Int J Legal Med (2010) 124:249–256
DOI 10.1007/s00414-009-0383-2

CASE REPORT

Forensic hair analysis to identify animal species on a case of pet animal abuse

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Received: 28 March 2009 / Accepted: 14 October 2009 / Published online: 18 November 2009

Abstract As part of an investigation of a case of pet animal abuse, we attempted to identify small mammalian species by morphological analysis and single-nucleotide polymorphism (SNP) typing of the cytochrome *b* gene using guard hairs as an analytical material. Guard hair samples from several species were measured for length, width, medulla formation, and cuticle scale pattern under a light microscope or scanning electron microscope. These samples were also analyzed for SNPs in the cytochrome *b* gene using a multiplex single-base primer extension reaction. Morphological analysis of cuticle scale pattern and medulla formation was able to discriminate ferret hairs from other hair samples that included rabbit, gerbil, degu, and Djungarian hamster. However, this also revealed a similarity of the guard hairs of the ferret (*Mustela putorius furo*) and Japanese weasel (*Mustela itatsi*). Although at three sites, the nucleotide color signals of SNPs in the cytochrome *b* gene could be used to discriminate completely among human, dog, and gerbil, the signals for cat, ferret, and Japanese weasel occurred at the same nucleotide sites. Unfortunately, no signals were obtained from degu, Djungarian hamster, and rabbit hairs. Although the discriminated hair samples were 100% identical to those of the ferret, there was only a 5% difference from Japanese weasel in the partial sequence of the cytochrome *b* gene. Construction of a database of mammalian hairs would be useful not only in forensic science, but also for investigating smuggling of endangered species in contravention of the Washington Convention.

Keywords Animal hair · Morphology · Mitochondrial DNA · Cytochrome *b* · SNP

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Introduction

In forensic investigations, hair samples are useful as trace evidence of the movements and behavior of suspects. Hair falls out of the body and secondarily attaches to the clothing of others; in this way, it is interchanged between victim and suspect by Locard' exchange principle [1, 2]. Although the hair of domestic animals, including the dogs and cats, can be identified by morphological methods, the hair of mammalian wildlife has not yet been sufficiently investigated with respect to morphological information [3–5]. It is nevertheless important that such samples be

tested because recent environmental changes have meant that some forest-dwelling mammalian wildlife (i.e., bear, monkey, deer, and wild boar) is being increasingly found close to areas of human habitation [6]. Additionally, several exotic mammalian species (ferret, monkey, and also some species of mouse) are now being used as companion animals for humans [7]. Recent studies have demonstrated the utility of analyses of mitochondrial DNA (mtDNA), including the ribosomal RNA (rRNA) and cytochrome *b* genes, for the discrimination of animal species [8–10]. For these reasons, it is important to forensic science that hair data is collected from mammalian wildlife. Here, we describe morphological and single-nucleotide polymorphism (SNP) typing of the cytochrome *b* gene as an analytical material using guard hairs.

Case report

This case involved an owner of small pet mammals. The owner had recorded his manual strangulation of ferrets on video (*Mustela putorius furo*) and uploaded the videos to the bulletin board of a pet lover's Internet website (Fig. 1a). The suspect had subsequently been arrested for violating Japan's Animal Protection and Control Law. No carcasses had been found at the suspect's home because all of them had been disposed off with the household garbage. We attempted to prove that the suspect had been keeping ferrets on the premises by identifying ferret guard hairs from among bulk samples of hair collected from a carpet that had appeared in one of the videos uploaded by the suspect (Fig. 1b). The suspect owned about 30 small mammals of species other than *M. putorius furo* (i.e., Djungarian hamster, rabbit, gerbil, and degu).

Materials and methods

Guard hair samples from the domestic dog (*Canis lupus familiaris*), cat (*Felis silvestris catus*), ferret (*M. putorius furo*), rabbit (*Oryctolagus cuniculus*), gerbil (*Meriones unguiculatus*), degu (*Octodon degus*), and Djungarian hamster (*Phodopus sungorus*) were obtained from animals kept in pet shops. Guard hair samples of Japanese weasel (*Mustela itatsi*) and muscle samples of wolf (*Canis lupus lupus*) were obtained from the Department of Wildlife Science, College of Bioresource Science, Nihon University, Fujisawa, Japan.

Morphological analysis

Thirty-eight of the mammalian guard hairs from the bulk hair samples were selected by the naked eye (Fig. 1c). The selected hair samples were measured for length, width, medulla formation, and cuticle scale pattern under a light microscope or scanning electron microscope (SEM; JXA-8200, JEOL, Tokyo, Japan) for comparison with cross-sections or vertical sections of hair samples from the domestic dog, cat, ferret, Japanese weasel, rabbit, gerbil, degu, and Djungarian hamster. These microscopic analyses

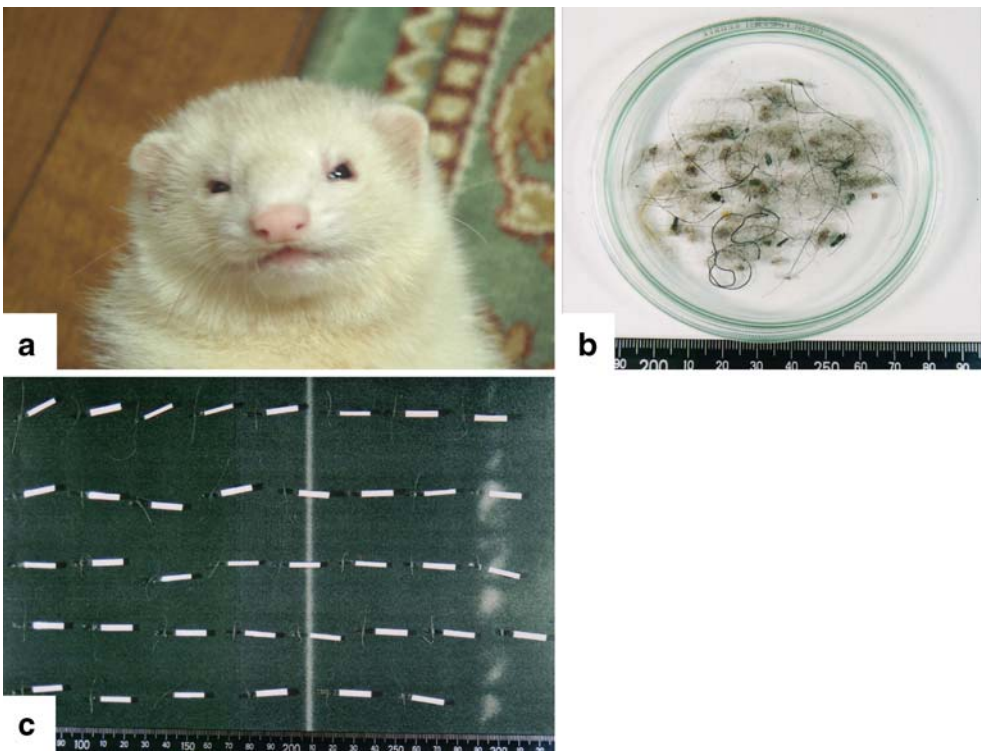

Fig. 1 Casework samples. **a** Scene in the video of animal abuse. **b** Bulk hair sample from the carpet identified in the background of panel **a**. **c** Thirty-eight hair samples obtained randomly from **b**

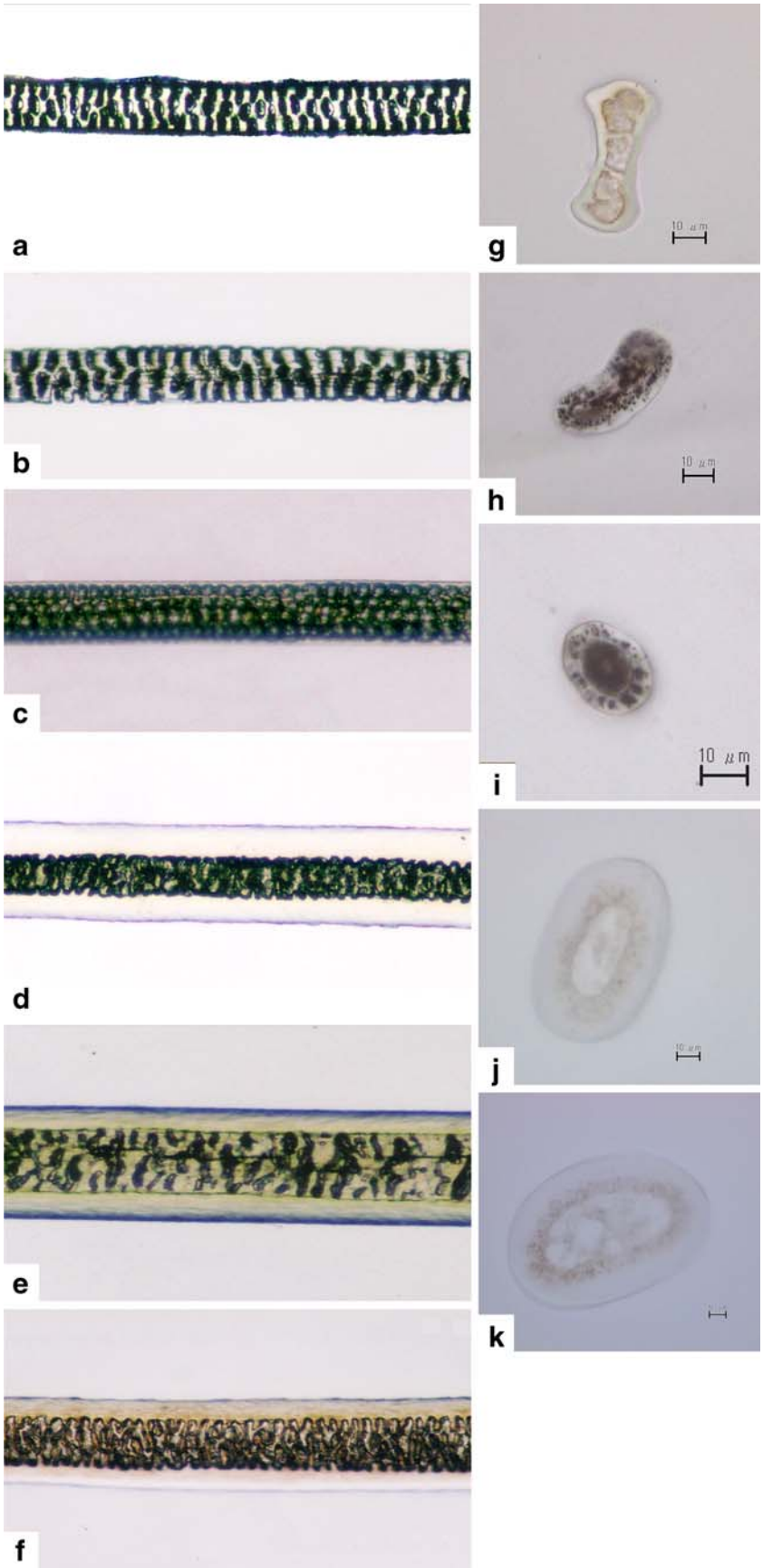

Fig. 2 Photomicrographs of hair shapes in longitudinal section (**a–f**) and medullary patterns in cross-section (**g–k**). Rabbit (**a** and **g**), gerbil (**b**), degu (**c** and **h**), Djungarian hamster (**d** and **i**), ferret (**e** and **j**), and Japanese weasel (**f** and **k**)

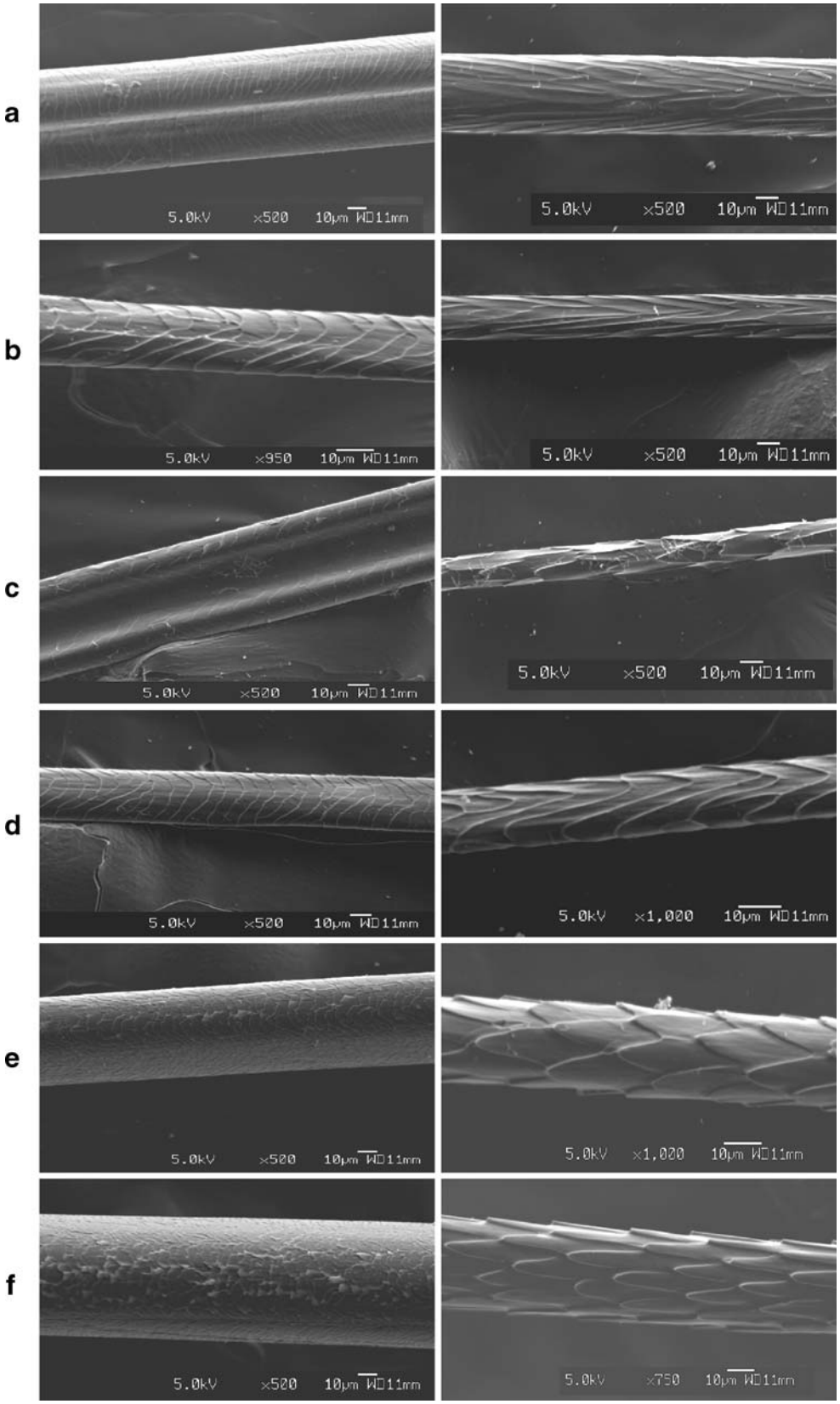

Fig. 3 Scanning electron micrographs of guard hairs from mammalian species. **a** *Left column* cuticle pattern on the hair shaft. *Right column* cuticle pattern on root. Cuticle patterns on the hairshaft and root from the *right* and *left* sides, respectively. **b** Longitudinally cut sections of the hairshaft. Animal species are as follows: rabbit (**b**, **d**, and **f**), gerbil (**b**), degu (**c** and **e**), Djungarian hamster (**d** and **a**), ferret (**e** and **b**), and Japanese weasel (**f** and **c**)

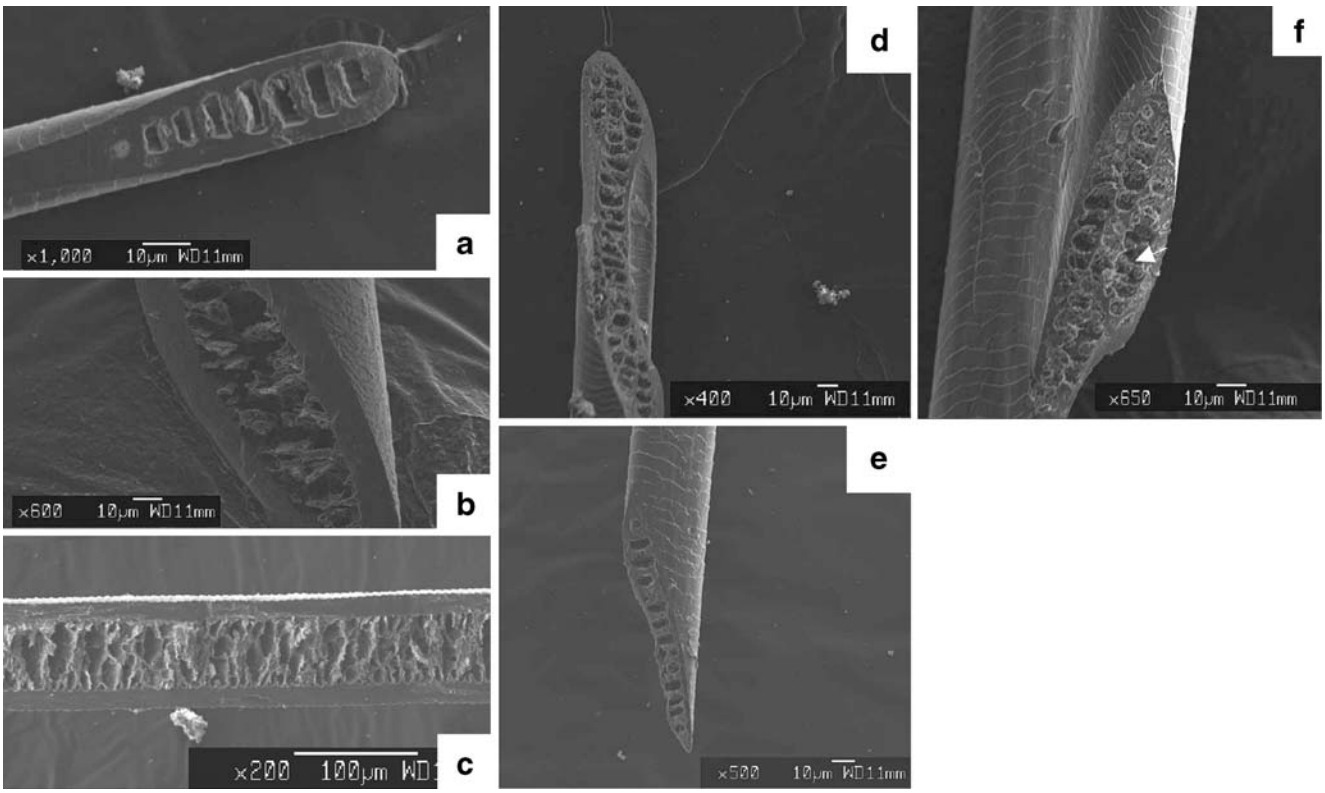

Fig. 3 (continued)

were done in accordance with practical guidelines and manual for the identification of human and animal hairs [3–5].

SNPs in cytochrome b gene analysis

Nine guard hair samples (n=3) were rinsed thoroughly with 0.05% (v/v) Tween-20 in distilled H_2O using a sonicator. The DNA was then extracted from a 1- to 2-cm length of hair shaft using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany) and 0.04 M dithiothreitol was added to each sample to solubilize the hair protein containing keratin. These DNA samples were analyzed for SNPs in the cytochrome *b* gene using a multiplex single-base primer extension reaction [10]. Polymerase chain reaction amplification and electrophoresis for detection of the SNPs were performed in accordance with a method described previously in detail [10]. Sequencing was performed using the primer pair, 5'-TCTTC AATAT TCTTT ATCTG CCT-3' and 5'-TCTTT GATTG TGTAG TAAGG GTG-3' and an ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA). The cycle-sequencing products were separated by capillary electrophoresis on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and analyzed by Sequencing Analysis ver. 5.2 software (Applied Biosystems). A phylogenetic tree based on the partial sequence of the

Table 1 Comparative study of hair parameters in several mammal species

Index		Length	Hair		Medulla	
Species	*n*	cm	Form	Index (%)	Form	Index (%)
Rabbit	5	1.6	Dumb-bell	23.6	Multiserial ladder	94.2
Gerbil	3	0.8	ND	ND	Wide aeriform lattice	95.4
Degu	7	1.1	Kidney	38.8	Wide aeriform lattice	97.7
Hamster	5	1.7	Oval	69.7	Narrow aeriform lattice	67.2
Ferret	9	3.0	Oval	60.7	Narrow aeriform lattice	62.4
Weasel	6	2.8	Oval	57.9	Narrow aeriform lattice	60.8
Sample	15	3.2	Oval	59.3	Narrow aeriform lattice	65.6

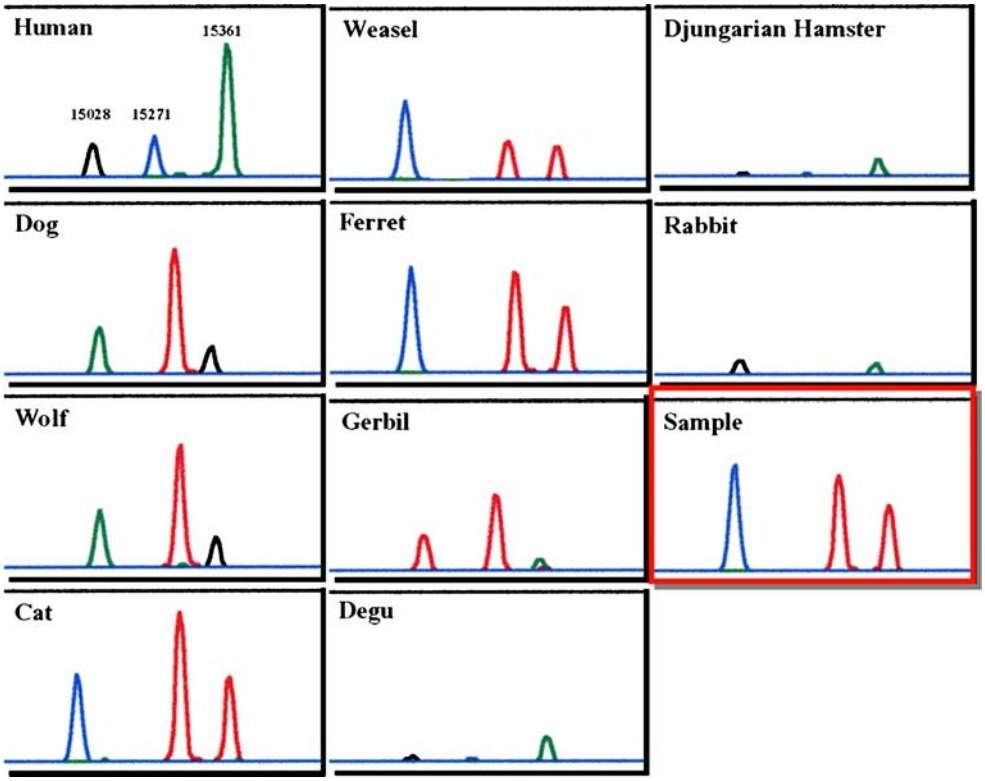

Fig. 4 Electropherograms compiled from multiplex single-base primer extension reactions applied to analysis of the guard hairs of several mammalian species and to the casework sample

cytochrome *b* gene, in this study, was constructed by the neighbor-joining method with 10,000 bootstrap replications on GENETYX ver.6 software (Software Development, Tokyo, Japan).

Results

Analytical data obtained by light microscopy and SEM for several mammalian species are shown in Figs. 2 and 3. Examination of the cuticle scale pattern of the hairshaft revealed differences between the middle and proximal parts of the shaft in each species. However, these patterns in the middle and proximal parts were similar in the ferret and Japanese weasel but differed from those in the other species (rabbit, gerbil, degu, and Djungarian hamster). Equally, medulla formation in the hairshaft of the ferret was similar to that in the Japanese weasel but differed from those in the other species. The medulla indices in the ferret and Japanese weasel were lower than those in the rabbit, gerbil, and degu (Table 1). Therefore, morphological analysis of cuticle scale pattern and medulla formation was able to discriminate ferret hairs from the other hairs in the bulk samples, which were found to belong to the rabbit, gerbil, degu, and Djungarian hamster.

Although three sites gave signals for SNPs in the cytochrome *b* gene that could be used to discriminate completely among human, dog, and gerbil, signals for two groups were demonstrated at the same nucleotide sites in positions 15028, 15271, and 15361 (Fig. 4). One group consisted of the dog and wolf, and the other consisted of the cat, ferret, and Japanese weasel. Thus, the ferret and Japanese weasel hair samples were both in the latter SNP group. Unfortunately, faint peaks were also obtained from the degu, Djungarian hamster, and rabbit.

The SNPs successfully detected in the five species mentioned above were sequenced in the region covering the three sites in the cytochrome *b* gene. A phylogenetic tree was constructed by the neighbor-joining method for a

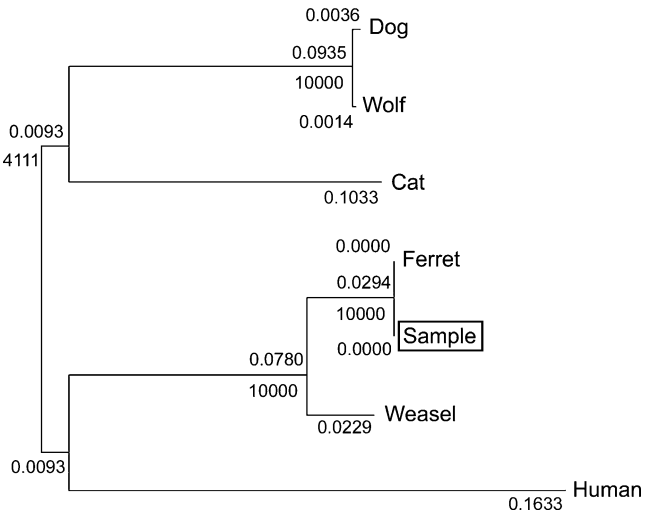

Fig. 5 Phylogenetic tree constructed by the neighbor-joining method for the sequence fragment from the cytochrome *b* gene

partial sequence (450 bp) on the cytochrome *b* gene (Fig. 5). The partial sequence in the hairs that had been discriminated morphologically from among the bulk samples as ferret hairs was 100% identical to the sequence in the hair samples from the pet shop ferrets, and there was a 5% difference in this sequence from that of the Japanese weasel (Fig. 6 and Table 2).

Discussion

These results demonstrated the feasibility of hair analysis by numerical morphology and by SNP typing of the cytochrome *b* gene for mammalian species identification. Although traditional methods, including light microscope and SEM observations enable the identification of cuticle

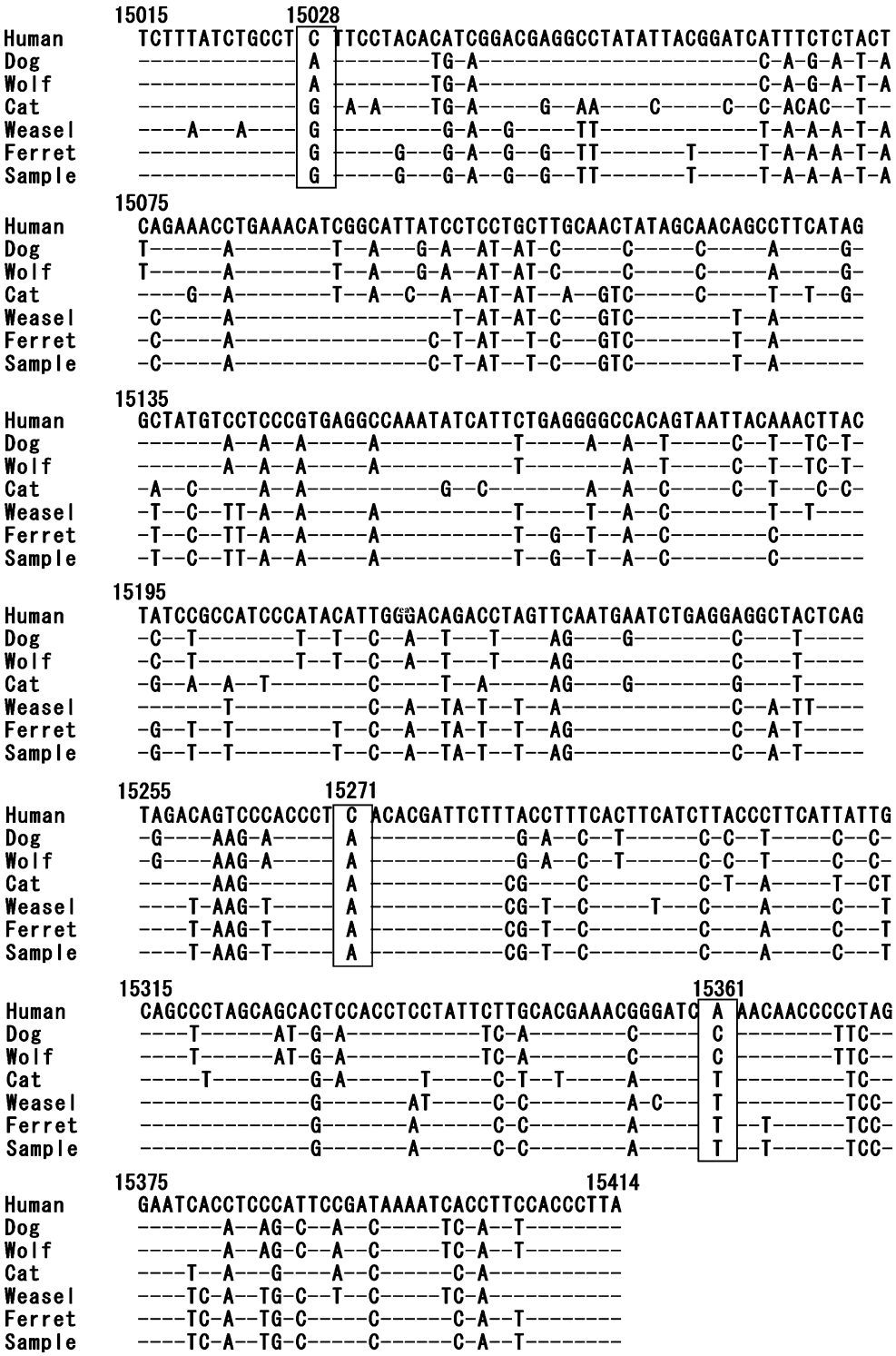

Fig. 6 The partial sequence of the cytochrome *b* gene of human, dog, wolf, cat, weasel, ferret, and the casework hair sample. Numbering is based on the human mtDNA sequence. *Empty square* target nucleotide in the present of animal identification using a multiplex single-base extension reaction. Nucleotide of L15028 and L15361 were detected by forward primers, nucleotide of L15271 was detected by reverse primer

Table 2 Homology (%) of the sequence fragment in the cytochrome *b* gene

	Dog	Wolf	Cat	Weasel	Ferret	Sample
Human	77.8	78.3	77.5	77.8	77.5	77.5
Dog		99.5	83.0	82.5	82.0	82.0
Wolf			82.5	82.8	82.2	82.2
Cat				82.0	81.5	81.5
Weasel					95.0	95.0
Ferret						100.0

scale shape or medullary patterns, which vary considerably among subspecies and species, these parameters cannot be used conclusively to identify individual species [11]. The morphological parameters of the guard hairs of the ferret and Japanese weasel were similar. Although recent studies have reported on hair follicle structure and morphogenesis, the reasons behind the differences in shape or development of the hair cuticle and medulla, as well as the functions of these tissues in the differentiation of mammalian species, have not yet been characterized [12–15]. Some medullary granules and other cellular debris could be observed in the air vacuoles of mature medullary cells in rabbit guard hairs (arrowhead in Fig. 3b) [5]. However, no cellar debris was observed in the hairs of the other species and it was unclear whether these observations were specific to the rabbit.

In the cytochrome *b* gene analysis, the SNPs could not be used to differentiate some subspecies (i.e., the dog from the wolf) or species (i.e., the Japanese weasel from the ferret; Fig. 4). Moreover, the three nucleotide positions in the cat had the same pattern as in the weasel and ferret, and the SNPs in the degu, Djungarian hamster, and rabbit barely had detectable patterns. The unique SNP detection method that uses the multiplex single-base primer extension reaction was developed for the convenient identification of only three species: human, dog, and cat in general forensics. Dogs and cats are universal pets and are often seen in forensic casework [10]. However, this method has some disadvantages in that it loses specificity in the cat and cannot be applied to other species such as mammalian wildlife because the primer sets were designed only for identifying humans, dogs, and cats. [10]. Also, the faint SNP peaks that appeared in the degu, Djungarian hamster, and rabbit had the same nucleotide color patterns as in humans (Fig. 4). This finding suggests that human mtDNA is easily transferred to the guard hairs of domestic animals in this case, pets by the human hand and then remains tightly adhering to the inner layer of the cuticle. Therefore, for species differentiation by the extraction of mtDNA from the guard hairs of pets, human mtDNA should first be removed from the hair samples by thorough washing.

Our analysis of sequence data from the cytochrome *b* gene in guard hair samples was able to discriminate individual species and even subspecies (e.g., ferret and Japanese weasel; dog and wolf; Figs. 5 and 6, Table 2). The ferret was domesticated from the European polecat (*Mustela putorius*) and is widely raised as a pet. The 5% difference between the ferret and Japanese weasel might have been caused by hybridization during domestication from the wildlife species.

Construction of a database by using a combination of morphological analysis and cytochrome *b* gene analysis of the hairs of mammalian wildlife could be a useful tool in the future, not only in forensic science, but also in the control of smuggling of endangered species in contravention of the Washington Convention.

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