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TECHNICAL NOTE

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Species identification by means of pyrosequencing the mitochondrial 12S rRNA gene

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Abstract The aim of this study was to develop a new method for species identification based on the analysis of a very short nucleotide sequence. For this reason, the mitochondrial 12S rRNA gene, together with the new method of pyrosequencing, was used. The detection of only 20 nucleotides, following the sequencing primer within a 149-bp fragment by pyrosequencing, was sufficient to identify the biological origin of the samples by alignment with a reference sequence database. A case example with a piece of skin is presented, and the question whether this piece of skin came from a missing wife or from an animal could be answered.

Keywords Species identification · Pyrosequencing · Mitochondrial DNA · Sequence analysis · 12S rRNA gene

Introduction

Most of the stain material analysed in forensic genetics is of human origin [2, 9]. However, in some cases, the identification of the biological origin of a specimen is necessary. Traditionally, tests for species identification are based on immunological methods with antibodies against soluble proteins specific for various species. Therefore, these methods are limited to stain material containing extractable proteins. Furthermore, only species for which antibodies are available can be identified.

Today, DNA typing allows the reliable identification of a species by comparative sequence analysis. Several methods using restriction fragment length polymorphism (RFLP) or sequence variations of the mitochondrial cytochrome *b* gene have been proposed [8, 15]. Sequence determination is commonly carried out by using dideoxy chain termination technology [11]. Pyrosequencing is a new sequencing methodology for the determination of a short DNA sequence. This technique is based on the detection of pyrophosphate (PPi) that is released from dNTPs during the DNA synthesis. Based on a cascade of enzymatic reactions, visible light is generated and detected. The amount of light is directly related to the number of incorporated nucleotides [10].

In this study, we amplified and sequenced a 149-bp fragment of the mitochondrial 12S ribosomal RNA (rRNA) gene from 11 different species including seven mammals, one fish and three birds. The sequence information was used for species identification. The gene for the 12S rRNA is between 945 and 975 bp long (depending on the species). In the secondary structure of the 12S ribosomal RNA, single-stranded loop regions and double-stranded regions alternate. Both regions are subjected to different selective pressure. The loop regions evolve much faster and display a high degree of variation between species, whereas the double-stranded regions are highly conserved [7, 14]. Due to this property, rRNA is extremely well suited for species identification, and the 12S rRNA gene is already used for nonforensic species identification [3, 4]. The highly conserved regions can be used as primer-binding sites. These primers bind to the 12S rRNA gene of many different species without any mismatch.

Furthermore, a case example with a piece of skin is presented. The question was whether this piece of skin came from a missing wife or from an animal.

Materials and methods

Extraction

Carnal specimens were obtained from 93 different animals. The DNA from 2 g of each sample was extracted using the phenol/chloroform method.

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Amplification

We analysed a 149-bp-long fragment from the mitochondrial 12S rRNA gene. The forward primer was 5′-CAAAC TGGGATTAGATACC-3′, which binds from position 1,067 to position 1,085 in the human mtDNA. The reverse primer was Biotin-5′-TAGAACAGGCTCCTCTAG-3′, which binds from position 1,194 to position 1,211 in the human mtDNA. The DNA extract (1 μl) was amplified in a final volume of 50 μl containing 1× PCR reaction puffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl), 200 μM of dNTP, 2.5 U AmpliTaq gold polymerase (Applied Bio Systems), 2 mM $MgCl_2$ and 0.1 μM each primer. PCR was carried out with 30 cycles at 95°C for 60 s, 60°C for 60 s and 72°C for 30 s in a 2,400 Gene Amp Cycler (Applied Bio Systems). The initial denaturation step was 95°C for 10 min and the final extension step was 72°C for 10 min.

Template preparation for pyrosequencing

The biotinylated DNA strands were immobilised at room temperature on streptavidin-coated sepharose beads (streptavidin sepharose, Amersham Bioscience Ltd.), 20 μl of the biotinylated PCR product was transferred into a tube containing 40 μl of water and 2 μl streptavidin sepharose beads suspended in 40 μl binding buffer (10 mM Tris–HCl, 2 M NaCl, 1 mM EDTA, 0.1% Tween 20, pH 7.6). This solution was incubated under gentle agitation at room temperature using a shaker for 5 min. Subsequently, NaOH was added for denaturation of DNA and the nonbiotinylated strands were removed by using the Vacuum Prep tool and the PSQ HS 96 system (Pyrosequencing AB, Uppsala) according to the manufacturer's instructions.

Sequencing

The sequencing primer was 5′-ATTAGATACCCCACTA TGC-3′. This primer was annealed for 2 min at 80°C to the template strand. The sequence reaction was carried out by using the PSQ 96 reagent kit (Pyrosequencing AB) according to the manufacturer's instructions and was analysed in the PSQ 96 HS pyrosequencing instrument.

Table 1 Overview of mammals, fish, and birds 12S-rRNA gene sequences in public nucleotide databases (January 2005)

Species	Accession no.	Length (bp)	Position 12S gene
Man (*Homo sapiens*)	AY275537	16,568	650–1,603
	AY275536	16,568	
Cow (*Bos taurus*)	AY684273	16,322	431–1,385
	MIBTXX	16,338	
Sheep (*Ovis aries*)	AY858379	16,620	69–1,026
	AJ849534	711	
	AF091699	959	
Hare (*Lepus timidus*)	AY292702	709	68–1,023
	ABO58613	832	
	ABO59258	832	
	LEU421471	17,734	
	NC001913	17,734	
Rabbit (*Oryctolagus cuniculus*)	AY292691	709	70–1,026
	AY001588	17,245	
	OCRPTRG	3061	
Horse (*Equus caballus*)	X79547	16,660	71–1,045
	AY584828	16,596	
	AY012147	1,040	
Pig (*Sus scrofa*)	AY334492	16,751	1,246–2,205
	AY337045	16,585	
Chicken (*Gallus gallus*)	MIGGX	16,775	1,297–2,272
	AP003318	16,785	
	AP003317	16,788	
	AB086102	16,784	
	AJ849444	450	
Duck (*Anas anser*)	AY164530	989	69–1,056
	AFU88732	984	
	APU59666	982	
	DUKMTRG	538	
	DUKTGRG	3,919	
Turkey (*Meleagris gallopavo*)	MGA490508	403	69–1,056
	MGU83741	971	
Salmon (*Salmo salar*)	AF133701	16,669	69–1,015
	U12143	16,660	
	NC001960	16,660	

Results

In this study, the 12S gene sequence was investigated as a marker for the identification of 11 different species from three different classes: mammals, fishes and birds. By comparison from the 12S sequences that could be found in the public sequence databases (Entrez from the National Center of Biotechnology Information, http://www.ncbi.nlm.nih.gov/BLAST), we were able to define highly conserved regions in the 12S gene that can be used as primer-binding sites. Table 1 shows the accession numbers and the position of the 12S rRNA gene in the database sequences. The primers were chosen in a way that the 20–25 bases adjacent to the sequencing primer displayed a high degree of polymorphism. These regions proved to be well suited for species differentiation. We used pyrosequencing for the determination of these short stretches, which is a new sequencing method that is well suited for the sequencing of short stretches located next to the sequencing primer.

We collected our own sequence data so that we could compare our results. The tissue samples from 93 different animals (ten humans, 22 cows, seven sheep, 15 pigs, five rabbits, five hares, two horses, 11 chickens, five ducks, six turkeys, five salmon) were amplified and sequenced. Next, all 93 samples were aligned and compared with the public sequence databases (Entrez from the National Center of Biotechnology Information) using the BLAST software package [12]. No differences could be found between the animals we analysed and the database entries. Table 2 shows

Table 2 The sequence of 20–23 bp after the sequencing primer for identification of the investigated species

Species (no. of probes)	Sequence	Position of first base in the 12S gene
Human (10)	TTAGCCCTAAACCTCAACAG	1,095
Rabbit (5)	TTAGCCCTAAACTTTGATAA	516
Sheep (7)	TTAGCCCTAAACACAAATAATTA	441
Hare (5)	TTAGCCTTAAACCTAAATAATTT	447
Cow (22)	TTAGCCCTAAACACAGATAATTA	870
Horse (2)	TTAGCCCTAAACTAAAATAGCTTA	447
Pig (15)	CTAGCCCTAAACCCAAATAGTT	1,690
Salmon (5)	CTAGCCGTAAACTTTGATGGAA	512
Turkey (6)	CTGGCCCTAAATCTTGATACTAA	551
Duck (5)	CTGGCCCTAAATCTTGATACTTAC	551
Chicken (11)	CTAGCCCTAAATCTAGATACCTCCC	1,756

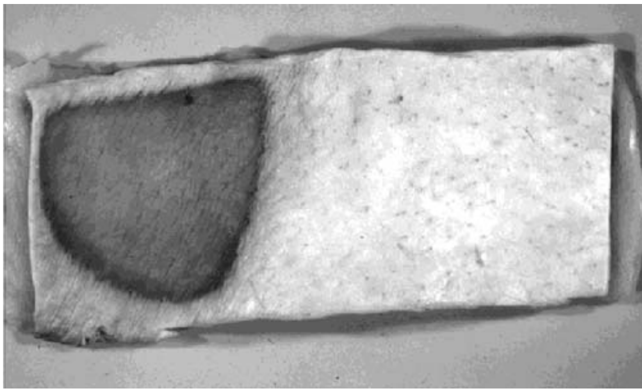

Fig. 1 Photo of the piece of skin. The discoloration on the *left side* is freezer burn

the sequences obtained by using our pyrosequencing method for the species under investigation.

Case example

In a case of a suspected cannibal, we received a piece of skin (Fig. 1), which was found frozen in the suspect's kitchen. The question was whether this piece of skin came from the missing wife of the suspect (and thus being of human origin) or from an animal. When the human-specific STR systems yielded no results after testing, pyrosequencing revealed a DNA sequence in the skin that was identical to the porcine sequence. Automatic base calling by the SQA software (version 2.0) gave a sequence in which the 21st base was evaluated as the last position, which furthermore could not be explicitly identified as CC and therefore was denoted as C (Fig. 2). On account of this the biological origin of this investigated skin was identified as a pig.

Discussion

Species identification using DNA analysis has already been described by many authors [1, 5, 6, 13, 15] and has replaced or at least supplemented species determination by biochemical test in many laboratories.

Mitochondrial DNA has become a very powerful tool in forensic identification because of the high number of copies in each cell and the lack of recombination with paternal mtDNA. The high copy number results in an increased sensitivity of mtDNA testing compared to nuclear DNA investigations. For species identification, the amplification and sequencing of whole genes like a part of the cytochrome *b* gene has been described [8]. We developed a new test based on pyrosequencing of a short stretch of the mitochondrial 12S rRNA gene. The amplification and sequencing primers we used bind to a highly conserved region showing no variation between species, even between mammals, fish and birds. However, adjacent to this primer, 20–25 bases can be found, which are not conserved, and showed a high degree of variation. Pyrosequencing of these bases allows for discrimination between different species.

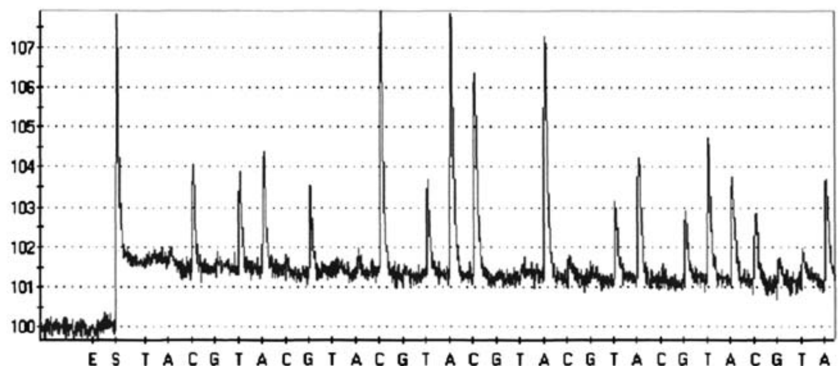

Fig. 2 Pyrogram of the piece of skin. The observed sequence was CTAGCCCTAAACCCAA ATAGT and was identical with the porcine sequence

We tested 93 individuals from 11 species, including seven mammals, one fish and three bird species. Interestingly, we did not find any single-nucleotide substitutions between individuals from the same species. Although there is a lot of variation between the species, no variation within one species is to be expected. However, all the species we investigated could be identified correctly except for turkey and duck. The reason for this is that the first sequence difference between these two species appears at the end of the analysed fragments (22 bases). The method of pyrosequencing is preferably suitable for the depiction of short sequences. As the dNTPs are added subsequently and a successful elongation immediately results in a light signal, the nonadded dNTPs have to be decomposed before the injection of the next base. The decomposition takes place due to the enzyme pyrophosphatase, which reduces during the progress of sequenceing and therefore the possible number of repetitions (=sequence length) limited. This may also be a limitation for the identification of closely related species such as duck and turkey.

One limiting factor in much forensic genetic analysis is the degradation of the DNA in the stain material. In such cases, the fragment length of the DNA extracted from the stain is reduced. The method we have developed requires only very short fragments and is therefore applicable even in cases with degraded template DNA. Due to the high degree of polymorphism of the rRNA, even very short fragments contain sufficient information for species identification. For the first time, the method of pyrosequencing was used for species identification in forensic casework. Pyrosequencing is very effective in sequencing short fragments. In contrast to "classical" DNA sequencing, even the first base after the primer can be determined with a high degree of accuracy.

During the process of pyrosequencing, an immediate signalling takes place. In case of a heteroplastic position or a mixed stain, both bases would be detected. Consequently, a shift in the reading frame for both sequences would result and a mixed stain would cause a signal by nearly every subsequent dNTP addition. The result would be a nonanalysable sequence. Therefore, a mixed stain or a heteroplasmic position are by means of pyrosequencing not identifiable.

In conclusion, the information content within the nucleotide sequence of a 20-bp fragment adjacent to the sequencing primer enabled the discrimination between all species used in this study to at least the level of the class (for duck and turkey).

In our case example, the origin of the unknown skin sample could be discovered with the database search. The result of our investigation was exonerating for the owner of the kitchen because the skin was porcine and not human.

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