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TECHNICAL NOTE

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Simple and Sensitive Method for Identification of Human DNA by Allele-Specific Polymerase Chain Reaction of *FOXP2*

ABSTRACT: The forkhead box P2 (*FOXP2*) gene is specifically involved in speech and language development in humans. The sequence is well conserved among many vertebrate species but has accumulated amino acid changes in the human lineage. The aim of this study was to develop a simple method to discriminate between human and nonhuman vertebrate DNA in forensic specimens by amplification of a human-specific genomic region. In the present study, we designed an allele-specific polymerase chain reaction (PCR) using primers to amplify smaller than 70-bp regions of *FOXP2* to identify DNA as being of human or nonhuman, including ape, origin. PCR amplification was also successfully performed using fluorescence-labeled primers, and this method allows a single PCR reaction with a genomic DNA sample as small as 0.01 ng. This system also identified the presence of human DNA in two blood stains stored for 20 and 38 years. The results suggested the potential usefulness of *FOXP2* as an identifier of human DNA in forensic samples.

KEYWORDS: forensic science, allele-specific polymerase chain reaction, fragment analysis, human identification, vertebrates, forkhead box P2

Polymerase chain reaction (PCR)-based typing of short tandem repeats (STR) of nuclear DNA loci is the established method for identification of individual persons in forensic science (1). Recently, methods for detection of human species DNA in forensic specimens such as visible biological stains have been vigorously developed, and have replaced or at least supplemented biochemical methods in many laboratories. The targets are mainly mitochondrial loci such as cytochrome b and 12S and 16S ribosomal RNA because mitochondrial loci have more copy numbers than nuclear loci (2–7). In addition, nuclear loci such as Alu repetitive elements (8,9), TP53 (10), and β-actin (3) are used for species identification. Direct sequencing by a ribosomal RNA-based species-identification method is useful when a biological sample is from a single species, but it will amplify both species if the sample is a mixture of two species. Recently, the multiplex PCR-based species-identification method was developed using the mitochondrial cytochrome b gene (11). Multiplex PCR can identify 18 mammalian species, many of which are often associated with forensic investigations, but many species-specific primers are necessary. Therefore, development of useful genetic markers for human identification is desirable in the forensic field. We surveyed the DNA sequences in a database to find new nuclear markers for human identification and identified *FOXP2* as one of the candidate genes (12,13).

FOXP2 was the first gene identified that is related to the human ability to develop language (13). The human FOXP2 protein differs at only three amino acid positions from its ortholog in the mouse (12). Changes in the amino acid coding and a pattern of nucleotide polymorphism in human *FOXP2* strongly suggest that this gene has been the target of directional selection during recent human evolution (12). In this study, in order to develop a simple and effective method for determination that a tissue is of human origin, we designed an allele-specific PCR using primers of exon 7 of *FOXP2*, which is where the nucleotide differences between humans and other vertebrates are located, amplified the conserved exon 10 for a control, and evaluated *FOXP2* as a new genetic marker for identification of human tissues.

Materials and Methods

DNA Extraction

This study protocol was approved by the Ethical Committee of Kurume University. Blood samples were taken from 40 randomly selected individuals from various populations (10 Africans, 10 Europeans from South Africa, 10 Chinese from Guangzhou, and 10 Tamils and Sinhalese from Sri Lanka), and genomic DNA was prepared as described previously (14,15). Genomic DNA was extracted from peripheral blood or flesh from 52 different vertebrates (33 different species) by using phenol–chloroform (16) or a QIAamp DNA Mini Kit according to the manufacturer's protocol (QIAamp DNA Mini Kit handbook) (Qiagen K.K., Tokyo, Japan). The Tris-buffered phenol/chloroform/isoamyl alcohol (25:24:1) solution was purchased from Nippon Gene Co., Ltd. (Tokyo, Japan). Proteinase K was purchased from Qiagen K.K. DNA was also isolated from two blood stains stored for 20 and 38 years at room temperature using a QIAamp DNA Mini Kit (Qiagen K.K.). The DNA mixtures were prepared by mixing human and chicken DNA (final concentrations of 0.05 ng/μL each, 0.05 and

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0.5 ng/μL, and 0.5 and 0.05 ng/μL for human and chicken DNA, respectively). The DNA was quantified using a Quant-iT™ dsDNA HS assay kit and Qubit® fluorometer (Invitrogen Japan K.K., Tokyo, Japan).

Design of PCR Primer Sets

The DNA sequences of *FOXP2* homologs of several species were obtained from the Ensembl database (http://www.ensembl.org/index.html). The DNA sequences of part of *FOXP2* exon 7 from several species and primers are shown in Fig. 1*A*. Two forward primers were designed, one human-specific (FOXP2-humF) and the other nonhuman-specific (FOXP2-nonhumF). A common reverse primer (FOXP2-commonR) was designed for both forward primers.

We also designed primers based on the sequence of exon 10 of *FOXP2*, which is completely conserved among many vertebrates except zebrafish, as a positive control of the PCR amplification (Fig. 1*B*).

PCR Amplification and Detection of PCR Products

The amplification was performed in a final volume of 20 μL containing 1× PCR reaction buffer, 0.16 mM dNTP, 1 U TaqGold (Applied Biosystems, Tokyo, Japan), and 0.25 μM of each primer. We used 1 ng of genomic DNA as a template unless otherwise noted. The various annealing temperatures (54–62.8°C) and numbers of cycles (30–50 cycles) for a human-specific primer set and a nonhuman-specific primer set were tested to optimize the amplification that gave the best specificity without a reduction in yield. We selected the following PCR conditions: 35 cycles at 94°C for 30 sec, 55°C for 30 sec, and 65°C for 15 sec in a 9700 Gene Amp Cycler (Applied Biosystems). The initial denaturation step was 95°C for 9 min, and the final extension step was 60°C for 30 min.

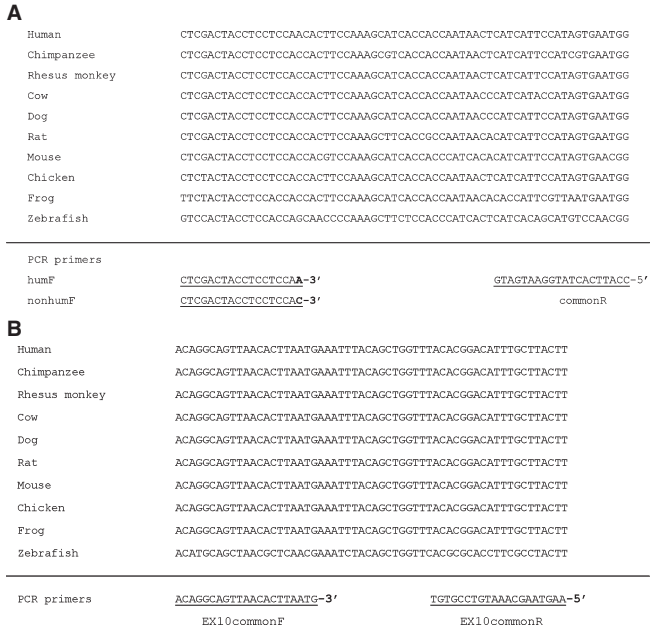

FIG. 1—*Sequences of primers and amplified regions in this study. (A) DNA sequences of FOXP2-humF (humF), FOXP2-nonhumF (nonhumF), and FOXP2-commonR (commonR) primers and amplified region of FOXP2 exon 7 from several species. (B) DNA sequences of FOXP2-EX10commonF (EX10commonF) and FOXP2-EX10commonR (EX10commonR) primers and amplified region of FOXP2 exon 10 from several species.*

The PCR amplification products were electrophoresed on 12% acrylamide gel and stained with 0.5 μg/mL ethidium bromide (Nacalai Tesque, Kyoto, Japan). All primers used for polyacrylamide gel-based PCR were synthesized by Operon Biotechnologies (Tokyo, Japan). A positive signal was defined as a product of the expected size made visible by ethidium bromide staining.

Multiplex PCR Using Fluorescence-Labeled Primers

To develop the multiplex sequence-specific PCR method, we used fluorescence-labeled primers (synthesized by Applied Biosystems). The amplification was performed in a final volume of 20 μL containing 1× PCR buffer containing 1.5 mM $MgCl_2$, 0.16 mM dNTP, 1 U TaqGold (Applied Biosystems), and various concentrations of each primer. We used 0.1 ng of genomic DNA as a template unless otherwise stated. The various annealing temperatures (55–60°C), primer concentrations (0.21–1 μM), and numbers of cycles (30–40 cycles) were tested to determine the amplification that optimized the specificity without a reduction in yield. The optimal profile of temperature and cycles was as follows: 31 cycles at 94°C for 1 min, 59°C for 1 min, and 65°C for 1 min in a 9700 Gene Amp Cycler (Applied Biosystems). Fluorescence and concentration of primers are as follows: 0.75 μM of 5′-FAM-FOXP2-humF, 0.40 μM of 5′-VIC- FOXP2-nonhumF, 1 μM of FOXP2-commonR, 0.21 μM of 5′-NED-EX10commonF, and 0.5 μM of EX10commonR. The initial denaturation step was 95°C for 10 min, and the final extension step was 60°C for 60 min. The PCR amplification products were detected using the ABI Prism® 310 Genetic Analyzer, and the electropherograms were analyzed using GENE MAPPER ver. 3.2 software (Applied Biosystems). A 310 Genetic Analyzer (Applied Biosystems) was used with filter set G5v2 to process the data from the five dyes 6FAM, VIC, NED, PET, and LIZ after an appropriate matrix had been created using materials from the matrix standard set DS-33 (Applied Biosystems). Each sample for analysis on the 310 was prepared by adding 1 μL of PCR product to 19 μL of Hi-Di™ formamide (Applied Biosystems) containing 0.20 μL of GeneScan 500 LIZ size standard (Applied Biosystems). Samples were injected for 5 sec at 15,000 V and separated at 15,000 V for 40 min with a run temperature of 60°C. Standard electrophoretic conditions were used including 310 Genetic Analyzer POP™-6, 1X Genetic Analyzer Buffer with ethylenediaminetetraaceticacid (EDTA), and a 47 cm × 50 μm capillary (Applied Biosystems). A positive signal was defined as more than 150 relative fluorescence units.

DNA Profiling

The DNA profiling was performed on DNA from the blood stains by using an AmpFℓSTR® Identifiler® PCR Amplification Kit (Applied Biosystems).

Results

To discriminate human and nonhuman vertebrate DNA, we designed primers based on exon 7 of the *FOXP2* gene (Fig. 1*A*). Using a human-specific primer set (humF and commonR), all amplifications of 40 samples of human DNA from four populations (Europeans, Africans, Indians, and Chinese) gave PCR products with the expected size of 66 bp, but no product for nonhuman animals. On the other hand, amplification of DNA extracted from nonhuman mammals and birds using the nonhuman-specific primer set (nonhumF and commonR) (Fig. 1*A*) gave PCR products with the expected size of 66 bp, while no PCR product was amplified from human, reptile,

amphibian, and fish samples. Using a common primer set designed on exon 10 of the *FOXP2* gene (Fig. 1*B*), a PCR product with the expected size of 58 bp was detected (positive result) for amplification of genomic DNA extracted from humans, nonhuman mammals, birds, reptiles, and amphibians, but no product was detected (negative result) for fishes. A summary of the results of the polyacrylamide gel-based method is shown in Table 1.

By using serial dilutions for the genomic DNA from fresh human blood, the minimum amount of DNA required for successful amplification using the human-specific primer set was found to be between 0.02 and 0.05 ng in our present experimental condition (Fig. 2). The lengths of the PCR products of human-specific and nonhuman-specific primers are the same (66 bp) in our system. If we were to introduce artificial oligonucleotides such as poly T into one of the primers, the sizes would be different, and we could perform multiplex PCR using polyacrylamide gel-based assay.

Instead of that method, in the present study, we developed the method of multiplex sequence-specific PCR using fluorescence-labeled primers to obtain multiple results from each sample with a single PCR. Human-specific PCR (FAM signal) and exon 10 PCR (NED signal) products were detected by PCR amplification of genomic DNA from human blood while nonhuman specific PCR (VIC signal) and exon 10 PCR (NED signal) products were detected by PCR amplification of genomic DNA from nonhuman mammals or bird blood (Fig. 3). Only the exon 10 PCR product (NED signal) was detected by PCR amplification of genomic DNA from reptiles or Xenopus blood (Fig. 3). On the other hand, no fluorescent signal was detected in PCR amplification of genomic DNA from fish blood (Fig. 3). All samples used for gel-based detection-system were analyzed by this multiplex system, and the results of both systems were fully consistent (Table 1). A series of samples of mixed human and chicken DNA (0.05 and 0.05 ng/μL, 0.05 and 0.5 ng/μL, and 0.5 and 0.05 ng/μL) was used to validate the test for mixed samples. Positive results were obtained from the three mixed samples with any set of primers (not shown).

In addition, by using serial dilutions of genomic DNA from fresh human blood, the minimum amount of DNA required for successful amplification using the human-specific primer set was found to be around 0.01 ng in our present experimental condition (not shown).

TABLE 1—*Amplification of human and various animal DNAs by the polyacrylamide gel-based and fluorescence-based methods.*

		Gel-Based			Genetic Analyzer-Based		
		Exon 7		Exon 10	Exon 7		Exon 10
	N	Human	Nonhuman	Common	Human	Nonhuman	Common
Mammals							
Homo sapiens	40	+	−	+	+	−	+
Pan troglodytes	1	−	+	+	−	+	+
Gorilla gorilla	1	−	+	+	−	+	+
Cercopithecus pygerythrus	1	−	+	+	−	+	+
Macaca fuscata	1	−	+	+	−	+	+
Papio hamadryas	1	−	+	+	−	+	+
Canis familiaris	6	−	+	+	−	+	+
Felis catus	6	−	+	+	−	+	+
Equus caballus	3	−	+	+	−	+	+
Bos taurus	4	−	+	+	−	+	+
Sus scrofa	5	−	+	+	−	+	+
Capra hircus	1	−	+	+	−	+	+
Cervus nippon	1	−	+	+	−	+	+
Mus musculus	1	−	+	+	−	+	+
Rattus rattus	1	−	+	+	−	+	+
Oryctolagus cuniculus	1	−	+	+	−	+	+
Balaenoptera acutorostrata	1	−	+	+	−	+	+
Birds							
Gallus gallus	1	−	+	+	−	+	+
Columba livia	1	−	+	+	−	+	+
Corvus corone	1	−	+	+	−	+	+
Anas platyrhynchos	1	−	+	+	−	+	+
Aythya fuligula	1	−	+	+	−	+	+
Bubulcus ibisibis	1	−	+	+	−	+	+
Ardea cinerea	1	−	+	+	−	+	+
Phasianus versicolo	1	−	+	+	−	+	+
Aptenodytes patagonicus	1	−	+	+	−	+	+
Reptiles							
Geochelone elegans	1	−	−	+	−	−	+
Pogona vitticeps	1	−	−	+	−	−	+
Amphibian							
Xenopus laevis	1	−	−	+	−	−	+
Fishes							
Thunnus orientalis	1	−	−	−	−	−	−
Pagrus major	1	−	−	−	−	−	−
Trachurus japonica	1	−	−	−	−	−	−
Sardinops melanostictus	1	−	−	−	−	−	−
Danio rerio	1	−	−	−	−	−	−

Positive (+) means a product with expected size was made visible by ethidium bromide staining or is defined as more than 150 relative fluorescence units (RFU) in the fluorescence-based method.

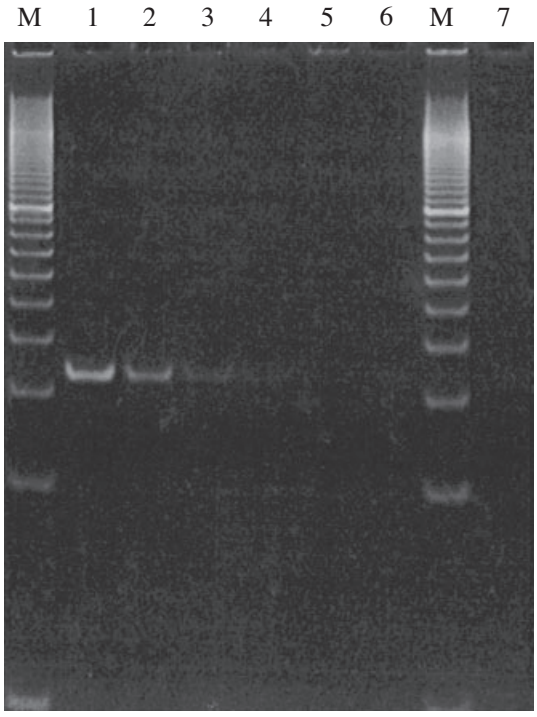

FIG. 2—*Detection limit of human-specific primers using serial dilutions of human DNA as a template. Lanes (1) 1.0 ng; (2) 0.5 ng; (3) 0.1 ng; (4) 0.05 ng; (5) 0.02 ng; (6) 0.01 ng; (7) NC, negative control; (M) 20-bp ladder marker (Takara Bio, Shiga, Japan).*

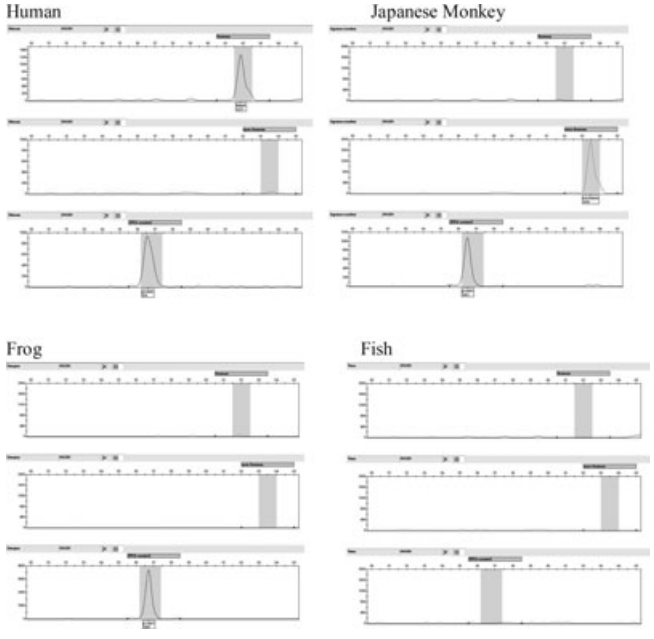

FIG. 3—*Electropherograms of sequence-specific PCR amplification using fluorescence-labeled primers. The results obtained from genomic DNAs from human (*Homo sapiens*), Japanese monkey (*Macaca fuscata*), frog (*Xenopus laevis*), and fish (*Trachurus japonica*) are indicated. Upper panel: human-specific PCR product (FAM signal); Middle panel: nonhuman-specific PCR product (VIC signal); Lower panel: PCR product of exon 10 (NED signal).*

We then examined the amplification of genomic DNA isolated from blood stains on cotton threads stored at room temperature for 20 and 38 years. Although the DNA concentrations of these samples were not determined using our system because they were out of range, we obtained positive results for them (Fig. 4). We determined all 15 STR loci of these blood stains by using an AmpFℓSTR® Identifiler® PCR Amplification Kit (data not shown). These results suggest that this multiplex PCR amplification is a potential method for forensic cases.

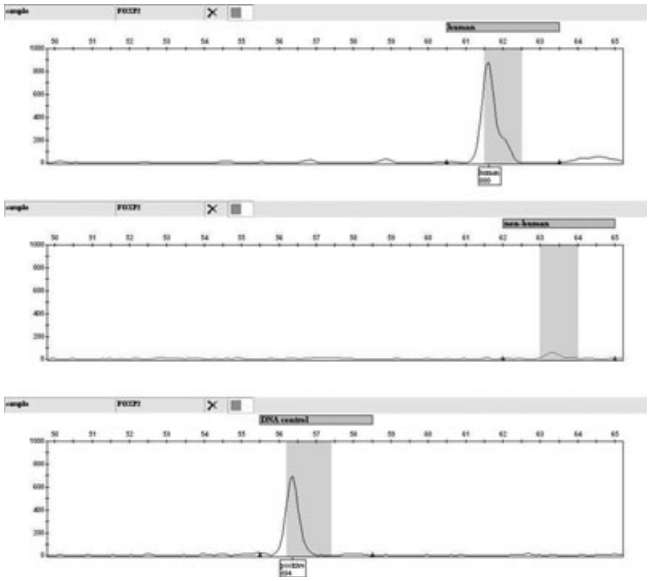

FIG. 4—*Sequence-specific PCR amplification using purified DNA from a blood stain stored for 20 years. Upper panel: human-specific PCR product (FAM signal); middle panel: nonhuman-specific PCR product (VIC signal); lower panel: PCR product of exon 10 (NED signal).*

Discussion

Language is a uniquely human trait that is likely to have been a prerequisite for the development of human culture (12). The ability to develop articulate speech relies on capabilities such as fine control of the larynx and mouth that are absent in chimpanzees and other great apes. *FOXP2* is so far the only human gene known to be specifically linked to the cognition of language and speech, based on genetic mapping and mutational analysis in a three-generation pedigree with an autosomal dominant form of a speech and language disorder (13). The protein is an evolutionarily conserved transcriptional repressor containing a zinc-finger motif, a forkhead DNA-binding domain, and a polyglutamine tract. In this study, we evaluated whether this locus was useful as a genetic marker for human species identification. We distinguished the DNA of humans from that of other primates, including chimpanzees and gorillas (Table 1).

Furthermore, this simple amplification system made it possible to classify the species of samples into four categories: human, nonhuman mammals and birds, reptiles and amphibians, and fishes and others outside the detection limit. In addition, because all the PCR products are smaller than 70 bp, the primers might be applicable to low-yield and degraded samples.

For the quantification and detection of human DNA, human-specific DNA probes and primers have been reported and used in the forensic community for many years (17–20). Among the commercially available systems, the QuantiBlot® Human DNA Quantitation Kit (Applied Biosystems) or AluQuant Human DNA Quantitation System (Promega) utilize human-specific probes, and the Quantifiler™ Human DNA Quantification Kit

(Applied Biosystems) is based on real-time PCR. It is likely that PCR-based methods have advantages over the probe-based method in the ability to indicate the existence of PCR inhibitors, which is not negligible for performing the following STR profiling. More recently, the Quantifiler® Duo kit and Plexor® HY System were introduced by Applied Biosystems and Promega, respectively. These kits can identify and quantify not only human but also male DNA from biological specimens by using a multichannel real-time PCR machine. However, we can perform both human identification and DNA profiling using multiplex STR systems with a genetic analyzer if we only introduce the present multiplex allele-specific PCR of *FOXP2*. We can also include a marker such as *SRY* in this system for identification of male DNA. Therefore, this simple and actually human-specific method using *FOXP2* might be a useful initial screening tool to identify human tissues from forensic specimens.

In this study, we developed a simple PCR method for discrimination of human tissues from those of nonhuman vertebrates. In addition, by multiplex sequence-specific PCR using fluorescence-labeled primers, human DNA can be identified by a single PCR reaction per sample for routine procedures.

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