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Abstract

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KEYWORDS: species identification, myoglobin, polymerase chain reaction

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Original Article

Human Identification from Forensic Materials by Amplification of a Human-Specific Sequence in the Myoglobin Gene

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We developed a method for human identification of forensic biological materials by PCR-based detection of a human-specific sequence in exon 3 of the myoglobin gene. This human-specific DNA sequence was deduced from differences in the amino acid sequences of myoglobins between humans and other animal species. The new method enabled amplification of the target DNA fragment from 30 samples of human DNA, and the amplified sequences were identical with that already reported. Using this method, we were able to distinguish human samples from those of 21 kinds of animals: the crab-eating monkey, horse, cow, sheep, goat, pig, wild boar, dog, raccoon dog, cat, rabbit, guinea pig, hamster, rat, mouse, whale, chicken, pigeon, turtle, frog, and tuna. However, we were unable to distinguish between human and gorilla samples. This method enabled us to detect the target sequence from 25 pg of human DNA, and the target DNA fragment from blood stored at 37 °C for 6 months, and from bloodstains heated at 150 °C for 4 h or stored at room temperature for 26 years. Herein we also report a practical application of the method for human identification of a bone fragment.

Key words: species identification, myoglobin, polymerase chain reaction

Studies on a method for human identification from forensic materials had long concentrated on the application of immunological reaction until the 1980s when hybridization methods with an *Alu* probe or a human repetitive 2.3 kb Hind III fragment probe were reported [1, 2]. In the 1990s, PCR was introduced in research on human identification [3-12]. However, these new methods also posed problems: they required the analysis of band patterns and/or size of amplicons; used expensive kits and instruments for analysis; involved troublesome procedures such as amplification using some sets of primers and/or subsequent digestion with endonuclease (s): and investigated only a few species of animals. Moreover, these reports, with only one exception, presented no rationale for human specificity (that is, no information on animals' DNA sequences), and species specificity of these methods were merely empirical. Although human specific *Alu* sequences are known [13], they have not been applied in forensic practice for human identification using PCR.

The primary structure of the myoglobins of human and a great many animal species have been determined and registered (SWISS-PROT). This means that a human-specific sequence in an exon of the myoglobin gene can be deduced from known interspecies differences in amino acid sequence, although the nucleic acid sequences of this gene

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have not been elucidated in many animal species. Thus, we developed a method for human identification by amplification of a human-specific sequence in the myoglobin gene. We also tested the usefulness of the method by applying it to forensic specimens that had been kept under various conditions.

Materials and Methods

Rationale and primer design. Table 1 lists the portion of the amino acid sequence of human myoglobin that is encoded by exon 3, along with the corresponding portions of the myoglobins of certain animals. A comparison of these sequences showed that amino acids at positions 110, 144, and 145 from the N terminal in many of the animals were different from those in human: cysteine in human at position 110 was substituted with alanine, isoleucine, or valine in animals; serine at position 144, with alanine or glutamic acid; and asparagine at position 145, with lysine, glutamine, or glutamic acid. According to the codons corresponding to the substituted amino acids, we determined the bases that were different between human and animals. For the cow, pig, mouse, seal, whale, tuna, and human, the reported sequences were used (Table 2). On this basis, we designed 3 primers, the 3'-terminals of which were located in the regions having interspecies differences: a forward primer (MbE3F1) and 2 reverse primers (MbE3R1 and MbE3R2) (Table 2). The primer sequences and 2 kinds of reaction system based on primer combinations were as follows:

Primer sequence
MbE3F1: 5'-CTGCAGTTCATCTCGGAATG-3'
MbE3R1: 5'-GGAAGCCCAGCTCCTTGTAG-3'
MbE3R2: 5'-CCCAGCTCCTTGTAGTTGGA-3'
Reaction system
3a: amplification using primer set MbE3F1-MbE3R1
3b: amplification using primer set MbE3F1-MbE3R2
PCR of a template DNA of human origin using these primers is expected to amplify a 145 bp DNA fragment in the 3a system and a 140 bp DNA fragment in the 3b system. In contrast, PCR of a template DNA of animal origin should fail to give an amplification product in either reaction system.

Human and animal DNA samples. Human DNA was isolated from the leukocytes present in 2 ml of peripheral blood of 30 healthy individuals by the phenol-chloroform (P-C) method and dissolved in 100 μl of Tris-EDTA buffer (TE). DNA from K562 cells was used as a human standard at concentrations of 1 ng-1 pg/μl.

Animal DNA was similarly extracted from about 30 mg of muscle of 22 animal species: gorilla, crab-eating monkey, horse, cow, sheep, goat, pig, wild boar, raccoon dog, dog, cat, rabbit, guinea pig, hamster, rat, mouse, whale, chicken, pigeon, turtle, frog, and tuna. Solutions containing 50 ng/μl of the extracted DNA were prepared for use in PCR.

Dilution or storage of blood and extraction of DNA. Serial 10-fold dilutions of up to 1:100,000 of peripheral blood from 3 healthy individuals were prepared with distilled water. DNA was extracted

Table 1 The amino acid sequences of the myoglobins of human and various animals (portions corresponding to exon 3).

	106-FISEC	IIQVLQSKHP	GDFGADAQGA	MNKALELFRK	DMASNYKELG	FQG-153
Human (P02144)						
Gorilla (P02147)	*****	**********	**********	**********	**********	***
Cow (P02192)	***DA	**H**HA***	SN*A******	*S*******N	*A*EK**V**	*H*
Horse (P02188)	***DA	**H**H****	*N********	*T*******N	*I*AK*****	***
Sheep (P02190)	***DA	**H**HA***	SN********	*S*******N	***AE**V**	***
Pig (P02189)	****A	**********	**********	*S*******N	***AK*****	***
Dog (P02158)	***DA	*********H	S*****TEA*	*K*******N	*I*AK*****	***
Rabbit (P02170)	****A	**H**H****	*********A*	*S*******N	*I*AQ*****	***
Mouse (P04247)	****I	**E**KKR*S	**********	*S*******N	*I*AK*****	***
Seal (P02162)	****A	**H**H****	AE*******A*	*K*******N	*I*AK*****	*H*
Whale (P02185)	****A	**H**H*R**	**********	**********	*I*AK*****	Y**
Chicken (P02197)	****V	**K*IAE**A	A*****S*A*	*K********D	****K***F*	***
Turtle (P02202)	**C*I	*VK*IAE***	S*****S*A*	*K********N	****K***F*	*L*
Tuna (P02205)	L***V	LVK*MHE*AG	L*--*GG*T*	LRNVMGIIIA	*LEA******	*S*

SWISS-PROT accession number is given in parenthesis.

from 10 μl of each dilution by the P-C method, and a fourth of the extracted DNA was used as a template for PCR.

Alternatively, peripheral blood from 3 healthy individuals was sealed in 5-ml vials and stored at 4 °C, room temperature, or 37 °C in the dark for up to 6 months. DNA was extracted from 10 μl of each sample of stored blood by the P-C method, and a fourth of the extracted DNA was used as a template for PCR.

Preparation of heated or aged bloodstains and extraction of DNA. Bloodstains were made on cotton cloth using 10 μl of peripheral blood from 3 healthy individuals. These bloodstains were heated at 150, 170, or 200 °C for up to 6 h. DNA was extracted from each bloodstain by the P-C method, and a fourth of the extracted DNA was used for PCR.

DNA samples of aged bloodstains were also obtained from one square centimeter of each of the three 26-year-old bloodstains on cotton cloth stored in the dark at room temperature by the P-C method, and a twentieth of the extracted DNA was used as a template for PCR.

Conditions of PCR and electrophoresis. Each PCR mixture (25 μl) contained 2.5 μl of a template DNA solution, 0.15 μl of 5 units/μl Ex Taq (Takara Shuzo Co., Ltd., Otsu, Japan), 2.5 μl of PCR buffer distributed with Ex Taq, 1 μl of 2.5 mM each of dNTP mixture, 0.25 μl of 20 mg/ml of bovine serum albumin, 0.25 μl of 50 μM each primer, and 18.1 μl of sterile distilled water. After preheating the mixture at 95 °C for 3 min, PCR was performed for 35 cycles of denaturation at 94 °C for 40 sec, annealing at 63 °C for 1 min, and extension at 72 °C for 1 min.

The PCR products were electrophoresed on an 8% polyacrylamide gel at 200 V for 70 min, and the gel was examined for the presence or absence of amplification products after being stained with ethidium bromide.

Sequencing of amplification products. Amplification products were electrophoresed on a 1% agarose gel and the target fragments were recovered from the gel. They were then sequenced with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Forster City, CA, USA) using the PCR primers as the sequencing primers.

Results

In reaction systems 3a and 3b, PCR of template DNA from 30 human subjects each gave an amplified product, which was observed as a single band on electrophoresis. Fig. 1 depicts the results of PCR of DNA from 10 different subjects in reaction system 3a. Sequencing of 5 amplification products showed that their sequences all

Table 2 The deduced sequences of exon 3 of the myoglobin gene in human and various animals and the regions for which the primers were designed.

[a]Human (X00373)	TTCATCTCGGAATGCATCATC	~	ATGGCCTCCAACTACAAG ~
[b]Gorilla (P02147)	**Y**HWSN**R**Y**H**H	~	*****NWSN**YWMN**R ~
[a]Cow (D00409)	**********TGC*******	~	GC****GAG**G****** ~
[b]Horse (P02188)	**Y**HWSN**YGCN**H**H	~	**Y**NG*N**RWMN**R ~
[b]Sheep (P02190)	**Y**HWSN**YGCN**H**H	~	*****NG*NG*RWMN**R ~
[a]Pig (M14433)	********A***GC*******	~	*****GG****G****** ~
[b]Dog (P02158)	**Y**HWSN**YGCN**H**H	~	**H**NG*N**RWMN**R ~
[b]Rabbit (P02170)	**Y**HWSN**RGCN**H**H	~	**H**NG*NC*RWMN**R ~
[a]Mouse (X04417)	**********ATT******	~	**T***G****G****** ~
[a]Seal (V00473)	********A***GC*******	~	**C**TG****A****** ~
[a]Whale (J03566)	**********GC*******	~	**C**TG****A****** ~
[b]Chicken (P02197)	**Y**HWSN**RGTN**H**H	~	*****NWSN**RWMN**R ~
[b]Turtle (P02202)	*******GY***ATH***G*N	~	*****NWSN**RWMN**R ~
[a]Tuna (AF291838)	**G*********GT*C**G**	~	C***A*G**********A ~
	→ MbE3F1		← MbE3R2
			← MbE3R1

[a]GenBank or [b]SWISS-PROT accession number is given in parenthesis.
H: A, C, or T; M: A or C; N: A, C, G, or T; R: A or G; S: G or C; W: A or T; Y: C or T.

agreed with that of human myoglobin exon 3 reported by Weller *et al.* (GenBank accession number X00373). In the supplemental experiment using DNA obtained from the muscle of 2 autopsied bodies, expected amplified products were also observed.

As with PCR of human DNA, PCR of gorilla DNA in reaction systems 3a and 3b yielded an observable amplification product. None of the DNAs from the remaining 21 kinds of animals gave a detectable amplification product (Fig. 2, Table 3).

When we amplified the human-specific sequence in the myoglobin gene using various concentrations of human standard DNA, we were able to identify a 140–145 bp amplified DNA fragment per reaction system resulting

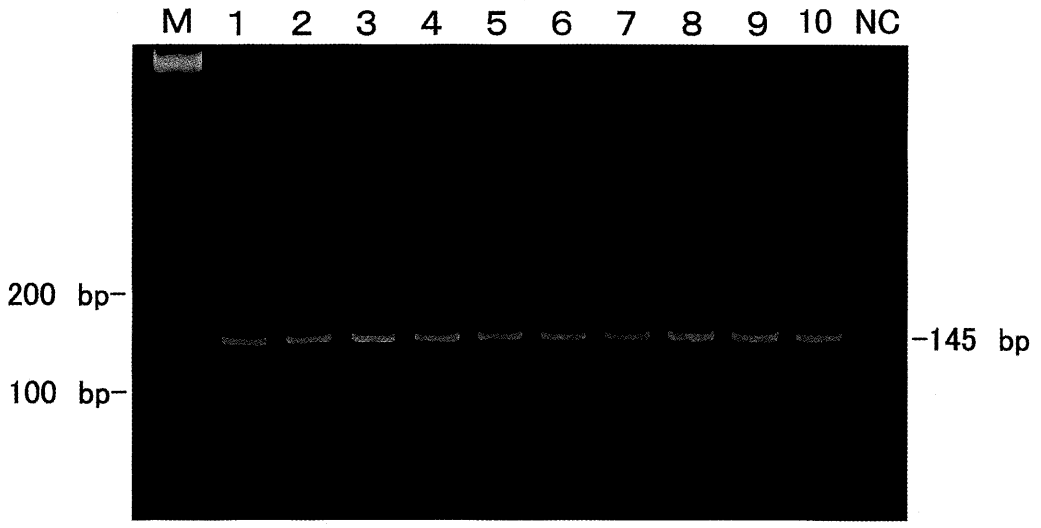

Fig. 1 Detection of a human-specific sequence in the myoglobin gene using human lymphocyte DNA from 10 different subjects (Reaction 3a). NC: negative control (no template DNA was added); M: 100 bp ladder.

Fig. 2 Amplification of a human-specific sequence in the myoglobin gene from DNA samples of various animals (Reaction 3b). 1: human; 2: gorilla; 3: crab-eating monkey; 4: horse; 5: cow; 6: dog; 7: cat; 8: rat; 9: chicken; 10: tuna; NC, negative control (no template DNA was added); M: 100 bp ladder.

Table 3 Amplification of a human-specific sequence in the myoglobin gene from DNA samples of various animals.

	145 bp fragment (Reaction 3a)	140 bp fragment (Reaction 3b)
Human	+	+
Gorilla	+	+
Crab-eating monkey	−	−
Horse	−	−
Cow	−	−
Sheep	−	−
Goat	−	−
Pig	−	−
Wild boar	−	−
Raccoon dog	−	−
Dog	−	−
Cat	−	−
Rabbit	−	−
Guinea pig	−	−
Hamster	−	−
Rat	−	−
Mouse	−	−
Whale	−	−
Chicken	−	−
Pigeon	−	−
Turtle	−	−
Frog	−	−
Tuna	−	−

from a template DNA of at least 25 pg (Fig. 3). When we used DNA extracted from serial dilutions of human blood from 3 different subjects for PCR, we were able to detect the target DNA sequence from at least a 1:1,000 dilution of blood.

When we used blood samples stored at 3 different temperatures in the dark, we were able to clearly detect the human myoglobin gene-specific sequence after storage for up to 4 months at 4 °C, room temperature or 37 °C. In samples stored for 6 months at room temperature or 37 °C, electrophoretic bands of amplified products were still discernible, although less intense than those after storage at 4 °C. Experiments with the DNA from 3 different subjects produced the same result (Fig. 4).

In experiments with bloodstains heated at 150 °C, the target DNA was amplifiable in both reaction systems 3a and 3b up to 4 h after heating. However, at 170 °C and 200 °C, the human myoglobin gene-specific sequence was detectable up to 30 min and up to 3 min after heating, respectively (Fig. 5). The same result was observed among bloodstains derived from 3 different subjects.

When we amplified the human myoglobin gene-specific sequence using 3 bloodstains that had been stored at room temperature for 26 years, we were able to detect clear

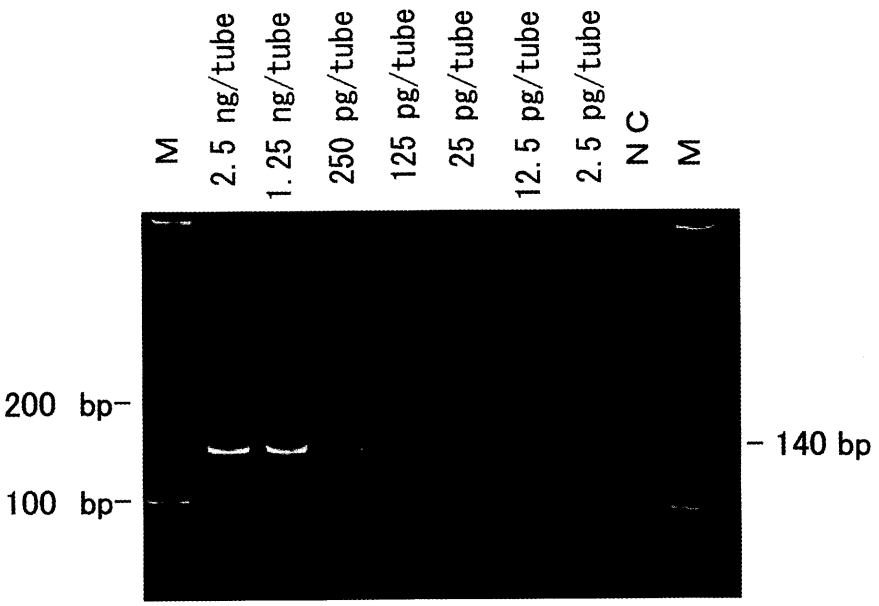

Fig. 3 Detection of a human-specific sequence in the myoglobin gene using human DNA of various concentrations (Reaction 3b). NC: negative control (no template DNA was added); M: 100 bp ladder.

bands by electrophoresis from all samples (Fig. 6).

Case Report

A 54-year-old man was strangulated. An accomplice of the murderer was asked to dispose of the victim's body, which he carried to a beach and burned all night, sprinkling kerosene on it and beating it bluntly. The burnt remains were dumped into the sea. A month later, a broken bone fragment, partly charred, was recovered from the sea. Human identification of this bone fragment became an important forensic issue. We filed the bone into powder first, extracted DNA from about 80 mg of the powder, and dissolved it in 10 μl of TE. Using this sample, we were able to detect the human-specific sequence in reaction systems 3a and 3b (Fig. 7).

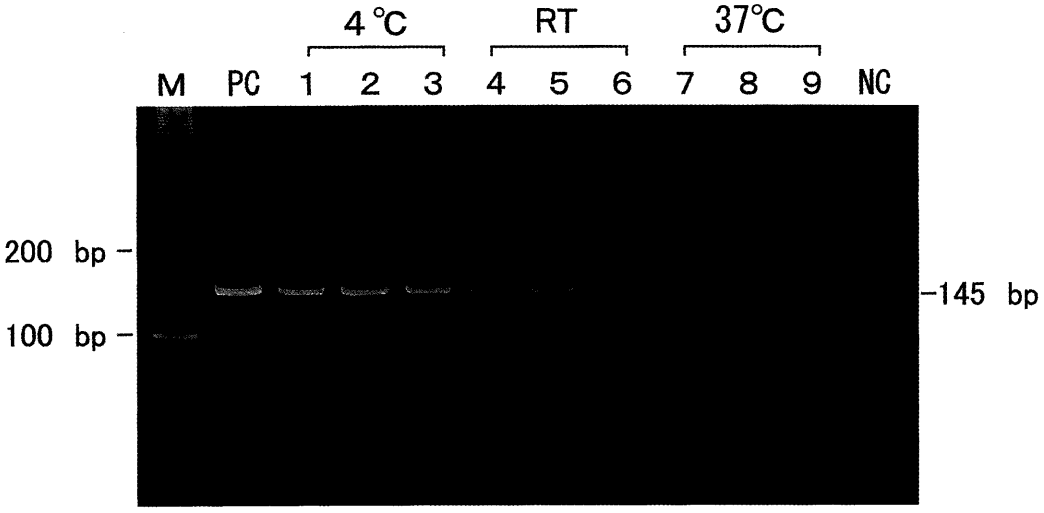

Fig. 4 Detection of a human-specific sequence in the myoglobin gene from human blood samples preserved at 3 different temperatures for 6 months (Reaction 3a). 1, 4, 7: Subject A; 2, 5, 8: Subject B; 3, 6, 9: Subject C; PC: untreated human blood; NC: negative control (no template DNA was added); M: 100 bp ladder; RT: room temperature.

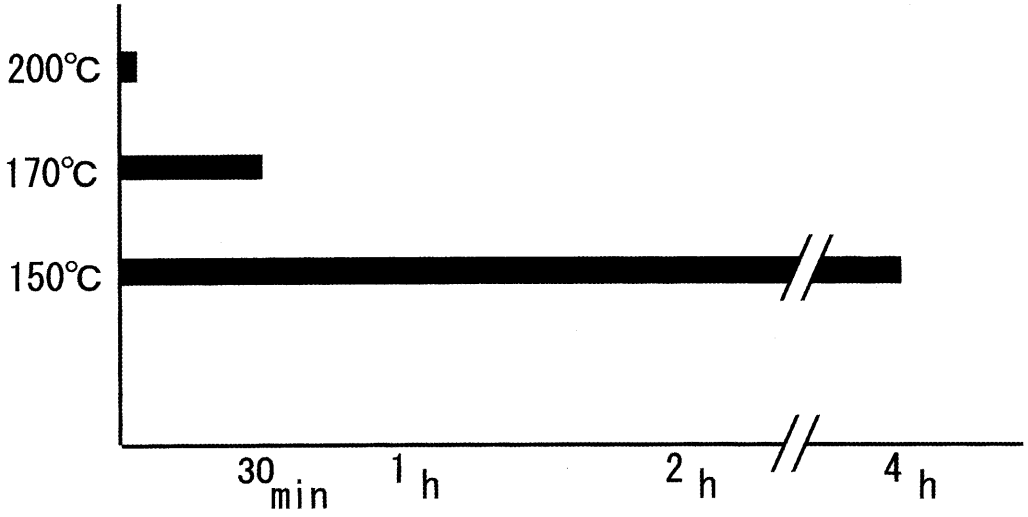

Fig. 5 Detectability of a human-specific sequence in the myoglobin gene from burnt bloodstains.

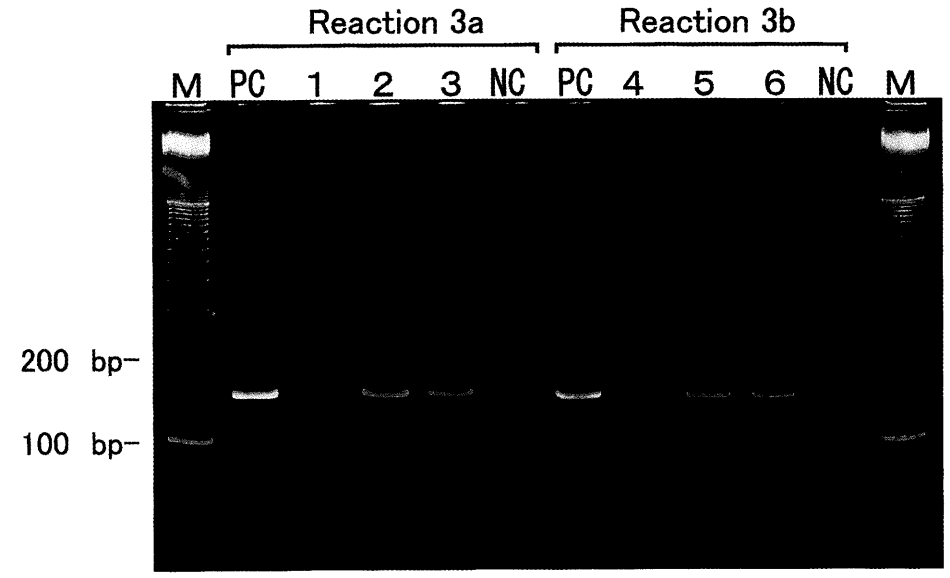

Fig. 6 Detection of a human-specific sequence in the myoglobin gene from 3 human bloodstains preserved at room temperature for 26 years. 1, 4: Specimen A; 2, 5: Specimen B; 3, 6: Specimen C; PC: human DNA; NC: negative control (no template DNA was added); M: 100 bp ladder.

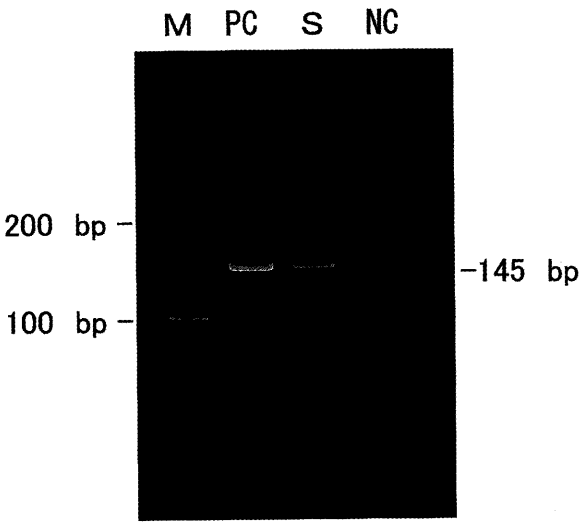

Fig. 7 Detection of the human-specific sequence in the myoglobin gene from a bone fragment recovered from the sea (Reaction 3a). The body to which the bone belonged had been burned and the remains had been dumped into the sea a month earlier. S: DNA extracted from the bone fragment; PC: human DNA; NC: negative control (no template DNA was added); M: 100 bp ladder.

Discussion

In this study we took special note of the difference in amino acid sequences between human and animal myoglobins, and deduced the human-animal base substitutions in the myoglobin gene. Then we attempted to develop a simple and highly specific method for human identification by PCR with primers complementary to the region of these substitutions.

Using this method to amplify DNA from 30 healthy subjects in reaction systems 3a and 3b, we were able to electrophoretically detect single bands of approximately 140–150 bp amplicons, as was expected theoretically. In fact, sequencing of amplicons obtained from 5 different DNA samples showed that their sequences were in complete agreement with the reported one (X00373). These results indicate that our method provides theoretically correct amplicons.

Amplification of DNA from 22 animal species by our method gave no products in either reaction system 3a or 3b in 21 species. However, the gorilla sample could not

be distinguished from the human sample. Immunological discrimination between anthropoids and humans has been considered difficult. In our experience with immunological detection of human myoglobin, it has not been possible to distinguish a gorilla sample from a human sample [14]. Concerning the discrimination between anthropoids and humans by DNA analysis, when applying the analysis of VNTR or STR polymorphisms, the distinction was possible by simultaneous analysis of multiple loci [7, 9, 10], but analysis of a single locus could not give reliable results. Applying the ABO genotyping reported by Crouse and Vinek, an orangutan sample was distinguishable from a human sample, but chimpanzee and gorilla samples were not [8]. On the other hand, Lin *et al.* claimed that they could distinguish chimpanzee and gibbon samples from a human sample. However, their ABO genotyping procedure was very complicated in that it combined PCR with RFLP analysis using 2 restriction enzymes [11].

The method described here is very simple and does not require a special kit or analytical instruments. However, it is not likely to show specificity for gorilla, chimpanzee, and gibbon. This is due to the fact that the amino acid sequences in the region for which the primers were designed are the same in humans and these anthropoids, and, further, that these species are close in phylogeny. Our experimental test on 22 kinds of animal DNA proved that the gorilla sample was not distinguishable from the human sample. Orangutan, which belongs to the class of anthropoids including gorilla, chimpanzee, and gibbon, has a myoglobin in which the amino acid at position 110 differs from that in human myoglobin at this position: cysteine in humans and serine in orangutan. This suggests the possibility of discriminating orangutan from human. The amino acids of gorilla, chimpanzee, and gibbon at positions 22, 116, and 23, respectively, differ from those in humans at these positions. Therefore, if new primers were designed for these regions, these anthropoids could be distinguished from humans.

A review of reports on the myoglobin amino acid sequences of primates excepting anthropoids shows that they have replacements at position 110 (cysteine → serine or alanine), at position 144 (serine → alanine), and at position 145 (asparagine → lysine). In addition, our method was able to discriminate between the crab-eating monkey and the human. These facts suggest that our method has the potential to distinguish these primates from human.

Animals other than primates used in this study, including the horse, cow, sheep, pig, dog, rabbit, rat, mouse, whale, chicken, turtle, and tuna, had their myoglobin amino acid sequences reported. The myoglobin amino acid sequences of all of these animals differ from that of human at positions 110, 144, and 145. Therefore, as was expected, samples from these animals were easily distinguishable from those from humans. On the other hand, the myoglobin amino acid sequences of the wild boar, raccoon dog, cat, guinea pig, hamster, pigeon, and frog, also included in this study, have not been documented. However, it was presumed that these animals could be distinguished from human by the present method, considering that they are related to animals for which the myoglobin amino acid sequences are known, and this presumption was experimentally proved.

Myoglobin amino acid sequences have been documented for many animals unavailable for the present study, including the deer, elephant, fox, seal, fur seal, dolphin, lace monitor lizard, crocodile, shark, *etc.*, and amino acids at positions 110, 144, and 145 differ from those of human. Therefore, by the present method, amplification of the human-specific base sequence in the myoglobin gene would not occur using DNA samples from these animals.

In light of the foregoing, we consider that the present method can distinguish all the animals except gorilla, chimpanzee, and gibbon from human. As it is exceptionally rare in forensic practice that one must distinguish anthropoid samples from human samples, the present method can be said to have excellent species specificity.

The quantity of the template DNA required for PCR-based human identification has been reported to be 100 or 500 pg in methods using STR polymorphism analysis [4, 10]. Naito *et al.* and Lin *et al.* reported that they were able to identify human origin using 1 and 10 pg of template DNA, respectively [3, 11]. The present method detecting the human-specific sequence required 25 pg of template DNA per tube, with either primer set. This sensitivity was lower than that reported by Naito *et al.* or Lin *et al.*, but was comparable to that for various single-locus DNA polymorphisms [4, 10, 15–20]. Assuming more practical cases, we examined the sensitivity of our method using dilutions of blood. From the results of this experiment, it was theoretically calculated that 0.01 μl of whole blood, or about 10 nucleated cells, was sufficient to amplify the target sequence. Considering the recovery in DNA extraction,

these amounts corresponded with the above-mentioned template DNA required, and the present method seemed to be sufficiently sensitive.

In forensic practice, biological specimens for human identification have often undergone DNA fragmentation as a result of putrefaction, heating, or aging. We therefore investigated the applicability of the present method to specimens kept under various environmental conditions.

When amplifying the human-specific sequence in the myoglobin gene from blood samples stored in the dark at 4 °C, room temperature, or 37 °C, we could detect the target DNA fragment after 6 months of storage at 37 °C without any problems. Lygo *et al.* reported that they were able to detect STR polymorphisms from bloodstains stored at 56 °C in an atmosphere of 100% humidity for up to 11 days, but not after 28 days of storage [4]. Dimo-Simonin and Brandt-Casadevall successfully detected the DNA loci of PolyMarker from cadaveric blood that had been stored at 4 °C for 10 days and subsequently left at room temperature for 30 days [19]. Sasaki *et al.* described that they were able, to some extent, to detect Y27H39 even from bloodstains, taken from cadaveric blood, kept at room temperature for 11 months. [17]. Our method of human identification bears similarity to theirs, and is considered to be well applicable to putrefied specimens.

We investigated the thermostability of DNA by sex determination based on simultaneous detection of DYZ3 and DXZ1, which are multicopy sequences, and reported that the target DNA fragments could be detected from bloodstains heated at 150 °C for 30 min, but not from those heated at 200 °C for 30 min [21]. This thermostability of DNA was similarly observed in the detection of alleles of IgA2, a single-copy gene [18]. In the present study we were able to detect the human-specific sequence in the myoglobin gene from bloodstains heated at 150 °C for up to 4 h, which was longer than the heating period yielding above-mentioned results. When heated at 200 °C, organic substances became rapidly charred, and the target DNA fragment was unamplifiable 4 min later. This thermostability did not conflict with that hitherto reported by us [21]. In addition, in the case of heating at 170 °C, the target DNA fragment was detectable for up to 30 min. These results show that the present method can be applied to heated specimens.

In many studies on the detection of DNA polymorphisms from aged bloodstains, satisfactory results have been reported [4, 10, 17, 18, 21]. In the present study we were also able to amplify the target DNA sequence, human-specific in the myoglobin gene, from 26-year-old bloodstains. Under the experimental conditions of the present study, a sufficient quantity of template DNA remained in a 5 mm^2 bloodstain. This suggests that human identification from older bloodstains by the present method will be possible if larger bloodstains are used.

The method for human identification described in this paper involves one round of 35 cycles of PCR followed by staining with ethidium bromide, and its detection sensitivity is not extremely high. Moreover, the region detected is a single-copy sequence. Nevertheless, the present method, which detects the human-specific sequence in the myoglobin gene, produced satisfactory results as described above, even when applied to forensic biological materials kept under various conditions. This is probably because the length of DNA detected was as short as 140–145 bp. The length of the target sequence has been considered relevant to whether a DNA fragment is amplifiable from forensic materials [22].

We consider the present method to be an excellent means of human identification, and therefore applied it to a bone fragment in a practical case. Reports on DNA analysis of bone specimens include the detection of the COL2A1 3' variable region from skeletal remains by dual PCR [23], detection of the D18S535 locus from a 10-year-old bone [20], successful sex determination of 20-year-old bones [17], and personal identification by detection of 9 STR loci from skeletal remains of 2 of Che Guevara's guerrillas who had died about 30 years earlier [24]. Concerning DNA analysis of burned bones, Cattaneo *et al.* reported that they were unable to detect the mtDNA region V from burned bones in practical cases or from the bones that had been experimentally burned at 800–1,200 °C for 20 min [25]. In the present case, a bone fragment was recovered under unusual circumstances: a body had been burned with kerosene all night and the burnt remains had been submerged in seawater for about a month. Morphological examination suggested that the bone fragment was part of the human left femur, but its species could not be identified by serological methods. By applying the present method, however, the bone fragment was identified as having a human origin, and demonstrated the practical utility of our method.

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