobcat (*Lynx rufus*), cougar (*Puma concolor*) and Erasian lynx (*Lynx lynx*) were 194 bp for *AMELY* and 214 bp for *AMELX*. The difference in the size of the amplified products may be due to the difference in the species of felids in these two studies. Nevertheless, the amelogenin region could be used to determine the sex of the felids in this study.

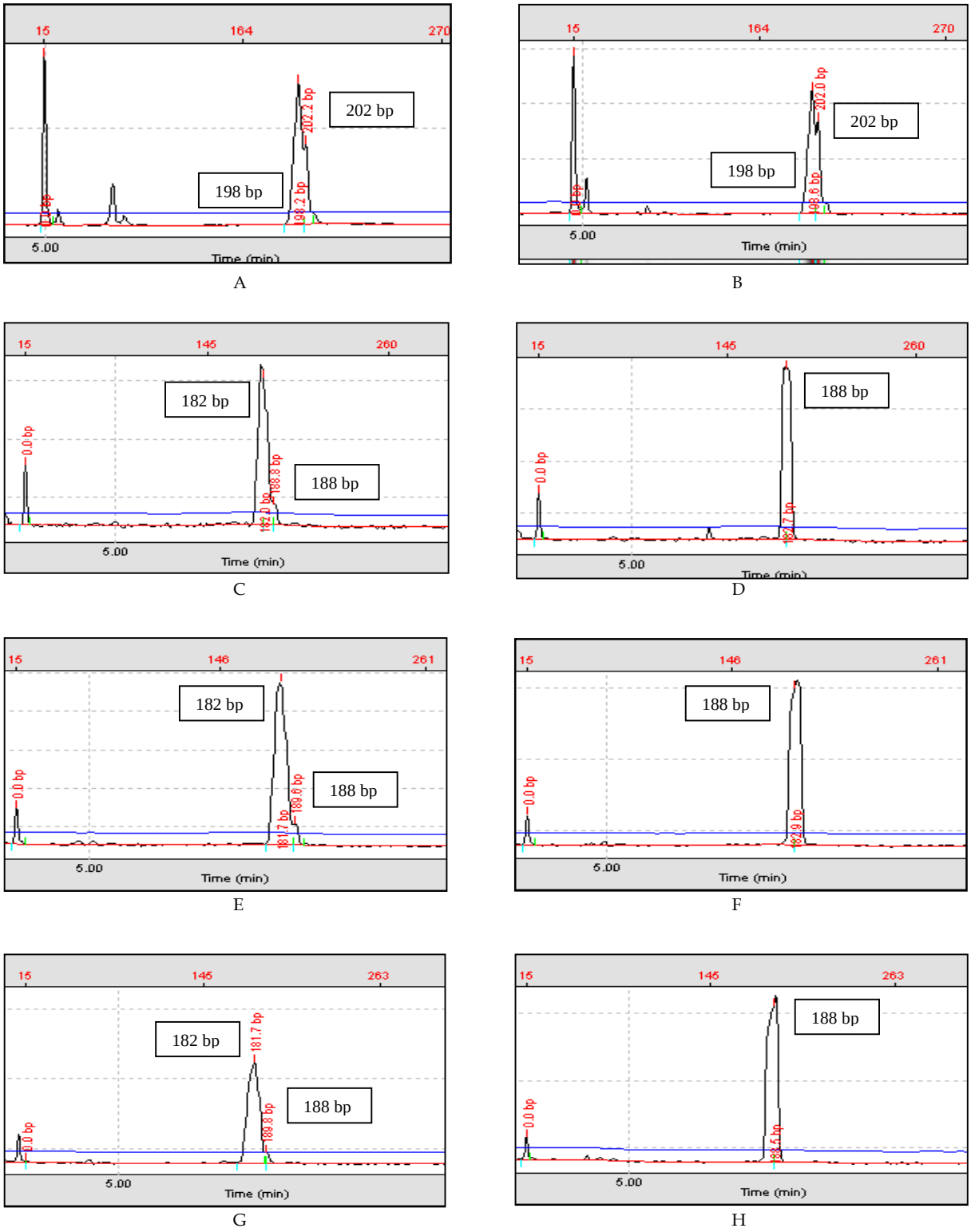

Figure 2 Photographs of peaks of amplified products of zinc-finger region in male (A, C, E and G) and female (B, D, F and H) fishing cats (A and B), Asiatic golden cats (C and D), Persian cats (E and F) and tigers (G and H)

The zinc-finger region was also successfully amplified and could be used to identify the sex for Asiatic golden cats, Persian cats and tigers. The sizes of the PCR products were 182 and 188 bp for males and 188 bp for females; however, it was not possible to differentiate between male and female for the fishing cats because the size of the male and female alleles were the same (198 and 202 bp). This result was

different from the previous report, which gave amplified products of 163 and 166 bp for males, and 166 bp for females (Pilgrim et al., 2005). This might be due to the differences in the species of the felids used in these two studies. By using the primers for zinc-finger regions in this study, non-felid species produced PCR products of 166 bp, but these products were not sex-specific (Pilgrim et al., 2005). Xu et al. (2007) determined the gender of giant panda by using a different set of primers to target the conserved region in the exon of the zinc-finger region; these primers successfully amplified PCR products which were three amplified products (447, 350 and 146 bp) in males and two amplified products (447 and 146 bp) in females. There have been several reports using different sets of primers for zinc-finger gene to determine sex in other species such as in the prehensile-tailed porcupine (*Coendou prehensilis*) (Woc-Colburn et al., 2013; Okuyama et al., 2014).

In the present study, the amelogenin primers could be used to identify the sex in Asiatic golden cats, jungle cats, Persian cats and tigers and the zinc-finger primers could determine the gender in Persian cats, tigers and Asiatic golden cats. However, the results showed that the identification of a homozygous genotype based on both the amelogenin and zinc-finger tests should be interpreted prudently. If there is a failure to amplify a Y-chromosome fragment, then the result will be interpreted as a false positive homozygous genotype, indicating a female. The amplification success of the samples is limited by the DNA quality. Pagès et al. (2009) used three sex-specific gene including amelogenin gene, zinc-finger gene and SRY gene to identify the sex of eight bear species to overcome the DNA quality problem from non-invasive samples and fossil remains. Therefore, in order to use genetic markers for sex identification, there should be more than one markers used to increase the potential accuracy of results.

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บทคัดย่อ

การประเมินการใช้เครื่องหมายพันธุกรรมในตำแหน่งยีน Amelogenin และ Zinc-finger ในการแยกเพศสัตว์ตระกูลแมวในประเทศไทย

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การศึกษาการใช้ยีนตำแหน่ง amelogenin และ zinc-finger ในการแยกเพศในสัตว์ตระกูลแมว ได้แก่ แมวเปอร์เซีย (*Felis catus*) แมวป่า (*Felis chaus*) แมวดาว (*Prionailurus bengalensis*) เสือไฟ (*Pardofelis temminckii*) เสือโคร่ง (*Panthera tigris*) และเสือปลา (*Prionailurus viverrinus*) จากการศึกษาพบว่าตำแหน่ง amelogenin ในเพศผู้ให้อัลลีลแบบเฮเตอร์โรไซกัสจีโนไทป์ (Heterozygous genotype) และในเพศเมียให้อัลลีลแบบโฮโมไซกัสจีโนไทป์ (Homogygous genotype) และสามารถแยกเพศในสัตว์ตระกูลแมวทั้งหมดที่ใช้ในการศึกษานี้ ยกเว้นแมวดาวที่ให้อัลลีลแบบเฮเตอร์โรไซกัสจีโนไทป์ทั้งในเพศผู้และเพศเมีย สำหรับตำแหน่ง zinc-finger สามารถเพิ่มสารพันธุกรรมและสามารถแยกเพศได้ โดยให้อัลลีลเป็นแบบโฮโมไซกัสจีโนไทป์ในเพศเมีย และเฮเตอร์โรไซกัสจีโนไทป์ในเพศผู้ได้ในแมวเปอร์เซีย เสือไฟ เสือโคร่ง สำหรับเสือปลาสามารถเพิ่มสารพันธุกรรมได้แต่ให้อัลลีลเป็นแบบเฮเตอร์โรไซกัส (Heterozygous) ทั้งเพศผู้และเพศเมีย ดังนั้นตำแหน่ง zinc-finger สามารถใช้ในการแยกเพศในแมวเปอร์เซีย เสือไฟ และเสือโคร่ง ส่วนตำแหน่ง amelogenin เป็นตำแหน่งที่สามารถใช้แยกเพศได้ในเสือปลา เสือไฟ แมวป่า แมวเปอร์เซีย และเสือโคร่ง

คำสำคัญ: ตำแหน่ง amelogenin สัตว์ตระกูลแมว การระบุเพศ ตำแหน่ง zinc- finger

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