Studies on Forensic Species Identification by Fluorescent Multiplex PCR

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Studies on Forensic Species Identification by Fluorescent Multiplex PCR

(蛍光マルチプレックス PCR による法科学的な動物種識別に関する研究)

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Chapter 1

General introduction

In forensic science, much attention has been paid to the identification of human individuals (Butler, 2012; Parson et al., 2016). However, the identification of non-human animal species is also important in many areas, for example, criminal investigations (Naue et al., 2012; Savolainen and Lundeberg, 1999; Schulz et al., 2006), wildlife forensics (Iyengar, 2014; Johnson et al., 2014; Linacre and Tobe, 2011), forensic entomology (Amendt et al., 2004, 2011; GilArriortua et al., 2013), and food authentication (Galimberti et al., 2013; Mafra et al., 2008). In particular, the identification of non-human mammalian species is useful in criminal investigations. When non-human biological material (e.g., hair) unrelated to the victim or the surrounding environment is found at a crime scene, information about its origin can narrow down the range of suspects and increase the possibility of identifying the true culprit particularly when human DNA evidence related to the culprit is unavailable.

Several different approaches have been used in forensic science to identify mammalian species. Immunological tests (Anhalt and Yu, 1975; Ouchterlony, 1949) have traditionally been adopted to discriminate one mammalian species from another. These tests target particular species-specific proteins and detect them via antigen–antibody reactions, without the need for expensive equipment. However, species which are phylogenetically closely related cannot be discriminated immunologically. Immunological reactions also depend on the state and type of samples. For example, antibodies can barely recognize protein antigens if they have been subjected to heating.

Histological methods can distinguish human from non-human bones, even in the case of partial

fragments that have lost their gross morphology (Inoi et al., 1994; Mulhern and Ubelaker, 2001). Some studies have also reported the possibility of distinguishing species among a group of a limited number of mammalian species by comparing the shapes of osteons (Gudea and Ștefan, 2013; Hidaka et al., 1998). However, it is difficult to identify a large number of species from histological structures alone. In addition to the limitation of their discrimination power, these methods require expensive equipment (e.g., for X-ray microradiography) as well as highly skilled personnel to prepare the samples (Inoi et al., 1994; Yoshino et al., 1994).

Due to recent advances in molecular biology, DNA analysis is becoming increasingly appropriate for species identification compared with the conventional methods described above. Polymerase chain reaction (PCR)-based DNA analysis requires only a small amount of sample; therefore it can be used to identify species from samples to which immunological or histological methods are not applicable, for example, partial bone fragments, hairs, and old bloodstains.

However, DNA-based species identification methods also have limitations when they are applied to mixtures of DNA from multiple species, because interpretation of the results becomes difficult. This problem can occur when analyzing meat products, wildlife feces containing ingested material from other species (Deagle et al., 2005; Wasser et al., 1997), and during forensic casework where animal samples are contaminated with human DNA (Tobe and Linacre, 2008b). Another problem for DNA analysis is DNA degradation, which is common in forensic samples and prevents the successful amplification of long DNA fragments.

To overcome these problems, several methods for mammalian species identification have been developed over the last two decades (Arulandhu et al., 2017; Pereira et al., 2010; Tillmar et al., 2013; Tobe and Linacre, 2008a). However, none of them has yet been standardized in the forensic field. The use of standardized methods improves the reliability of test results obtained from forensic laboratories and enhances their acceptance in court. Thus, there is a strong need for the

standardization of methods used to identify mammalian species for forensic purposes.

In this study, the reliability and versatility of recent mtDNA-based techniques were evaluated to reveal obstacles to the establishment of standard analytical methods, with a particular focus on DNA mixtures and DNA degradation. Furthermore, a multiplex PCR assay was developed to simultaneously identify numerous species even from a DNA mixture of multiple species. The developed assay was validated for forensic species identification.

Chapter 2

Current issues for mammalian species identification in forensic science

2.1. Introduction

For species identification, the analysis of mitochondrial DNA (mtDNA) has become a powerful tool in forensic contexts (Branicki et al., 2003; Bravi et al., 2004; Dawnay et al., 2007; Imaizumi et al., 2007; Kitano et al., 2007; Nakamura et al., 2009; Parson et al., 2000; Pereira et al., 2010; Ramón-Laca et al., 2013; Tobe and Linacre, 2008a) because of its high copy number per cell compared with the nuclear genome (Holland and Parsons, 1999; Robin and Wong, 1988; Tobe and Linacre, 2008b). Mammalian mtDNA is a circular genome, typically 16 kbp in length. As shown in **Fig. 1**, the typical mitochondrial genome includes 13 genes which encode protein subunits of enzymes involved in oxidative phosphorylation and ATP synthesis, 12S and 16S ribosomal RNAs (rRNAs), and 22 transfer RNAs (tRNAs). This genome also has a non-coding control region, known as a D-loop, between tRNA[Pro] and tRNA[Phe]. Compared with sequences in the coding regions, sequences in the non-coding control region are highly variable among individuals (Anderson et al., 1981; Andrews et al., 1999; Boore, 1999). Therefore, forensic scientists use two hypervariable regions (HV1 and HV2) in the D-loop to identify human individuals, particularly when common individual identification tests based on nuclear DNA cannot be applied, as is the case with highly degraded samples (Ginther et al., 1992; Holland et al., 1993; Wilson et al., 1995). In contrast to the identification of human individuals, variable regions of mtDNA, such as the cytochrome *b* (cyt *b*)

gene, the cytochrome *c* oxidase I (COI) gene, the 12S and 16S rRNA genes, and the D-loop region, have all been used for species identification (Dawnay et al., 2007; Kitano et al., 2007; Nakamura et al., 2009; Parson et al., 2000). this chapter reviews the use of mtDNA analysis for the identification of mammalian species in the field of forensic science.

2.2. Sequencing analysis for species identification

Sequencing analysis of mtDNA is one of the most commonly used methods for species identification. Sequence data obtained from unidentified samples can be compared with those of known species using a BLAST (Basic Local Alignment Search Tool) search of GenBank (http://www.ncbi.nlm.nih.gov/genbank/) or a similar, publicly available genetic sequence database. To enable a wide range of species, including closely related ones, to be distinguished from one another, the target regions sequenced for species identification must have wide interspecies variation and minimal intraspecies variation. If the flanking regions on both sides of target loci are highly conserved, the loci can be amplified in various species by universal primer sets designed to anneal to the conserved regions. In their study on the dynamics of mtDNA evolution in animals, Kocher et al. (1989) developed universal primer sets for the cyt *b* gene, the 12S rRNA gene, and the D-loop region. They reported that partial cyt *b* sequences obtained using cyt *b* universal primers showed high interspecies variation in vertebrates. Since then, the cyt *b* gene has been widely studied for forensic applications (Branicki et al., 2003; Ewart et al., 2018; Hsieh et al., 2001; Johns and Avise, 1998; Parson et al., 2000) as well as for molecular taxonomy and phylogenetics, although the 12S and 16S rRNA genes have also been studied (Imaizumi et al., 2007; Karlsson and Holmlund, 2007; Kitano et al., 2007). To improve the reliability of sequence-based species identification in mammals, Naidu et

al. (2012) made an attempt to obtain the complete sequence of the cyt *b* gene (more than 1,000 bp) in

mammals, using a single universal primer pair. The availability of complete cyt *b* sequence data for a

large number of mammalian species can lead to a high accuracy of species identification based on

BLAST matching. However, PCR amplification of long DNA fragments, such as the complete cyt *b*

gene, often ends in failure in forensic cases where samples are highly degraded. The demand for a

method capable of analyzing highly degraded DNA resulted in the development of a new, universal

cyt *b* primer pair to amplify a short fragment (194 bp) that covers a highly variable region of the cyt

b gene (Lopez-Oceja et al., 2016).

Although most of the sequencing analysis for species identification rely on GenBank, the

database contains data which are not quality controlled for forensic standards. For accurate species

identification in forensic contexts, DNA sequences from casework samples need to be compared to

authenticated reference DNA sequences attached to voucher specimens. Recently, a method for

analyzing the COI gene, known as DNA barcoding, has been promoted as a simple, universal

procedure for identifying species across the entire animal kingdom (Frézal and Leblois, 2008; Hebert

et al., 2003; Ratnasingham and Hebert, 2007). The aim of the Barcode of Life Data System (BOLD)

is to provide a reference sequence database for the identification of animal species, using an

approximately 650-bp section of the COI gene sequence produced by standard protocols. The

International Barcode of Life project (iBOL) is now extending the geographic and taxonomic

coverage of DNA barcode reference libraries. As a result, the use of the COI gene has become

popular in wildlife forensic science, where the identification of a broad range of species is required

for wildlife crime investigations (Dawnay et al., 2007; Wilson-Wilde et al., 2010).

Although the sequencing-based methods using universal primers described above are powerful

techniques that can be used to identify species in single-source samples, there are many cases where

materials from multiple-species sources constitute a single sample, with a mixture of DNA. In such

cases, DNA from various species are co-amplified using universal primers, with resultant PCR products from different species often being the same size. The mixed DNA profiles from subsequent sequencing are difficult to interpret and lead to database matching of low reliability (Dawnay et al., 2007).

2.3. Species-specific fragment length polymorphisms

Mitochondrial DNA-based techniques that require no sequence information have also been used for species identification. PCR amplification using universal primers and subsequent restriction fragment length polymorphism (PCR-RFLP) analysis can distinguish several species by differences in the digested patterns of the PCR products (Bravi et al., 2004; Chen et al., 2012; Girish et al., 2005; Haider et al., 2012; Murray et al., 1995; Murugaiah et al., 2009; Pfeiffer et al., 2004). This method is simple, quick, and cost effective. However, target species are limited by the availability of restriction enzyme recognition sites in amplified fragments. Another disadvantage of PCR-RFLP is, as with sequencing analysis, the inability to analyze a mixture of DNA sources from multiple species. The outcome of PCR-RFLP on a DNA mixture is an overlap of the digested patterns derived from each source. It is difficult to determine the contributors to a DNA mixture because the possibility that the pattern observed is formed by a combination of unexpected or unknown species cannot be eliminated.

Fragment length analysis using species-specific primers enables the identification of more species than PCR-RFLP (Dalmasso et al., 2004; Matsunaga et al., 1999; Ramón-Laca et al., 2013; Tobe and Linacre, 2008a). These species-specific primers are designed to bind exclusively to target species sequences due to interspecies sequence variation, yielding PCR products of different sizes.

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An individual species can be identified based on the species-specific product length through gel or capillary electrophoresis without further sequencing analysis. In addition, multiplex PCR containing several primers together in one reaction mixture enables simultaneous identification of target species and can save time, reagents, and template DNA. This greatly improves the success rate of species identification when using challenging samples from which only a small amount of DNA can be extracted. Another big advantage of such multiplex PCR assays is their applicability to a DNA mixture. Each species in a DNA mixture can be easily identified by species-specific PCR products in a multiplex PCR assay, in contrast to the sequencing-based methods described above (Dalmasso et al., 2004; Matsunaga et al., 1999).

The use of fluorescent-labeled primers and capillary electrophoresis can dramatically increase the number of simultaneously identifiable species. The development of a combination of fluorescent-labeled universal forward primers and non-labeled species-specific reverse primers enabled the simultaneous identification of up to 19 mammalian species from mixed DNA samples (Ramón-Laca et al., 2013; Tobe and Linacre, 2008a). The identification capability depends on numerous species-specific primers that target a partial region of the cyt *b* gene. Hence, the conditions for successful amplification when using multiplex PCR assays can be complicated, and it may be difficult to add new primer sets to increase the number of simultaneously identifiable species.

Another approach is to design universal primer sets to amplify a region which contains interspecies length polymorphisms as a result of nucleotide insertions or deletions. For example, Nakamura et al. (2009) reported a multiplex method based on size variations in one of the HV regions of the D-loop. Using three universal primer sets, the method can identify 18 mammal, 4 bird, and 19 fish species. The size of the PCR products derived from target species ranges from approximately 350 to 900 bp; this is because some species have long insertion sequences in this region. Therefore, if this method were applied to highly degraded samples, the amplification of such

long fragments might be difficult (Deagle et al., 2006).

Pereira et al. (2010) reported that a combination of seven universal primer sets for highly variable regions spanning from the 12S to the 16S rRNA gene enable species discrimination among broad taxonomic groups, including eukaryotes, prokaryotes, and viruses. A SPInDel (Species identification by Insertions/Deletions) profiling kit provided by them was experimentally validated for the identification of ten mammalian species. Their primer sets were designed to amplify fragments less than 500-bp long. A unique profile for each species can be defined by the pattern of fragments amplified by multiplex PCR using the primer sets. SPInDel appears to be a reliable method for simultaneous identification from single-source samples or processed food products made from a mixture of a small number of predictable species.

Table 1 summarizes the mammalian species simultaneously identified by the fluorescent multiplex PCR assays described above. All of the assays can identify common domestic mammals such as livestock (cattle, goat, horse, pig, and sheep) and companion animals (cat, dog, and European rabbit). However, most of the methods cannot identify a broad range of wild animal species because they were specialized for the species found in the geographical areas of their developers. The SPInDel profiling kit (Pereira et al., 2010) is the only assay that does not limit its target species to a specific geographical area. It was designed to discriminate among a broad spectrum of eukaryotic species, although it has not been experimentally validated for a broad range of wild or domesticated mammals, with the exception of ten common species.

2.4. DNA metabarcoding

Recently, massively parallel sequencing (MPS) technologies, also called next-generation

sequencing (NGS) technologies, have brought revolutionary changes to the field of DNA analysis. MPS technologies enable millions of DNA sequences to be read in parallel. DNA metabarcoding, which couples the principles of DNA barcoding with MPS, enables the barcoding of each PCR fragment combined with highly sensitive, high-throughput analysis of DNA samples, including mixtures originating from multiple species (Arulandhu et al., 2017; Bertolini et al., 2015; Coghlan et al., 2012; Ribani et al., 2018; Ripp et al., 2014; Tillmar et al., 2013). DNA metabarcoding uses universal PCR primers to massively amplify taxonomically informative genetic regions commonly used in conventional DNA barcoding. DNA metabarcoding offers significant advantages over other methods for species identification. In theory, it can identify minor contributors to levels as low as 1% of a mixed DNA sample and determine the relative contribution of each species included in the sample (Arulandhu et al., 2017; Ripp et al., 2014; Tillmar et al., 2013). The application of PCR primers designed to amplify shorter DNA barcode regions to DNA metabarcoding also enables the successful analysis of highly degraded DNA (Arulandhu et al., 2017; Bertolini et al., 2015; Tillmar et al., 2013). Therefore, DNA metabarcoding is a powerful tool for overcoming some of the problems encountered during the analysis of DNA mixtures and/or degraded DNA.

Despite these advantages, there are some issues when performing DNA metabarcoding in the forensic field. At present, MPS platforms have not been adopted in many places, such as local police laboratories, because such laboratories routinely use a conventional capillary sequencer to conduct short tandem repeat (STR) analysis for human identification. The cost of introducing DNA metabarcoding to these laboratories is prohibitive. MPS platforms and their associated running costs remain expensive compared with those of other techniques used for species identification. Although the cost per sample of MPS continues to decline in large-scale analysis (Meier et al., 2016), there are few cases where local forensic laboratories are required to analyze numerous samples simultaneously for species identification. In addition, a local police laboratory is usually required to

analyze forensic evidence by itself and is prohibited from sending samples outside so as to maintain their evidential value. In such laboratories, DNA metabarcoding is not cost-effective. Another issue is the volume of data generated by MPS. The processing and interpretation of massive volumes of MPS data is much more complicated and therefore requires more time than conventional DNA analyses (Staats et al., 2016). It may be a rare occurrence that a local forensic laboratory needs to analyze a complex DNA mixture that requires DNA metabarcoding. The reasons described above suggest that DNA metabarcoding is not suitable for local police laboratories and is unlikely to be widely used for species identification in forensic investigations.

2.5. Other techniques

2.5.1. *Multiplex real-time PCR*

In the area of food control, multiplex real-time PCR analysis for species identification has been developed to identify the species of meat present in processed food products (Köppel et al., 2013; Şakalar and Abasiyanik, 2012; Thanakiatkrai and Kitpipit, 2017; Wadle et al., 2016; You et al., 2014). It has also been applied to forensic species identification (Ishida et al., 2018; Kanthaswamy et al., 2012; Kitpipit et al., 2016; Naue et al., 2014). Real-time PCR detects the amplified signal using fluorogenic probes or intercalating dyes. For multiplex reactions, the former requires species-specific fluorescent probes of different wavelengths and multiple detection channels provided by instruments. The latter only need a single channel for monitoring the intensity of the intercalating dye, while a melting curve analysis is necessary to distinguish different amplicons derived from non-fluorescent species-specific primer sets. Some multiplex real-time assays that

amplify mtDNA genes have been developed (**Table 2**). However, the number of species that can be simultaneously identified in a single reaction is limited, because it depends on the number of detection channels in the fluorogenic probe assays, while the development of multiple species-specific primer sets with different melting temperatures is difficult in the intercalating dye assay.

Köppel et al. (2013) reported a pentaplex real-time PCR system with five sets of species-specific primers and TaqMan probes. The assay has been further developed, resulting in mediator probe PCR (Faltin et al., 2012; Wadle et al., 2016). **Fig. 2A** illustrates the mediator probe PCR principle. A mediator probe consists of a tag sequence (mediator) and a target-specific sequence (probe), with no fluorescent label. During each PCR cycle, the annealed mediator probe is cleaved by the polymerase's 5'-nuclease activity, the mediator tag is released, anneals to a fluorogenic universal reporter, and is then quenched by a fluorophore–quencher interaction because of its hairpin-shaped secondary structure. Mediator elongation by the polymerase leads to dequenching of the fluorophore, and thus real-time PCR amplification can be detected. The independent synthesis and evaluation of fluorogenic probes for each assay becomes unnecessary because the optimized fluorogenic universal reporters can be used in different mediator assays. Therefore, mediator probe PCR is more cost-effective in comparison with other fluorogenic probe assays. Due to the recent increase in large-scale assays, mediator probe PCR may replace current assays that use sequence-specific fluorogenic probes.

2.5.2. Post-PCR analysis

The choice of post-PCR analysis method is also important because it directly affects the cost, time, and sensitivity of tests used to identify species. Gel electrophoresis has been widely used to analyze

PCR products because this method does not require expensive reagents or equipment. Capillary electrophoresis is more expensive than gel electrophoresis but is useful for highly precise separation of amplified fragments that cannot be separated by gel electrophoresis. Single-tag hybridization (STH) chromatographic printed array strip (PAS), a novel method, has some advantages over both gel and capillary electrophoresis (Monden et al., 2014; Tian et al., 2014). In this method, biotin-labeled PCR products containing tag-spacer sequences can be detected by hybridization with anti-tags printed on the strip membrane (**Fig. 2B**). STH chromatographic PAS is a rapid and cost-effective technique. The chromatography developing reaction takes just 15 minutes and does not need any preparation or specialized instrumentation. Ito et al. (2016) applied this method for the identification of ten mammals following species-specific multiplex PCR. The disadvantage of this method is that the number of simultaneously identifiable species is limited by the space available on the PAS membrane when PCR products are analyzed on the same strip.

2.6. Summary

This chapter described the advantages and limitations of current techniques used for mammalian species identification in forensic science, with a view to facilitating their standardization. The advances in mtDNA-based techniques have made a considerable contribution to forensic science. Although the number of studies that have investigated species identification has increased over the last two decades, the problem remains of how to reliably and cost-effectively identify species from degraded samples containing a mixture of DNA from multiple species.

DNA metabarcoding using MPS technologies is one option to overcome the problems arising from a DNA mixture. However, this is not suitable for the type of small-scale analysis routinely

performed by local forensic laboratories because MPS technologies remain costly and are more time-consuming than other techniques used for species identification. Therefore, cost-effective, rapid, and reliable methods that can simultaneously identify all mammalian species from challenging samples are desirable. In contrast to DNA metabarcoding, fluorescent multiplex PCR analysis enables cost-effective identification of DNA from such samples, although the number of simultaneously identifiable species remains limited. The further development of multiplex PCR analysis, sufficient for forensic laboratories to identify a wide range of mammalian species, is therefore also highly desirable. As seen with the increase in newly developed multiplex assays (Ewart et al., 2018; Ishida et al., 2018; Kitpipit, Thanakiatkrai, et al., 2016; Lee et al., 2018; Prusakova et al., 2018; Thanakiatkrai et al., 2019), it is a promising platform and will therefore play a key role in the standardization of mammalian species identification methods.

Table 1 List of mammalian species identified by species-specific fragment length polymorphisms using fluorescent multiplex PCR assays

Common names	Scientific names	References			
		Tobe and Linacre (2008a)	Nakamura et al. (2009)	Pereira et al. (2010)	Ramón-Laca et al. (2013)
Focused geographical area		Europe	Japan	−	New Zealand
Cat	*Felis catus*	+	+	+	+
Cattle	*Bos taurus*	+	+	+	+
Dog	*Canis lupus familiaris*	+	+	+	+
Goat	*Capra hircus*	+	+	+	+
Horse	*Equus caballus*	+	+	+	+
House mouse	*Mus musculus*	+	+	+	+
Human	*Homo sapiens*	+	+	+	N/A
Brown rat	*Rattus norvegicus*	+	+	N/A	+
Pig	*Sus scrofa*	+	+	+	+
European rabbit	*Oryctolagus cuniculus*	+	+	+	+
Sheep	*Ovis aries*	+	+	+	+
Donkey	*Equus assinus*	+	N/A	N/A	N/A
European Badger	*Meles meles*	+	N/A	N/A	N/A
Red fox	*Vulpes vulpes*	+	+	N/A	N/A
Guinea pig	*Cavia porcellus*	+	N/A	N/A	N/A
Harvest mouse	*Micromys minutus*	+	N/A	N/A	N/A
Hedgehog	*Erinaceus europaeus*	+	N/A	N/A	+
Red deer	*Cervius elaphus*	+	N/A	N/A	+
Antarctic minke whale	*Balaenoptera bonaerensis*	N/A	+	N/A	N/A
Asian black bear	*Ursus thibetanus*	N/A	+	N/A	N/A
Ferret	*Mustela putorius furo*	N/A	+	N/A	+
Japanese macaque	*Macaca fuscata*	N/A	+	N/A	N/A
Japanese weasel	*Mustela itatsi*	N/A	+	N/A	N/A
Raccoon dog	*Nyctereutes procyonoides*	N/A	+	N/A	N/A
Black rat	*Rattus rattus*	N/A	N/A	N/A	+
Brushtail possum	*Trichosurus vulpecula*	N/A	N/A	N/A	+
Least weasel	*Mustela nivalis*	N/A	N/A	N/A	+
Pacific rat	*Rattus exulans*	N/A	N/A	N/A	+
Stoat	*Mustela erminea*	N/A	N/A	N/A	+
Tammar wallaby	*Macropus eugenii*	N/A	N/A	N/A	+
Total number of species		18	18	10	19

+, Identified; N/A, not applicable; −, not defined.

Table 2 Multiplex real-time PCR for detection of mitochondrial DNA

| Principle of the assay (Fluorescent dye)	Identified species	Target genes	Reference
Multi-fluorogenic detection			
Mediator probe PCR (FAM, Rhodamin-6G, ROX, Cy5, BMN6)	Chicken, Duck, Goose, Pig, Turkey	16S rRNA, cyt *b*	Wadle et al. (2016)
TaqMan PCR (FAM, JOE, ROX, Cy5, DY681)	Chicken, Duck, Goose, Pig, Turkey	16S rRNA, cyt *b*	Köppel et al. (2013)
Single-fluorogenic detection			
Intercalating and melting curve analysis (SYBR Green)	(Reaction 1) Cat, Cattle, Chicken, Dog, Goat, Horse, Pig, European rabbit, Sheep (Reaction 2) Bear, Deer, Japanese monkey, Raccoon dog	ATP8, COIII, ND5, cyt *b*	Ishida et al. (2018)
Intercalating and melting curve analysis (EvaGreen)	Cattle, Chicken, Duck, Horse, Pig, Ostrich	16S rRNA, COI, cyt *b*	Thanakiatkrai and Kitpipit (2017)
Intercalating and melting curve analysis (SYBR Green)	Cattle, Chicken Deer, Donkey, Goat, Horse, Pig	12S rRNA, 16S rRNA, cyt *b*	You et al. (2014)
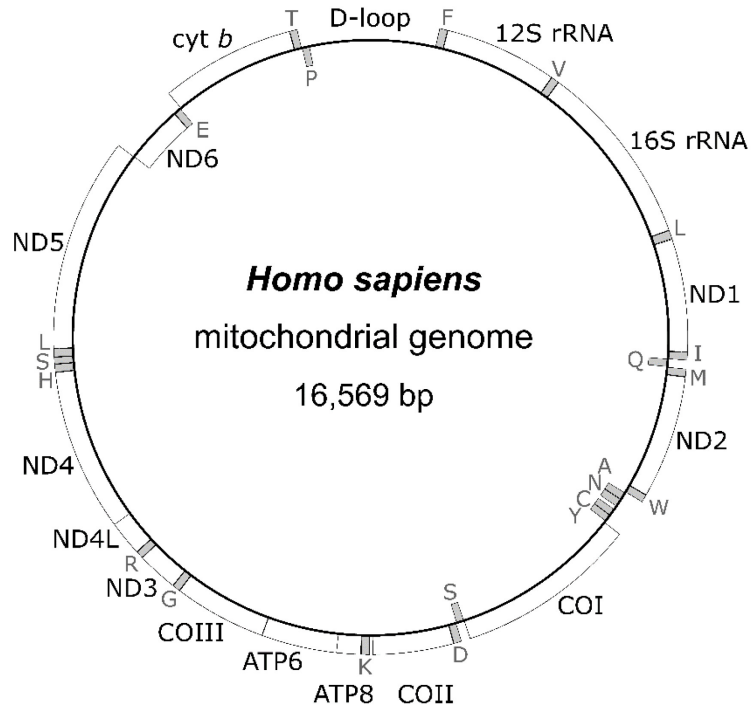
Fig. 1 Schematic representation of the human mitochondrial genome based on the revised Cambridge Reference Sequence (rCRS, NC_012920) (Andrews et al., 1999). Genes transcribed from the heavy-strand and the light-strand are shown outside and inside of the circle, respectively. Genes encoded are 12S and 16S ribosomal RNAs, subunits 1-6 of NADH dehydrogenase (ND1-6), subunits I-III of cytochrome *c* oxidase (COI-III), ATP synthase (ATP6, 8), cytochrome *b* (cyt *b*), and 22 transfer RNAs (indicated by a letter). The non-coding control region is shown as the D-loop.
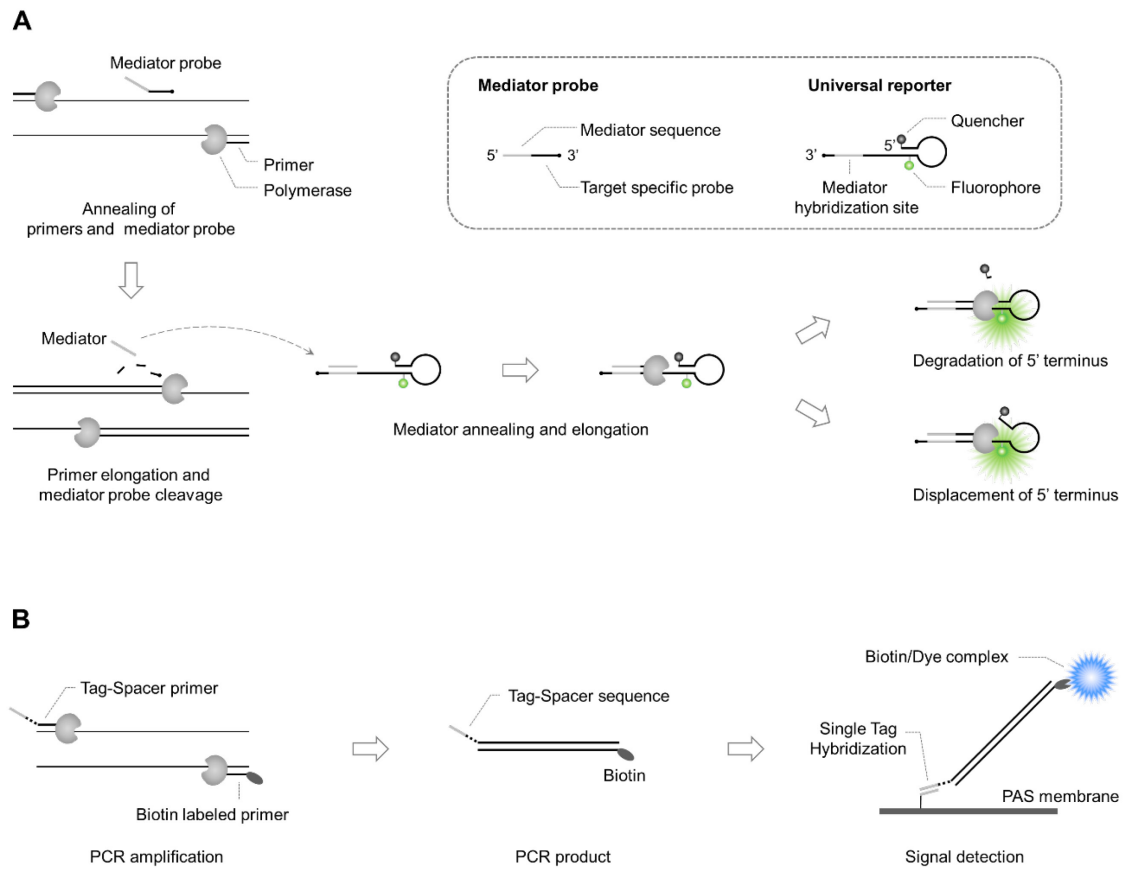
Fig. 2 Principles of signal detection in emerging techniques for real-time and post-PCR analysis. **(A)** Mediator probe PCR based on Faltin et al. (2012). Following denaturing of the target DNA in the first step of the PCR cycle, a mediator probe and primers anneal to the target sequences. A non-hybridized mediator sequence is cleaved by primer elongation and released. Annealing of the mediator to a universal reporter and subsequent mediator elongation results in fluorescence emission due to release of a quencher by sequence degradation of the 5' terminus or unfolding of the hairpin structure. **(B)** Single-tag hybridization (STH) chromatographic printed array strip (PAS) based on Monden et al. (2014). The target sequence is amplified using a primer set with a tag-spacer sequence or a biotin label. The tag sequence of PCR products hybridizes to the complementary oligonucleotides printed on the PAS membrane.

Chapter 3

Development and validation of simultaneous identification of 26 mammalian and poultry species by a multiplex assay

3.1. Introduction

Non-human biological samples are frequently encountered in routine forensic investigations. Forensic scientists are required to identify mammalian species in many forensic cases; for example, traffic accidents involving animals (Schneider et al., 1999), animal cruelty (Lorenzini, 2005; Sato et al., 2010; Stern and Lamm, 2011; Tillmar et al., 2013), livestock robbery (Bravi et al., 2004; Nakamura et al., 2009), animal attacks (Nakamura et al., 2009; Naue et al., 2012; Tillmar et al., 2013), murder cases (Savolainen and Lundeberg, 1999) and postmortem investigations (Schulz et al., 2006). In addition, species identification has become important in wildlife forensics (Alacs et al., 2010; Ewart et al., 2018; Johnson et al., 2014; Ogden and Linacre, 2015; Ouso et al., 2020; Summerell et al., 2019), conservation of endangered animals (Arulandhu et al., 2017; Conte et al., 2019; Hsieh et al., 2001; Iyengar, 2014), and the detection of food fraud (Dobrovolny et al., 2019; Galimberti et al., 2013; Mafra et al., 2008).

In forensic investigations, non-human biological samples recovered from crime scenes are often contaminated with human DNA (Tobe and Linacre, 2008b). In other cases, samples may contain DNA from more than one non-human species. Typical DNA mixture samples include feces containing materials from ingested species, mixed meat or meat products, and saliva collected from a bite mark on a victim's body. Species identification from these mixtures is a challenge for forensic

scientists.   As described in Chapter 2, fluorescent multiplex PCR involving capillary electrophoresis or real-time PCR allows for cost-effective species identification from such mixed samples. The target species of these assays, however, are limited by their primer sets (Köppel et al., 2020; Nakamura et al., 2009; Ramón-Laca et al., 2013; Tobe and Linacre, 2008a). Therefore, the usefulness of the assays critically depends on the number of species they can analyze simultaneously because forensic laboratories often encounter cases with little information on the source species of the recovered DNA samples. Furthermore, negative results from the assays with a large number of identifiable species are also useful to eliminate the necessity of further investigation. Therefore, there is still a need to develop a more cost-effective assay for simultaneous identification of a wide range of mammalian species.

This chapter describes a newly developed multiplex PCR assay that can simultaneously identify 22 mammalian and four poultry species, even from a biological sample containing DNA from multiple species. The target species are composed of common domestic animals and wild animals forensic laboratories in Japan often encounter. Partial regions of the D-loop and cyt *b* loci were used for the assay. Universal primer sets were designed to amplify the hypervariable region (HV) of the D-loop of most of the target species. The region is equivalent to the HV1 of human mtDNA and shows interspecific size variation, giving size differences in PCR products between species. Additional species-specific cyt *b* primer sets were also designed to identify the species of which the HV regions cannot be amplified by the universal primer sets or are too long to amplify from degraded mtDNA. Each PCR product was analyzed by capillary electrophoresis using an Applied Biosystems 3500xL Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA). The combination of the primer sets of the D-loop and cyt *b* loci enabled an increase of the number of identifiable species while the number of species-specific primer sets remained small. Developmental validation, which is a standard procedure to evaluate conditions and limitations of a new DNA methodology for

forensic analysis, was performed to demonstrate efficacy, reliability and robustness of the assay in accordance with the guidelines of Scientific Working Group on DNA Analysis Methods (SWGDAM, 2016).

3.2. Materials and methods

3.2.1. Primer design

MtDNA sequences of the target species were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). For each species, the NCBI reference sequence was used, if available. The species and the accession numbers used in this study are listed in **Table 3**. The sequences were aligned using MEGA7 software (Kumar et al., 2016) to identify variable and conserved regions of the D-loop and the cyt *b* gene. Two universal primer sets, one targeting mammals and the other targeting poultry, were used for D-loop. Another primer set, which consisted of one universal forward primer and 10 species-specific reverse primers, was used for the cyt *b* in 12 mammals. All the primer sets were designed to amplify fragments of different sizes among species below ~500 bp (**Tables 3, 4**). DL_UniF and CYTB_UniF were modified from Nakamura et al. (2009) and Pääbo et al. (1988), respectively, in order to increase the number of the target species. CYTB_PigR and CYTB_GoatR were modified from Tobe and Linacre (2008a) and Matsunaga et al. (1999), respectively. CYTB_RabbitR was identical with that in Tobe and Linacre (2008a). The remaining primers were newly designed for this study. All forward primers were labeled with 6-FAM, VIC, or NED dyes and purified by HPLC. The fluorescent primers were ordered from Thermo Fisher Scientific and the other primers were ordered from Rikaken Co., Ltd. (Nagoya, Japan).

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3.2.2. Sample collection and DNA extraction

Biological samples (blood, buccal cells, tissues, hair, and nails) were obtained from 28 animal species, including 12 domestic mammals, 12 wild mammals, and four poultry species (**Table 3**). Most of the species are often encountered in forensic investigations in Japan. Total DNA was extracted from each sample using the QIAamp DNA Investigator Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. The following commercial DNA samples were used as human samples: AmpFLSTR Control DNA 9947A (Thermo Fisher Scientific), AmpFLSTR Control DNA 007 (Thermo Fisher Scientific), 2800M Control DNA (Promega, Madison, WI), K562 Genomic DNA (Promega), and human genomic DNA (HeLa) (Takara Bio Inc., Shiga, Japan).

The mtDNA copy number of all the DNA samples tested in this study was quantified using a real-time quantitative PCR (qPCR) analysis described below because the copy number varies depending on the tissue type. A universal primer set (Forward: 5'-TACGACCTCGATGTTGGATCA -3'; Reverse: 5'-AGATAGAAACCGACCTGGATT-3') was newly designed to amplify a conserved region of the 16S rRNA gene of vertebrates (**Table 5**). As a qPCR standard DNA, the sequence of the conserved region corresponding to the human mtDNA position 2980-3104 of the revised Cambridge Reference Sequence (rCRS, NC_012920) (Andrews et al., 1999) was inserted into the plasmid pUCFA and cloned (commercially available from Fasmac Co., Ltd., Kanagawa, Japan). The concentration of the purified plasmid DNA solution was quantified by measuring the absorbance at 260 nm (A260) using Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific). The copy number (copies/µL) of the plasmid DNA in the solution was then calculated by dividing the concentration by the average molecular weight. The solution was serially diluted to the following concentrations and subsequently used for the standard curve analysis: 10^7, 10^6, 10^5, 10^4, 10^3, 10^2,

and 10^1 copies/µL. The qPCR analysis was performed using a SmartCycler II system (Cepheid, Sunnyvale, CA). Each 25 µL reaction contained 12.5 µL of SYBR Premix Ex Taq (Takara Bio Inc.), 0.2 µM of the primers, and 2 µL of sample DNA or standard DNA. The amplification conditions were as follows: 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s. Each standard DNA was quantified in triplicate.

3.2.3. Multiplex PCR

Multiplex PCR was performed using Multiplex PCR Assay Kit Ver. 2 (Takara Bio Inc.) in a 25 µL reaction mixture containing 5000 copies of sample DNA, 12.5 µL of 2x Multiplex PCR Buffer (Mg^{2+}, dNTP plus), 0.125 µL of Multiplex Enzyme Mix, and the primer mix listed in **Table 4**. Amplification was carried out on an iCycler (Bio-Rad Laboratories, Inc., Hercules, CA) using the following conditions: initial denaturation at 94 °C for 1 min followed by 27 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 10 min.

3.2.4. Allelic ladder

To construct an allelic ladder for electrophoretic analysis using the 3500xL Genetic Analyzer, fragments of each target species except for house mouse and rhesus macaque were amplified by singleplex PCR. The fragments were sequenced using unlabeled primers and BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) to confirm target-specific amplification. The amplified fragments by the singleplex PCR were mixed together so that all peak heights detected by the 3500xL Genetic Analyzer were balanced. The mix was subsequently purified using Performa DTR Gel Filtration Cartridges (Edge Biosystems, Gaithersburg, MD). The constructed allelic ladder

was stored at −20 °C until use.

3.2.5. *Electrophoresis and data analysis*

All PCR products were analyzed on the 3500xL Genetic Analyzer with a 36 cm array and POP-4 polymer (Thermo Fisher Scientific). Spectral calibration was initially performed using DS-36 Matrix Standard (Dye Set J6). 1 µL of the PCR product or the allelic ladder was added to 9.5 µL of Hi-Di Formamide and 0.5 µL of GeneScan 600 LIZ dye Size Standard v2.0, and subsequently denatured at 95 °C for 3 min. The samples and the allelic ladder were injected at 1.2 kV for 24 s and electrophoresed at 13 kV for 1600 s. Electrophoresis results were analyzed using GeneMapper ID-X Software v1.4 with a peak amplitude threshold of 175 RFU and customized panel and bin sets.

3.2.6. *Species specificity, sensitivity, and repeatability*

Species specificity and the intraspecific variability of the assay were assessed by analyzing all samples for each species listed in **Table 3**. Sensitivity of the multiplex assay was evaluated by testing serial dilutions (10,000, 1000, 500, 100, 10 copies of mtDNA) from cattle, human, pig, dog, and chicken samples. Each test was repeated in triplicate. Repeatability of the assay was also evaluated by testing one individual of each species in triplicate. In order to assess the sizing precision of capillary electrophoresis on the 3500xL Genetic Analyzer, one microliter of the allelic ladder was injected onto each of the 24 capillaries and electrophoresed. The mean peak size (bp) and standard deviation within a single run (i.e., $n = 24$) were calculated for each peak, and this was repeated five times.

3.2.7. Mixture studies

To evaluate the ability to identify species from DNA mixture, three sets of DNA mixture (human with cattle, human with dog, and human with chicken) were tested with a total input of 5000 copies of mtDNA and one of the following mixture ratios: 50:1, 20:1, 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10, 1:20, and 1:50. An additional two sets of DNA mixture (brown rat with house mouse and Japanese wild boar with pig) were analyzed in the same manner to evaluate whether two adjacent peaks were detectable by the GeneMapper ID-X software. An experimental mixture sample, which contained 5000 mtDNA copies of each 10 species (cattle, European rabbit, sheep, pig, horse, goat, sika deer, domestic turkey, duck, and chicken), was also tested.

3.2.8. Case studies

To demonstrate the utility of the assay in forensic casework, a total of 31 unknown samples collected from crime or accident scenes were tested. These samples were either bloodstains, a saliva stain, tissues, partial bones, hairs, or feces.

3.2.9. A 1 bp-InDel and phylogenetic relationship of wild boars and domestic pigs

To investigate the relationship between the presence of a 1 bp-insertion/deletion (InDel) in the D-loop region (NC_000845.1:m.137delC) and the phylogeny among European and Asian wild boars and domestic pigs, complete mtDNA sequences of 307 Eurasian wild boars and domestic pigs were downloaded from GenBank. A complete mtDNA sequence of desert warthog (*Phacochoerus africanus*) was also downloaded and used as an outgroup of the phylogenetic analysis. Multiple

sequence alignment and construction of a neighbor-joining phylogenetic tree were carried out using

MAFFT online version 7 (https://mafft.cbrc.jp/alignment/server/large.html) (Katoh et al., 2019). The

generated tree was visualized using iTOL v6.1.1 (https://itol.embl.de/) (Letunic and Bork, 2021).

3.3. Results

3.3.1. *Species specificity*

The target mtDNA regions of 24 mammalian and four poultry species were successfully

amplified in all tested samples using the designed primers. Some differences were observed between

the expected and observed size of the products, most likely because of differences in electrophoretic

mobility. Nevertheless, the PCR products of all species were distinguished from each other through

the capillary electrophoresis, except for Japanese macaque and rhesus macaque (**Fig. 3**). Although

the sizes of the peaks at the cyt *b* locus in the observed electropherograms were the same between

Bactrian camel and alpaca, or pig and Japanese wild boar, each species was identifiable based on the

D-loop results. The source species of each PCR product was confirmed by sequencing and

subsequent NCBI BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Intraspecific length variation of the PCR product was observed in several species. Three types of

peak size at the D-loop locus in the electropherograms were observed in rhesus macaque, and the

shortest peak was the same size as the peak of Japanese macaque (**Fig. 4**). Asiatic black bear had

four types of peak size at the D-loop locus, at most with 3 bp difference, although these peaks were

easily distinguished from those of other species. Some individuals of masked palm civet had two

peaks at the D-loop locus with 1 bp difference. Sequencing analysis indicated that the two peaks

were caused by length heteroplasmy. The longer peak was the same size as Bactrian camel. However, the two species were distinguishable by the presence or absence of the camel peak at the cyt *b* locus in the electropherogram. No intraspecific variation was observed in the remaining species.

A low non-targeted peak was observed at the D-loop locus in the electropherogram only when horse DNA was amplified. Because the size of the peak differed from those of the other species, it did not affect species identification.

3.3.2. *Sensitivity*

The sensitivity study showed successful identification from 10,000 copies down to 100 copies of input mtDNA for all the tested species except for chicken (from 10,000 copies down to 500 copies). No peak was observed from 100 copies of input chicken mtDNA. Based on a calculation using the human control DNA 9947A (0.1 ng/µL), 100 copies of mtDNA is equivalent to approximately 0.15 pg of total DNA.

3.3.3. *Repeatability*

The accuracy of species identification based on the peak size detected by capillary electrophoresis depends on the precision of the peak size measurement as well as species specificity of the peak size. To increase the precision, the assay used a high-density internal size standard, 600 LIZ Size Standard v2.0. The peak size data was collected five times by injections of the constructed allelic ladder (**Fig. 5**) for all 24 capillaries on the 3500xL Genetic Analyzer. The sizing precision of the fragment analysis was assessed by calculating the standard deviations (SDs) of the size measurement for each peak of the allelic ladder among the 24 capillaries in a single run. The

maximum SD of a peak was 0.09 bp (**Fig. 6**) and was lower than the threshold (0.15 bp) of the sizing

precision described in the performance check section of the User Guide of the 3500xL Genetic

Analyzer (ThermoFisher Scientific, 2018). To detect a 1-bp difference in the peak size between

species correctly, peak size measurements should lie within a ±0.5 bp "window" around the size

obtained for each peak in the allelic ladder with a sufficiently high probability (Smith, 1995). The

observed SD in this study (< 0.1 bp) indicates that the probability is over 99.99% because the five

SDs were still less than 0.5 bp.

The repeatability was evaluated by testing one sample from each species multiple times. The

maximum SD of the peak size among the target species was 0.06 bp. In addition, testing all samples

once showed that the SDs of the observed peaks for each species were less than 0.5 bp (max 0.23

bp).

3.3.4. *Mixture studies*

It is important to identify species from mixtures of DNA because casework samples are often

contaminated with human or environmental DNA. Three pairs (human with cattle, human with dog,

and human with chicken) of DNA mixture with ratios ranging from 50:1 to 1: 50 (50:1, 20:1, 10:1,

5:1, 2:1, 1:1, 1:2, 1:5, 1:10, 1:20, and 1:50) were tested to evaluate the assay's ability to identify both

major and minor contributors correctly. All major contributors were identifiable in all cases. The

peak of each minor contributor was detected and its source species was identified correctly in the

mixture ratios between 20:1 and 1:20 ($\geqq$ 240 copies minor contributor) for the human–chicken DNA

mixture, and in the ratios between 50:1 and 1:20 for the human–cattle and human–dog mixtures.

Another two pairs (brown rat with house mouse, and Japanese wild boar with pig) of DNA mixture,

which have 1 bp-adjacent peaks, were tested in a similar way. The peak of each minor contributor

was not detected in any ratios for the brown rat and house mouse DNA mixture because it was masked by the tail of the major contributor peak. Similarly, the peak of each minor contributor was not detected in any ratios for the DNA mixture of Japanese wild boar and pig, except for the case when pig and Japanese wild boar were mixed in the mixture ratios of 1:1, 1:2, or 1:5.

The assay's ability to identify multiple species in a sample was also demonstrated using the experimental DNA mixture sample composed of ten species. Each species in the sample was correctly identified (**Fig. 7**).

3.3.5. *Case studies*

In forensic casework, samples may be degraded by environmental exposure and/or contaminated with human or other environmental DNA. The utility of the developed assay was evaluated using a total of 31 forensic casework samples. The assay unambiguously identified the source of DNA in each sample (**Table 6**).

3.3.6. *A 1 bp-InDel and phylogenetic relationship of wild boars and domestic pigs*

The assay distinguishes Japanese wild boar and pig based on a 1-bp difference of the fragment length of the D-loop region (**Fig. 3**). To evaluate the assay's ability to identify Japanese wild boar and pig correctly, the phylogenetic relationship of Eurasian wild boar and pig was analyzed. The complete mtDNA sequences of the 307 Eurasian wild boars and pigs were aligned with that of desert warthog (*Phacochoerus africanus*). The phylogenetic analysis showed that they were mainly grouped into two lineage groups: the European and Asian lineages **(Fig. 8)**. An InDel was found in the part of the D-loop region amplified by the assay. The presence or absence of the 1-bp deletion

(NC_000845.1:m.137delC) corresponded well to the two lineage groups. Almost all the East Asian wild boars and indigenous pig breeds, as well as Japanese wild boars tested in this study, had the 1-bp deletion. There were some exceptions to this lineage–InDel relationship. Six individuals (Yorkshire breed, Chinese Meishan breed, or native breed of the Andaman and Nicobar Islands in India) were phylogenetically grouped into the Asian lineage although they didn't have the deletion. There were also some exceptional individuals whose geographic origin did not match the phylogenetic position. Eight individuals of European pig breeds (Berkshire, Yorkshire, or Large White) were grouped into the Asian lineage, while a Chinese Northeast wild boar was grouped into the European lineage. Besides, the multiple sequence alignment showed that 11 individuals had other InDels in the D-loop region amplified by the assay.

3.4. Discussion

The developed multiplex PCR assay enables simultaneous identification of 26 mammalian and poultry species from biological samples recovered from crime scenes. The assay is cost-effective because it does not require specific fluorescently labeled primers for each species and only three forward primers were labeled with fluorescent dyes. The species specificity test and the repeatability test demonstrated that the assay identifies species accurately based on peak detection with 1 bp-precision using the internal size standard and the allelic ladder. The sensitivity study indicated that the assay was highly sensitive, with a detection limit of 100 copies of mtDNA. This is sufficient to detect one mammalian cell because somatic cells have a high copy number of mtDNA ranging from hundreds to thousands of copies depending on tissue type (Holland and Parsons, 1999; Robin and Wong, 1988; Tobe and Linacre, 2008b). The sensitivity level was similar to that in a previous

study (Ramón-Laca et al., 2013) but was achieved using fewer PCR cycles (27 cycles compared with 30 cycles). It is important to note that this fewer PCR cycles leave room for further sensitivity improvement, because additional cycles might enable us to identify species from ultra-low-level samples containing less than 100 copies of mtDNA. The high sensitivity coupled with any PCR product below ~500 bp in size made the assay robust for degraded samples. The assay was able to identify species even from degraded casework samples such as partial bones, hairs, and feces. The assay also enables the analysis of DNA mixtures. The peaks of different species contained in a DNA mixture were distinguishable as long as they were separated by >1 bp (**Fig. 7**). Although species identification was difficult in the mixture test using brown rat and house mouse DNA or Japanese wild boar and pig DNA, forensic scientists rarely encounter mixtures of these species in casework.

The assay requires careful consideration for some species due to their intraspecific variation. The species specificity tests in the present study showed that rhesus macaque (*Macaca mulatta*) had intraspecific length variation in the amplified D-loop region. Some individuals of rhesus macaque had the same PCR product size as Japanese macaque (*Macaca fuscata*) (**Fig. 4**). According to NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), there are intraspecific length variations in crab-eating macaque (*Macaca fascicularis*) as well as rhesus macaque (data not shown) and some individuals from crab-eating macaque and rhesus macaque are expected to show the same PCR product size as Japanese macaque. Therefore, it cannot be concluded firmly that a sample is derived from Japanese macaque based on the assay result. However, there were clear differences in the PCR product size between these macaque species and the other species tested in this study. This indicates that these macaque species can be identified at least as one of the *Macaca* species.

No intraspecific variation of the D-loop region was observed in the human control DNA samples used in this study. However, the targeted D-loop region contains the HV1 in which intraspecific length variation called "C-Stretch" has been reported in humans (Bendall and Sykes, 1995; Chen et

al., 2009; Sekiguchi et al., 2008). Therefore, some human samples can be identified as *Macaca* species based on the PCR product size of the D-loop. Even in this case, the assay can correctly identify the sample as human by the presence of the cyt *b* human-specific peak because no cross-amplification of the cyt *b* region in *Macaca* species by the human specific primer was observed.

The tested domestic pig (*Sus scrofa domesticus*) samples, which were obtained from European breeds (Yorkshire and Duroc) reared in Japan, were successfully distinguished from Japanese wild boar (*Sus scrofa leucomystax*) samples even though they are phylogenetically closely related to each other. An InDel made the size of the D-loop peak derived from Japanese wild boar 1 bp shorter than that of the pig samples. However, identification of pig and Japanese wild boar requires careful consideration. Firstly, the multiple sequence alignment and the phylogenetic tree showed that almost all the East Asian wild boars and pig breeds had the 1 bp-deletion (**Fig. 8**), and therefore the D-loop peak derived from these East Asian breeds may be the same size as that of Japanese wild boar. Secondly, other InDels in the amplified region may affect the assignment of samples to pig or Japanese wild boar although the present analysis showed such InDels rarely occur. Thirdly, crossbreeding between European pigs and East Asian pigs complicates the interpretation of electrophoresis results. These crossbred European pigs such as Berkshire (Li et al., 2014) may have the 1-bp deletion if they inherit mtDNA from the Asian lineage. Therefore, even if the peak of Japanese wild boar is detected by the assay, the possibility that the DNA sample is derived from a pig cannot be excluded. Fourthly, genetic introgression of European domestic pig breeds into Japanese wild boars (Anderson et al., 2019; Takahashi, 2018) might also affect the conclusion from the assay. The hybrid individuals, called "Inobuta" in Japanese, will be identified as either of the two species, depending on their maternal lineage. To avoid this misidentification, forensic scientists should take the possibility that an analyzed sample originates from a hybrid individual into

consideration, as proposed by Amorim et al. (2020).

3.5. Summary

In this study a cost-effective, highly sensitive, and straightforward assay for simultaneous species identification was developed. The validation studies demonstrated the efficacy, accuracy, and reliability of the assay. The present study markedly reduced the species-specific primers in the multiplex by using the D-loop universal primers while keeping the number of identifiable species high. In addition, the present study minimized the number of fluorescent dyes used in the assay: the multiplex reaction requires only three fluorescent dyes for the peak detection on a 3500xL Genetic Analyzer. These will facilitate further development of the assay, for example, depending on the geographical origin of samples, by adding new primers to identify species untested in this study. One of the advantages of the assay is that it can identify numerous species even from mixed biological samples for which the analysis based on the conventional sequencing is inappropriate. This advantage makes it easy for forensic laboratories to conduct species identification tests on the evidence left unanalyzed because it contains DNA mixture from multiple source species. Although DNA metabarcoding using MPS technologies is now an option for researchers handling mixed DNA samples, the assay is much more cost-effective than DNA metabarcoding. Furthermore, the procedure of the assay is time-saving: the time required from multiplex PCR to the interpretation of results is only two hours. The assay is therefore suitable for local forensic laboratories that have no MPS platform. Previous methods based on real-time PCR coupled with species-specific probes are indeed simple, cost-effective, and timesaving, but the number of target species is limited by the number of detection channels of the instruments (Köppel et al., 2020). Another type of real-time

PCR methods using melting curve analysis (Ishida et al., 2018; Lopez-Oceja et al., 2017; Ouso et al., 2020) can increase the number of target species compared with the probe-based methods. However, this type of methods is not appropriate for DNA mixture because the melting curve profile of a particular species can change depending on mixing of another species and its mixture ratios. In the case of forensic casework samples that are suspected of DNA mixture, researchers may not conclude whether the profile was obtained from single source or not. In comparison with such real-time PCR methods, the developed assay can identify numerous species in any mixture ratios as long as their peaks are detectable.

The results also indicate that the developed assay has a potential for application not only to routine forensic investigations but to other fields such as food authentication and textile authentication.

Table 3 List of the species and the GenBank accession number used in this study.

Common name	Scientific name	Number of samples	Expected amplicon length (bp)		Observed Mean size (bp)		Accession number
			cyt *b*	D-loop	cyt *b* (SD)	D-loop (SD)	
Cattle	*Bos taurus*	5	118	481	110.5 (0.04)	476.3 (0.06)	NC_006853
European rabbit	*Oryctolagus cuniculus*	8	187	503	181.3 (0.03)	496.8 (0.05)	NC_001913
Human	*Homo sapiens*	5	197	485	190.8 (0.02)	479.5 (0.06)	NC_012920
Sheep	*Ovis aries*	5	205	–	199.5 (0.005)	–	NC_001941
Pig	*Sus scrofa domesticus*	5	225	431	217.0 (0.04)	426.1 (0.06)	NC_000845
Japanese wild boar	*Sus scrofa leucomystax*	5	N/A	N/A	217.0 (0.02)	425.0 (0.08)	AB015070, AB015085
Horse	*Equus caballus*	5	246	–	240.8 (0.04)	–	NC_001640
Goat	*Capra hircus*	5	261	–	256.0 (0.05)	–	NC_005044
Cat	*Felis catus*	5	283	–	278.3 (0.05)	–	NC_001700
Sika deer	*Cervus nippon*	5	429	–	424.4 (0.02)	–	NC_006993
Common raccoon	*Procyon lotor*	10	–	278	–	273.7 (0.08)	NC_009126
Japanese badger	*Meles anakuma*	5	–	285	–	281.2 (0.05)	NC_009677
Siberian weasel	*Mustela sibirica*	5	–	295	–	291.6 (0.04)	NC_020637
Red fox	*Vulpes vulpes*	5	–	313	–	309.1 (0.05)	NC_008434
Raccoon dog	*Nyctereutes procyonoides*	7	–	326	–	322.0 (0.10)	NC_013700
Dog	*Canis lupus familiaris*	5	–	336	–	332.6 (0.10)	NC_002008
Masked palm civet	*Paguma larvata*	5	–	356	–	351.2–352.2 (0.02–0.06)	NC_029403
Bactrian camel	*Camelus bactrianus*	5	370	359	365.9 (0.04)	352.0 (0.03)	NC_009628
Alpaca	*Vicugna pacos*	4	370	360	366.1 (0.23)	354.2 (0.07)	KU168760
Asiatic black bear	*Ursus thibetanus*	12	–	367	–	361.6–364.5 (0.06–0.1)	NC_009971
Brown rat	*Rattus norvegicus*	5	–	373	–	369.8 (0.04)	NC_001665
House mouse	*Mus musculus*	5	–	374	–	370.4 (0.03)	NC_005089
Rhesus macaque	*Macaca mulatta*	6	–	487	–	481.6–483.7 (0.005–0.16)	NC_005943
Japanese macaque	*Macaca fuscata*	6	–	488	–	481.6 (0.07)	NC_025513
Japanese quail	*Coturnix japonica*	5	–	280	–	276.5 (0.05)	NC_003408
Domestic turkey	*Meleagris gallopavo*	5	–	285	–	281.0 (0)	NC_010195
Duck	*Anas platyrhynchos*	5	–	317	–	311.3 (0.04)	NC_009684
Chicken	*Gallus gallus*	5	–	344	–	340.2 (0.05)	NC_001323

N/A: The length is not available because the registered sequences (AB015070 and AB015085) do not cover the primer binding sites.

Table 4 List of primers used in the multiplex PCR.

Primer name	Sequence (5'-3')	Conc. (μM)	Target region	5' label
DL_UniF	CACCATCAGCACCCAAAGCT [a]	0.10	D-loop	VIC (Green)
DL_UniR	ATGGGCCCGGAGCGAGAAGAG	0.10		−
DL_Bird_UniF	TCGTGCATACATTTATATTCCACATA	0.50	D-loop	NED (Yellow)
DL_Bird_UniR1	GTGTACGATTAATAAATCCATCTGGTAC	0.20		−
DL_Bird_UniR2	GTGGACGATCAATAAATCCATCTGATAC	0.40		−
CYTB_UniF	GACCAATGATATGAAAAATCATCGTTGT [b]	0.80	cyt *b*	6-FAM (Blue)
CYTB_CattleR	GGCTGGAAGGTCGATGAATGTA	0.15		−
CYTB_RabbitR	GTGAAAATTTGAATTATAAGGCACAG [c]	0.20		−
CYTB_HumanR	ATAGTCCTGTGGTGATTTGGAGGATC	0.10		−
CYTB_SheepR	TGCTAGGAATAGGTCTGTTGGAATC	0.20		−
CYTB_PigR	GTCTGATGTGTAATGTATTGCTAAGAAC [d]	0.15		−
CYTB_HorseR	ACGGATGAGAAGGCAGTTGTC	0.06		−
CYTB_GoatR	CGACAAATGTGAGTTACAGAGGGA [e]	0.08		−
CYTB_CatR	TGATTCAGCCATAATTAACGTCG	0.20		−
CYTB_CamelR	GTAGGAGCCGTAGTAAAGCCCA	0.10		−
CYTB_SikaR	GCTGTGGCTATAACTGTAAATAGGACA	1.60		−

a) Modified from Nakamura et al. (2009).

b) Modified from Pääbo et al. (1988).

c) Same as Tobe and Linacre (2008a).

d) Modified from Tobe and Linacre (2008a).

e) Modified from Matsunaga et al. (1999).

Table 5   Primer binding sites of the 16S rRNA gene for vertebrate universal qPCR.

Common name	Scientific name	Sequence (5'-3')		Accession number
		Forward	Reverse	
Human	*Homo sapiens*	TACGACCTCGATGTTGGATCA	AATCCAGGTCGGTTTCTATCT	NC_012920
House mouse	*Mus musculus*	. . . .	. . . .	NC_005089
Chicken	*Gallus gallus*	. . . .	. . . .	NC_001323
Carolina anole	*Anolis carolinensis*	. . . .	. . . .	NC_010972
Burmese python	*Python bivittatus*	. . . .	. . . . .A.	NC_021479
Western clawed frog	*Xenopas tropicalis*	. . . .	. . . . .A.	NC_006839
Zebrafish	*Danio rerio*	. . . .	. . . . .A.	NC_002333

Table 6 List of casework samples.

Sample type	Number of samples	Identified species (number of samples)
Bloodstain	13	Cat (5), Common raccoon (3), Macaque (1), Raccoon dog (1), Sika deer (2), Wild boar (1)
Saliva stain	1	Red fox (1)
Tissue	4	Cattle (1), Chicken (1), Sika deer (2)
Partial bone	9	Chicken (3), Sika deer (2), Wild boar (4)
Hair	2	Common raccoon (1), Red fox (1)
Feces	2	Common raccoon (1), Red fox (1)
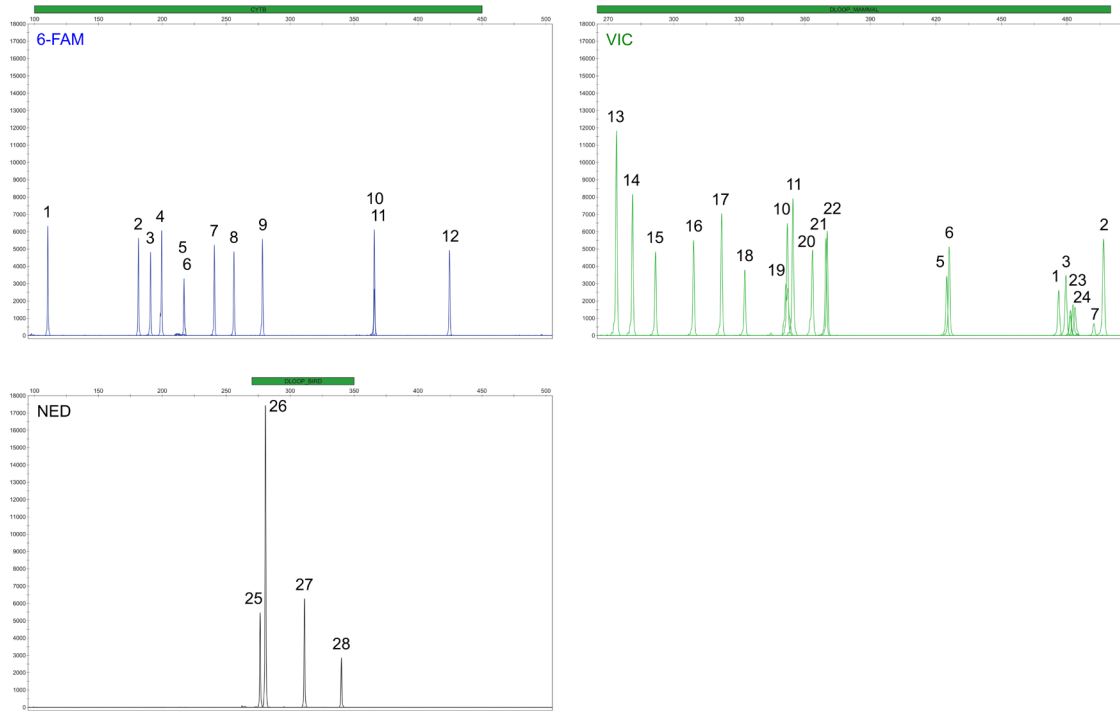
Fig. 3 Electropherograms of each tested species shown by "Overlay All" plots using GeneMapper ID-X Software v1.4. Peaks are 1: Cattle, 2: European rabbit, 3: Human, 4: Sheep, 5: Japanese wild boar, 6: Pig, 7: Horse, 8: Goat, 9: Cat, 10: Bactrian camel, 11: Alpaca, 12: Sika deer, 13: Common raccoon, 14: Japanese badger, 15: Siberian weasel, 16: Red fox, 17: Raccoon dog, 18: dog, 19: Masked palm civet, 20: Asiatic black bear, 21: Brown rat, 22: House mouse, 23: Japanese macaque, 24: Rhesus macaque, 25: Japanese quail, 26: Domestic turkey, 27: Mallard, and 28: Chicken.
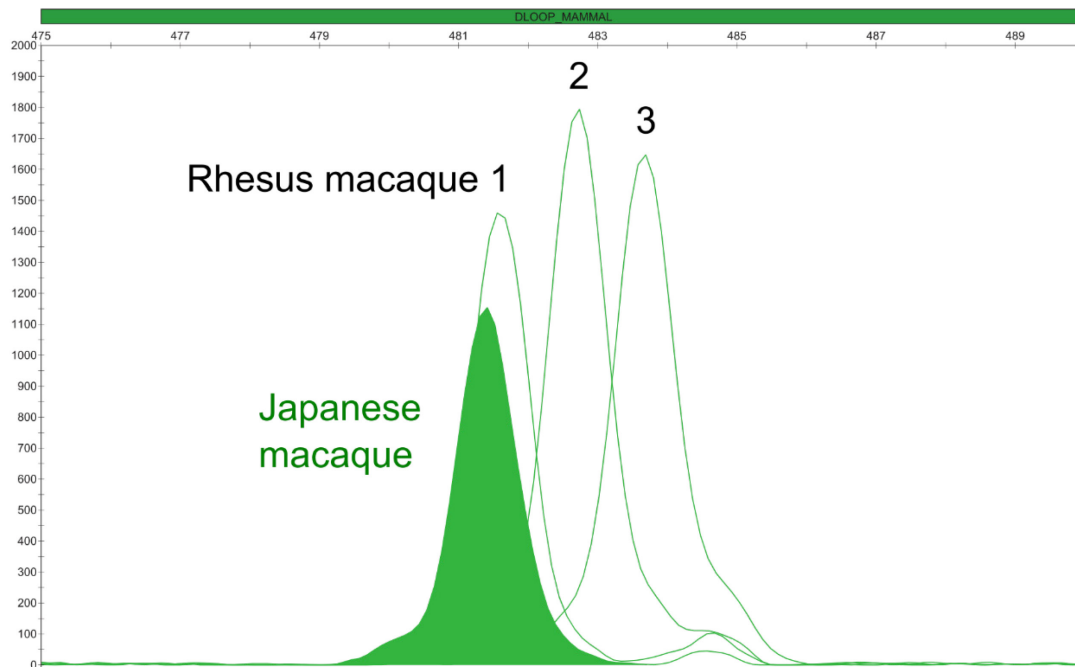
Fig. 4 Peak positions of Japanese macaque and individuals of rhesus macaque (1–3).
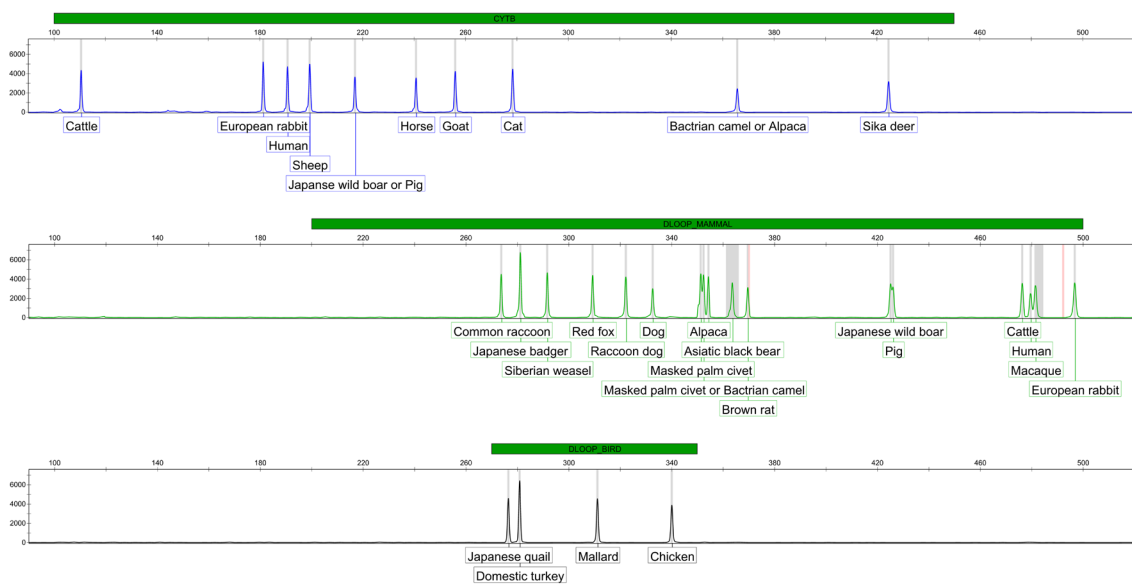
Fig. 5   An electropherogram of the allelic ladder. The three panels correspond to 6-FAM, VIC, and NED dye-labeled peaks from top to bottom.
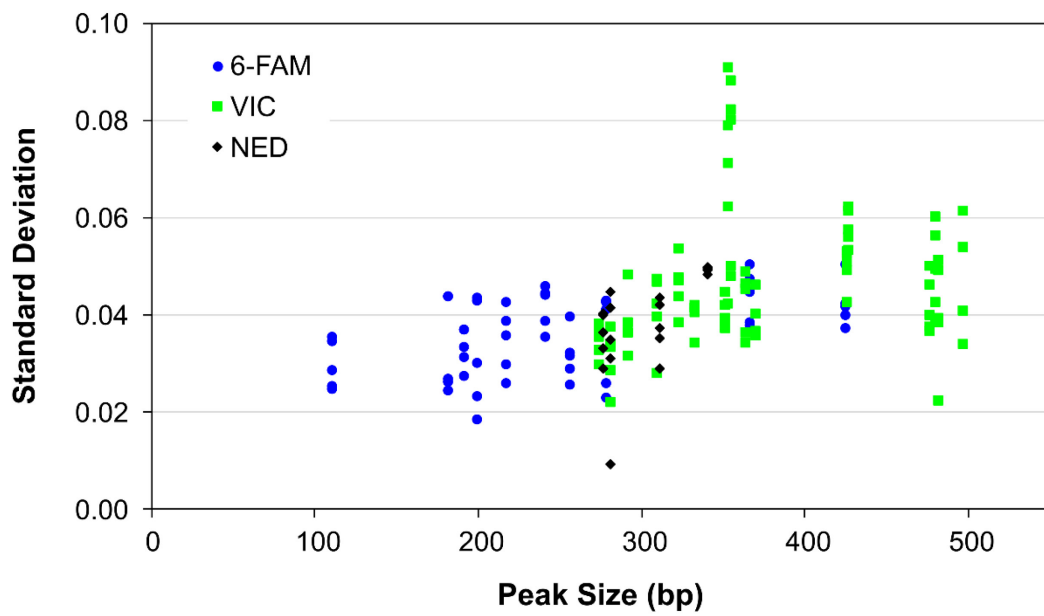
Fig. 6 Sizing precision of the allelic ladder across five runs on a 3500xL Genetic Analyzer. Each dot indicates the standard deviation of the observed peak sizes on 24 capillaries within a single run.
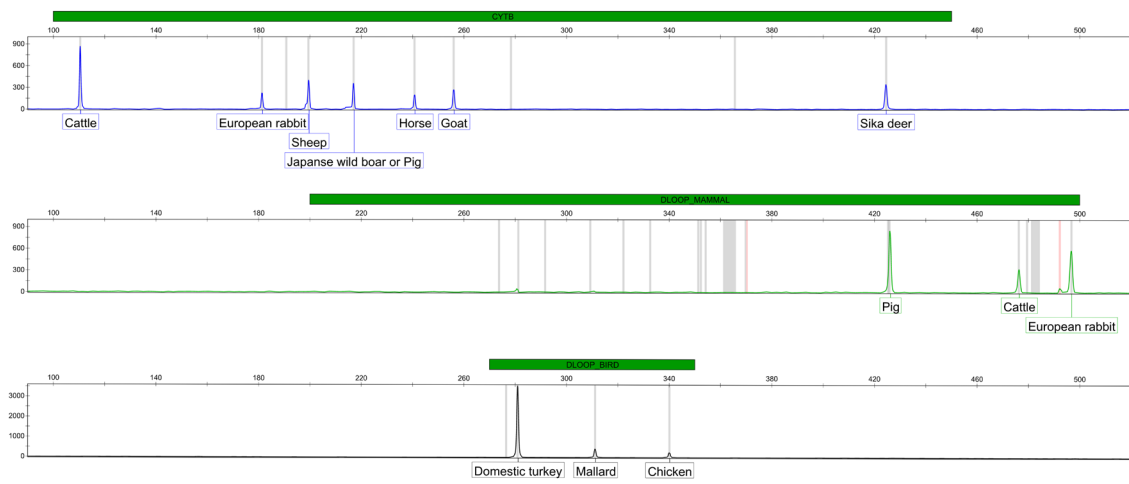
Fig. 7 Amplification of an experimental mixture sample consisting of 10 species (cattle, European rabbit, sheep, pig, horse, goat, sika deer, domestic turkey, mallard, and chicken).
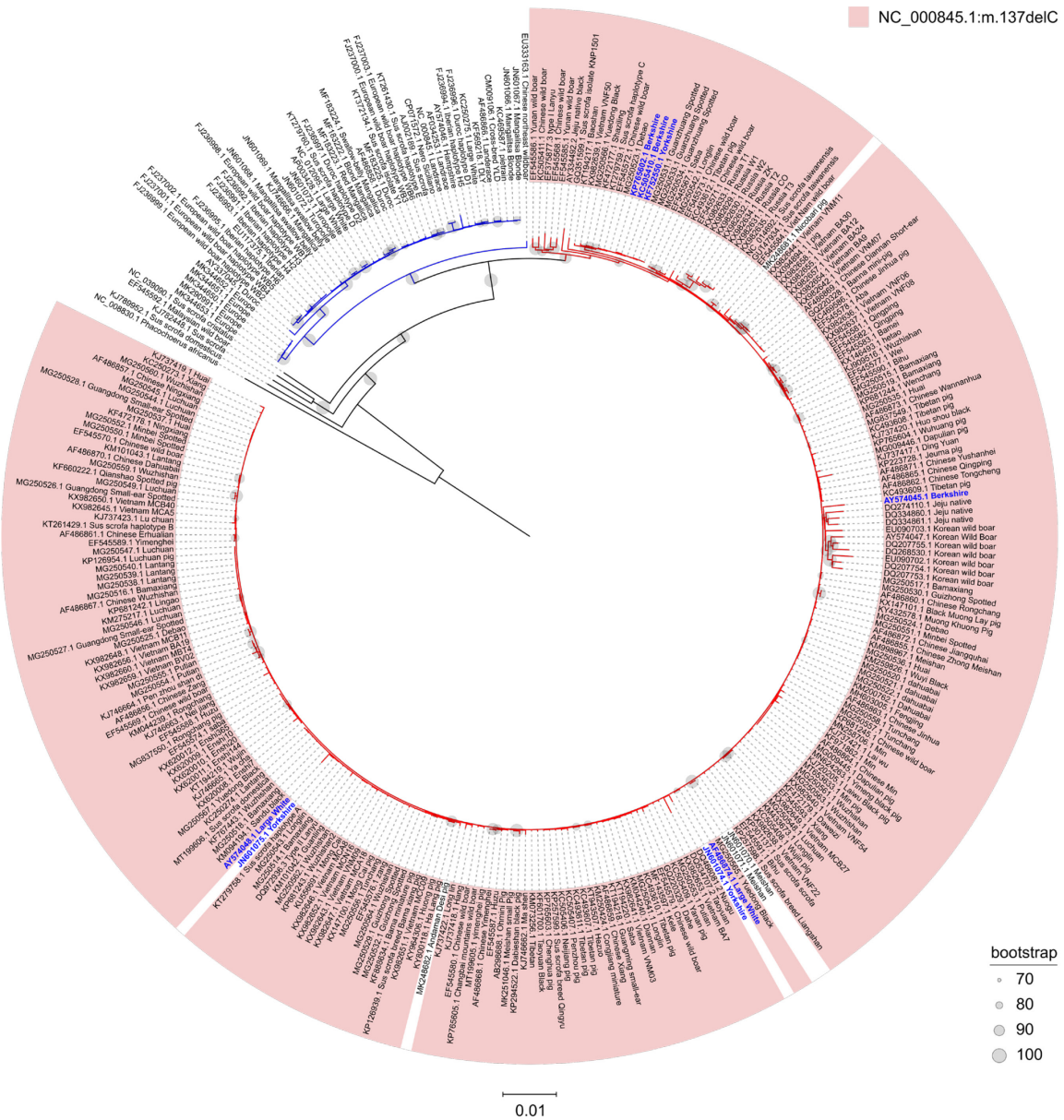
Fig. 8 Neighbor-joining phylogenetic tree based on the complete mtDNA sequences of 307 wild boars and domestic pigs. A sequence of desert warthog (*Phacochoerus africanus*) was used as an outgroup. Bootstrap values (1000 replicates) of 70% or more are shown by circles at the branch nodes. The blue and red lines represent the European and Asian lineages, respectively. The taxa names highlighted in light red indicate sequences with a 1 bp-deletion in the D-loop region amplified in this study compared with the pig reference sequence (NC_000845.1:m.137delC). The taxa names in blue and bold indicate possible descendants of crossbreeding between a male European pig and a female East Asian pig.

Chapter 4

Conclusion

Forensic investigations require not only human individual identification from casework samples but species identification from non-human biological samples. With recent advances in molecular biology, many mtDNA-based methods have been developed for species identification. However, there is still no method that can cost-effectively identify enough species for routine investigations even from degraded samples containing a mixture of DNA from multiple species. This has prevented the standardization of the species identification test for forensic purpose.

In this study, a new multiplex PCR assay was developed and validated for the identification of common domestic animals and wild mammals in Japan. The assay can simultaneously identify 26 mammalian and poultry species, which is sufficient for routine forensic investigations. The results of the assay are robust to DNA mixtures and the assay is much more cost-effective and timesaving than DNA metabarcoding. Furthermore, local forensic laboratories can easily introduce the assay without any additional instruments. it is therefore expected that the assay will be used as a standard method for species identification in routine forensic analysis in Japan.

The assay is also useful for other countries. The universal D-loop primers of the assay can be applicable for other untested species. Moreover, it is easy to add new primers to identify other wild animals depending on the geographical region. Because of its extensibility, the assay also has a potential to be standardized in other geographical regions.

I hope this study helps local forensic laboratories and makes a certain contribution for the standardization of forensic species identification.

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