DESIGN OF SPECIES SPECIFIC PRIMERS FOR AMPLIFICATION OF MITOCHONDRIAL DNA OF CROATIAN WILD ANIMALS

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Abstract

The goal of this paper was to design species specific primers for wolf (Canis lupus), fox (Vulpes vulpes), bear (Ursus arctos), roe deer (Capreolus capreolus), red deer (Cervus elaphus) and wild boar (Sus crofa). Primers have been designed using sequences of the listed species from the GenBank, using software BioEdit and Primer 3. We have defined the size and location of primers for each species, and size of the PCR product. With this species specific primers animal species can be identified using minimal quantities of different tissues using only DNA isolation, PCR and electrophoresis, so they can be used in a variety of ecological researches and in forensic cases.

Keywords: primer, mitochondrial DNA, control region, wild animal

Introduction

Scientific data about the presence of species in a specific area is fundamental ecological parameter and foundation for understanding population ecology. Signs of animal presence like feces, urine or hair are often the only source of information. To overcome those problems, noninvasive genetic research method was developed, using samples with low DNA quantity as a research objective for identification of the species.

MtDNA is present in all animal tissues, it has a small genome of a simple structure, no coding parts (introns) and it has different rates of evolution in all parts, which is important for solving philogenethic questions on different taxonomical levels (ZHANG and HEWITT, 1996). MtDNA is a circular, two-stranded molecule which varies in size from 15 000 to 20 000 base pairs (bp). Control region is non-coding part of the mtDNA, 1000 bp long with sequences that are acting in the beginning of replication and transcription of the mitochondrial genome (HARRISON, 1989; ODAK, 2004). MtDNA is prone to speed evolution and it is extremely valuable genetic marker in population and evolution biology. Control region doesn't code for protein synthesis and therefore is not part of a natural selection, which is one more reason why it is suitable for phylogenetic research.

Primer is a short, synthesized oligonucleotide which is used in molecular research. They are designed to recognize the precise sequence of DNA nucleotides, which is afterwards used as a model for PCR and amplifies the specific part of the strand. One of the most important factors for successful DNA amplification is a proper design of primers that are species specific.
The goal of this paper was to develop a noninvasive method for identification of animal species from samples with low quantity DNA. To reach that, our goal was to design primers for amplification of control region of mtDNA for different species of wild animals that are most common inhabitants of Croatian forests (red deer, fox, brown bear, wolf, roe deer and wild boar). Based on those primers we should be able to identify animal species using only DNA isolation, PCR and electrophoresis. That method would ease ecological researches and management of different animal species.

Materials and methods

For development of primers we have used sequences of mtDNA of mentioned animal species from the internet gene database - GenBank (www.ncbi.nlm.nih.gov/GenBank/). We have used three sequences of mtDNA control region of red deer (Cervus elaphus) (GenBabnk No EU004016, EU004017, EU004018), fox (Vulpes vulpes) (AF338801, AF338789, AF338800) and brown bear (Ursus arctos) (AB055141, AB055136, AB055139), and four sequences of mtDNA control region of wolf (Canis lupus) (FJ213916, FJ213913, FJ213914, FJ213915), roe deer (Capreolus capreolus) (DQ114763, DQ114760, DQ114761, DQ114762) and wild boar (Sus crofa) (EU979215, EU979212, EU979213, EU979214).

Using the program extension in Microsoft Word, MBCS1.2, we have transformed the mtDNA sequences into fast format. Using the programs BioEdit Sequence Aligment Editor, Version 7.0.9.0 (HALL, 1999) and ClustlW Multiple alignment (THOMPSON et al., 1994), we have compared the sequences of different animal species using the multiple alignment. Using internet program „Primer 3" http://biotools.umassmed.edu/bioapps/primer3_www.cgi we have designed primers obeying the rules for primer design (INNIS and GELFAND, 1990).

Results

Using computer programs and obeying rules for primer design, we have created primers for amplification of the mtDNA control region of THE red deer, fox, brown bear, wolf, roe deer and wild boar (Table 2 and Table 3).

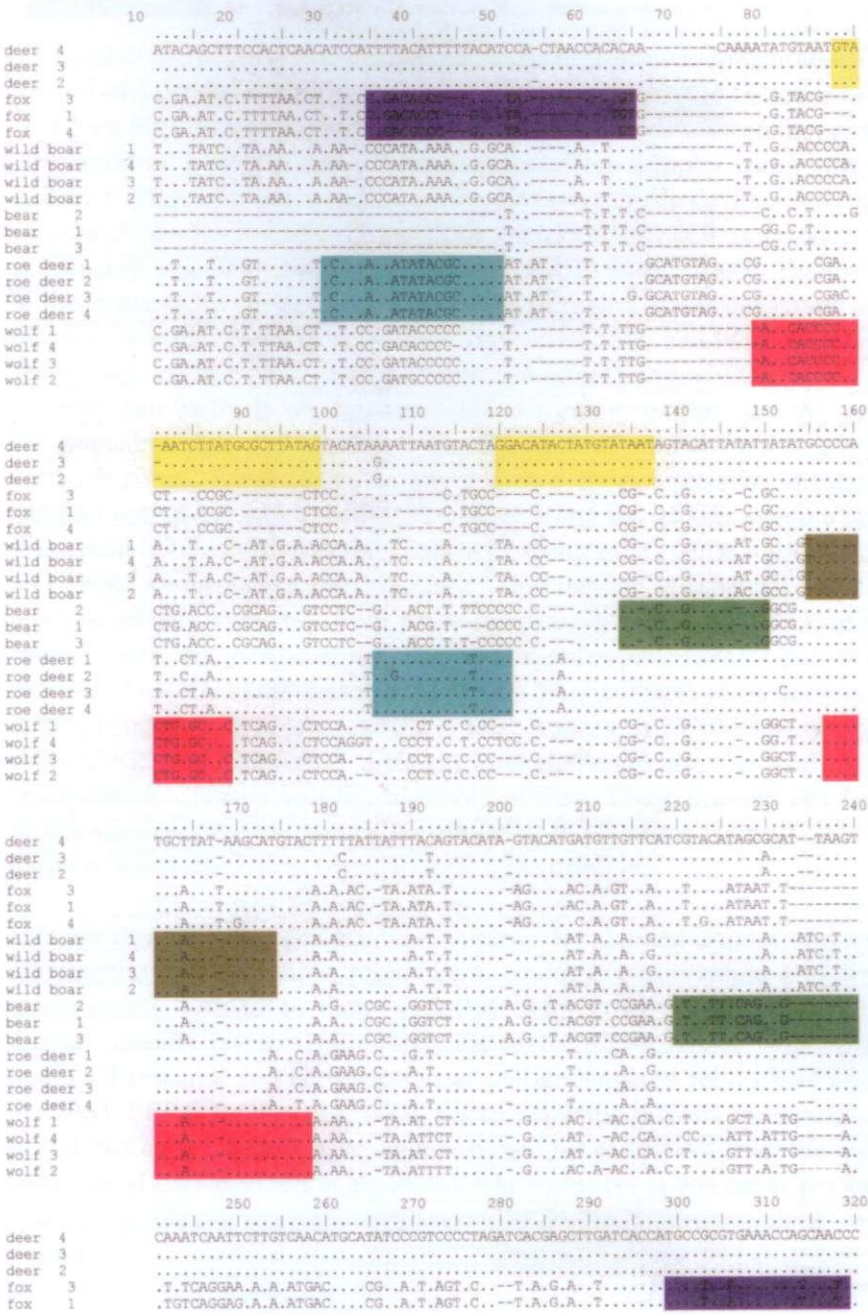
Table 2. Specific primers for defined species with nucleotide sequence, denaturation temperature, primer length and the expected length of PCR product.

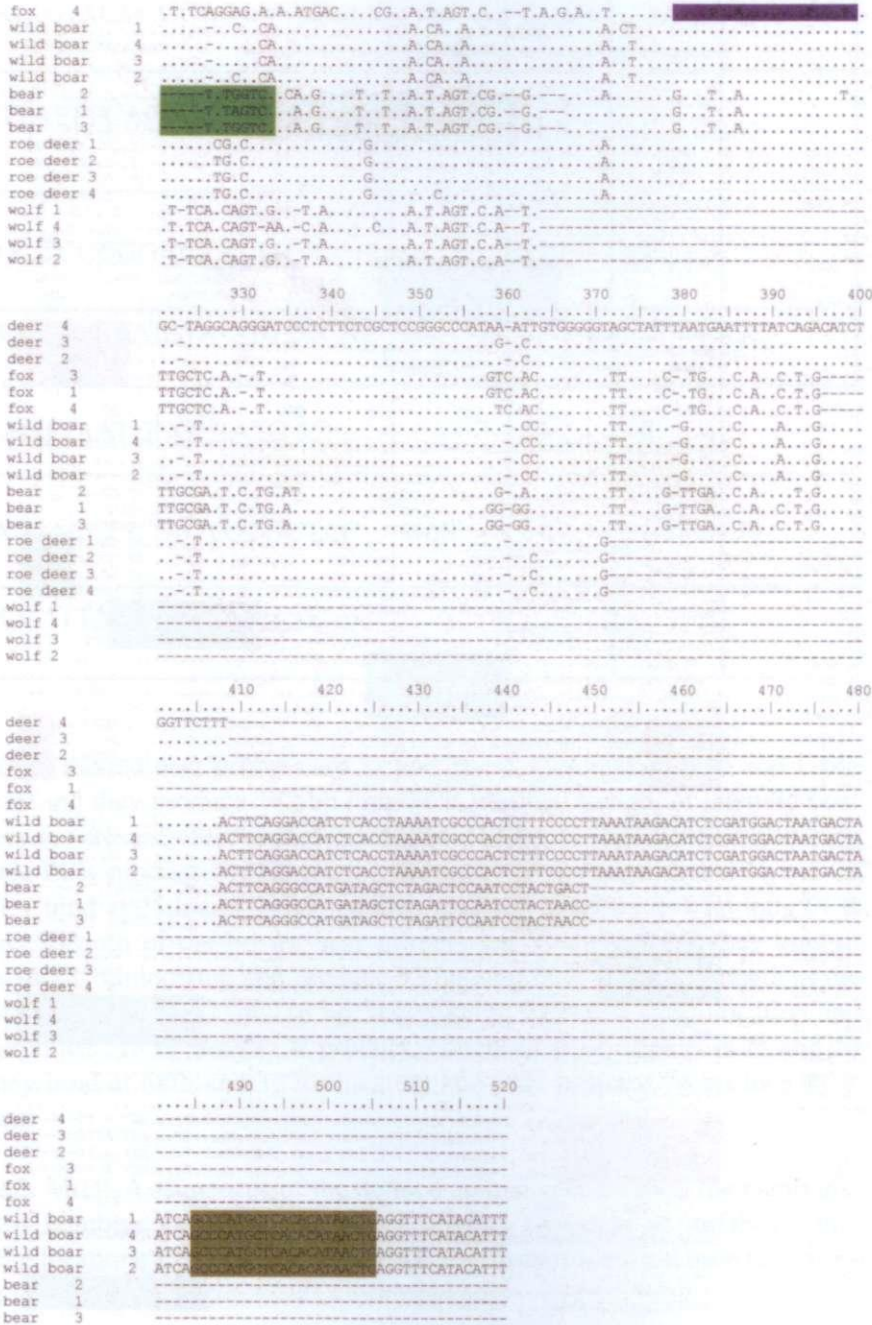
Nucleotide sequence in the primer	Animal species	Tm °C	Length of base pairs	Length of PCR product
5' GTAAATCTTATGCGCTTATAG 3'	Red deer	57.7	21	192
3' GGACGGGATATGCATGTT 5'		65.2	18	
5' GCCCATGCTCACACATAACTG 3'	wild boar	70.0	21	185
3' GTCCCGTAACCATTGACTGA 5'		66.9	20	
5'CTTGCCCTATGTACGTCGTGC 3'	fox	71.3	21	241

3' TAGAAACCCCCACGTTGACA 5'		69.8	20	
5' CTTATTTCAGGCGTATGGTCT 3'	bear	64.5	21	52
3' AGCTCCCGGACTAAGTG 5'		62.1	17	
5' ACCCAATTATATACGCTACAT 3'	roe deer	59.2	21	218
3' GACTTAATGCGCTATG 5'		53.2	16	
5' GAATCACCCCTACTGTGCTAC 3'	wolf	64.7	21	74
3' GCCATTAATGCACGACGTAC 5'		67.5	20	

Length of the red deer primers are 21 and 18 bp, they bind at 78th and 120th location and they produce 192 bp long PCR product. Length of the wild boar primers are 21 and 20 bp, they bind at 485th and 155th location and they produce PCR product 185 bp long. Length of the fox primers are 21 and 20 bp, they bind at 25th and 299th location and they produce 241 bp long PCR product. Length of the brown bear primers are 21 and 17 bp, they bind at 134th and 220th location and produce 52 bp long PCR product. Length of the roe deer primers are 21 and 16 bp, they bind at 21th and 106th location and they produce 218 bp long PCR product. Length of wolf primers is 21 and 20 bp, they bind at 68th and 157th location and they produce 74 bp long PCR product.

Table 3. MtDNA sequences of the defined animal species from the GenBank. Vertical numbers are the locations of aligned sequence of mtDNA. Only variable locations are shown, while dash (-) represents identical base according to the first sequence. Primers are color marked.





Discussion

The goal of this paper was to design species specific primers for amplification of the mtDNA control region of the most common inhabitants of Croatian forests (red deer, fox, brown bear, wolf, roe deer and wild boar). Based on those primers we should be able to identify animal species from samples using only DNA isolation, PCR and electrophoresis. Noninvasive methods for identification of animal species are mostly based on detection of DNA sequences, using sequencing and phylogenetic analysis (FARREL et al., 2000). Primers used in this process are designed for sequencing of region that is conserved among different species. Those primers aren't species specific and PCR can't be used for species identification, and therefore the sequencing of PCR product and phylogenetic analysis are required. By designing species specific primers we wanted to ease and accelerate identification of animal species present in Croatian forest, while using sample acquired by noninvasive methods and samples with low DNA quantity. Therefore, using the species specific primers it is possible to identify animal species by using PCR, while more expensive and longer sequencing can be avoided. This method is especially useful for analysis of carnivore diet from feces samples, in which the DNA of donor species, pray, microorganisms, parasites and arthropods usually can be found together. Analysis of those samples by using classical methods is not precise, it is time consuming and often results in false reading. If we use species specific primers for identification of different animal species for diet analysis from feces samples, results will be extremely precise. Those primers can be used in forensics and in cases where the subject of the research is small quantity of tissue in which the DNA degradation has occurred.

Primers that we have designed are specific for defined animal species and are important in evolution researchers, ecology, conservation and wildlife management. Similar to that, PALOMARES et al. (2002) designed four species specific primers for researches of the Iberian lynx, which greatly upgraded research and conservation of that endangered felid (JOHNSON et al., 2004; PERTOLDI et al., 2005). Species specific primers for amplification of mtDNA cytochrome b have been designed for carnivores from the Ibearian peninsula (FERNANDES et al., 2008), while primers in this paper are the first species specific primers for amplification of mtDNA control region of Croatian wildlife.

Conclusion

1. We have designed six pairs of species specific primers for amplification of mtDNA control region of red deer, fox, brown bear, wolf, roe deer and wild boar.
2. By using those primers it is possible to identify animal species using only DNA isolation, PCR and electrophoresis, which allows faster and cheaper research.
3. Primers have wide use in ecological researches, researches of diet and in forensic cases.

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