SHORT COMMUNICATION

CAPABILITY OF MITOCHONDRIA DNA D-LOOP MARKERS FOR SHARK SPECIES IDENTIFICATION

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ABSTRACT

Identification of dry-fin shark was conducted by mitochondria DNA (mtDNA) D-loop markers. Eighteen of thirty samples have been successfully amplified the mtDNA D-loop region. The average total length of mtDNA D-loop was approximately 1790 bp. The differences among samples were clearly identified using polymorpishm of seven restriction endonucleases, *Alu*I, *Hinf*I, *Hae*III, *Hind*III, *Mbo*I, *Rsa*I, and *Taq*I.

KEYWORDS: **dry-fin shark, identification, mtDNA, endonucleasis**

INTRODUCTION

One of the many animals threatened by human exploitation is shark. Shark fishing has become a booming business in the past decade, especially for their fin consumption. This activity has decreased the number of shark in the world. Their mortality was also due to "accidental" caught by commercial fishing with drift gill nets. More than half of the estimated 200 million annual killed shark are related to accidental gill net catches (Primack, 1993).

Declining production of shark species is becoming a serious threat for their future living. As well for other endangered species, assessing their genetic structure becomes one of the important aspects for consideration of their resource management in the future. A sensitive genetic marker is required to support this activity.

Application of the molecular genetic technique is dramatically increasing nowadays in conjunction with the discovery of Polymerase Chain Reaction (PCR). A number of easily assayable and highly variable genetic markers have been developed, such as microsatellite, minisatellite, and mitochondria DNA (Park & Moran, 1995). The aims should be addressed accurately to achieve the success of the application of those techniques. It has been noted that micro- and minisatellites are suitable for population genetic study and pedigree analysis because of their high polymorphism, while mitochondria DNA is also available to detect the population to divergence due its maternal inheritance of the haplotypes.

In this paper, the capability of mitochondria DNA D-loop marker was examined to identify shark species based on their fin as a preliminary activity to find an easy and applicable marker for the field activity of study on the genetic structure of shark, to support their future management and conservation.

MATERIALS AND METHODS

Samples

Thirty 'blind' dry-fin samples of shark were obtained from FAO collection. The crude DNA was extracted using standard phenol chloroform (Nugroho *et. al.*, 1998). Three samples from previous study (Taniguchi & Nugroho, 1999) were used as reference species *i.e.* Av-4 (*Alopias vulpinus*, from Ionian Sea), Pg-8 (*Prionace glauca*, Ionian Sea), and Pg-14 (*Prionace glauca*, South Adriatic).

Mitochondria D-loop analysis

The mtDNA D-loop region and parts of cytochrome b gene and 16SrRNA genes were amplified using PCR as described by Martin *et al.* (1992). The mtDNA D-loop region was amplified in volumes of 50 µL each consisting of 100 ng of template DNA, 1x reaction buffer (10 µM Tris-HCl pH 8.3, 50 µM KCl 1 µM MgCl2), 5 µL of 2.5 µM dNTP mixture, 2.5 µL of each 10 µM oligonucleotide primer and 0.5 unit of *Taq* polymerase. Amplification cycles consisted of 30 cycles for one minute at 94°C, one minute at 45°C, and one minute at 72°C, followed by one cycle for seven minutes at

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72°C. Primer sequences are as follows: L-15,560 (23 bases from 15,560th base of light strand, 5'à 3' CAT ATT AAA CCC GAA TGA TAT TT) and H1067 (25 bases from 1,067th base of heavy strand, 5'à3' ATA ATA GGG TAT CTA ATC CTA GTT T). The mtDNA D-loop regions were digested using the four- and five-base recognition endonucleases, i.e. *Alu*I, *Hinf*I, *Hae*III, *Hind*III, *Mbo*I, *Rsa*I, and *Taq*I. The fragments were separated onto 2% agarose gel in 1x TBE buffer, stained with ethidium bromide and photographed. Fragment patterns generated by each of restriction endonuclease were compiled for each individual as a haplotype. For shark species identification, the haplotypes of "blind" samples were compared to those of reference species.

RESULTS

Eighteen of thirty samples have been amplified the mtDNA D-loop region. The average total length of mtDNA D-loop was approximately 1790 bp. Polymorphic restriction fragment was observed among samples for all seven endonucleases. An example of fragment patterns is shown in Fig. 1. Digestion of the mtDNA D-loop region with *Taq*I revealed three fragment patterns, while the other endonucleases obtained two patterns. Restriction site of three patterns by endonuclease *Taq* I were 640, 390, 300, 230, and 230 bp (type A); 630, 480, 300, and 250bp (type B); and 640, 540, 300, and 250 bp (type C).

A total of three composite haplotypes, one haplotype consists of 7 letters representing the fragment patterns generated by each of the restriction endonuclease, were observed among samples. According to the species reference, four of the 18 amplified samples were included to haplotype I as *A. vulpinus* species, while 14 others can be suggested as *P. glauca* which can be further differentiated into six samples from Ionian Sea (haplotype II) and eight samples from South Adriatic (haplotype III) (Table 1).

DISCUSSION

About sixty percent of dry-fin samples have been amplified the mtDNA D-loop region. The reasons for un-amplified mtDNA D-loop region in several samples might be due to: i) sample was denatured and/or ii) un-matched with primers used. The first reason may influence the quality of whole DNA taken, therefore the decreasing of DNA purity was occured. The same case was noted for three spines stickle back from Hokaido and Fukushima, Japan. Consequently, other DNA extraction methods should be used or developed. The second reason is possibly the samples were other shark species. However, this possibility is not strong, as several primers have been used to amplify the mtDNA D-loop regions for these samples. If this reason is really happened, it may be suggested that the total mtDNA of this species should be observed firstly.

The length of mtDNA D-loop fragments of shark fin was comparable with greater amberjack (Nugroho *et al.*, 2000), red sea bream (Nugroho, 2001), and grouper (Nugroho & Taniguchi, unpublished) that used also the same primer for mtDNA D-loop amplification. *Taq*I revealed more fragment patterns than other endonucleases. As well in the previous study, this enzyme restricted three types of fragment, while two types were observed by other endonucleases. It means that all of the amplified samples are of shark from species *A. vulpinus* and *P. glauca*. According to the haplotype of species reference used, it is estimated that four from the total of 18 amplified samples (*i.e.* samples no 4, 13, 21, and 22) are classified as the species *A. vulpinus* and others are *P. glauca* (10, 15, 16, 17, 19, 20, 23, 24, 25, 26, 27, 28, 29, and 30) (see Table 1). The capability of mtDNA marker for species identification has also been evaluated among other species *i.e.* greater amberjack (*Seriola dumerili)*, kingfish (*S. lalandi*), and yellow tail (*S. quinqueradiata*) (Nugroho, 2001).

Even other technique such as AFLP (Amplified Fragment Length Polymorphism) is also useful for species identification as shown in previous experiment (Taniguchi & Nugroho, 1999). However higher skill and cost are relatively needed. Therefore, for field application it is unfavorable. It suggests that sequence of shark's mtDNA should be found out as much as possible in order to develop the mtDNA primer. Once the accurate primer is available, amplification of mtDNA D-loop is not difficult, and this can be widely used in the field.

CONCLUSIONS

Mitochondria DNA D-loop marker is available for 'blind' fin fish identification to an optimum degree of accuracy. The differences between shark species (*A. vulpinus* and *P. glauca*) detected by polymorphism of mtDNA D-loop sequences were generated using all of seven endonucleases, while differences between locations in *P. glauca* is possible to be found out by restriction endonuclease, *Taq* I.

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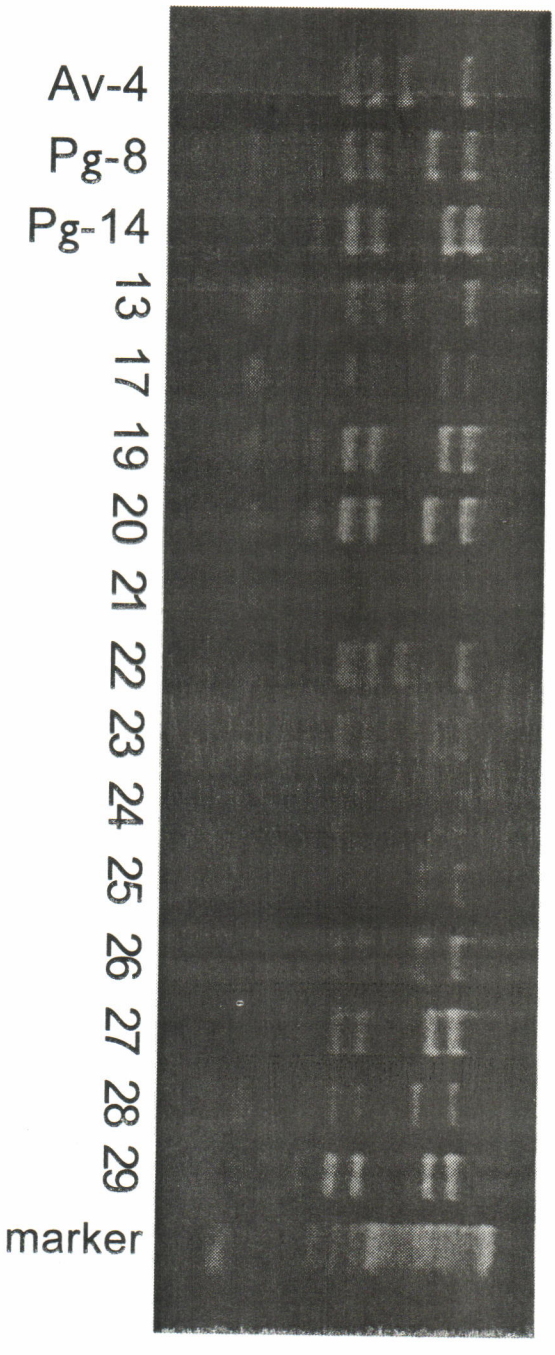

Figure 1. Restriction patterns of 'blind' shark samples (written as number) generated by *Taq*I endonuclease, with three 'reference' samples at left side (AV-4, Pg-4, and Pg-14), and a 50 bp marker ladder size at right size

Table 1. Species allocation of unknown shark samples using composite haplotype of mtDNA D-loop

Endonuclease	Species reference*)			Sample number**)																	
	Av-4	Pg-8	Pg-14	4	10	13	15	16	17	19	20	21	22	23	24	25	26	27	28	29	30
Taq-I	A	B	C	A	B	A	C	C	C	C	B	A	A	C	C	B	B	C	B	C	B
Mbo-I	A	B	B	A	B	A	B	B	B	B	B	A	A	B	B	B	B	B	B	B	B
Hae III	A	B	B	A	B	A	B	B	B	B	B	A	A	B	B	B	B	B	B	B	B
Hinf-I	A	B	B	A	B	A	B	B	B	B	B	A	A	B	B	B	B	B	B	B	B
Alu-I	A	B	B	A	B	A	B	B	B	B	B	A	A	B	B	B	B	B	B	B	B
Hind-III	A	B	B	A	B	A	B	B	B	B	B	A	A	B	B	B	B	B	B	B	B
Rsa-I	A	B	B	A	B	A	B	B	B	B	B	A	A	B	B	B	B	B	B	B	B
Haplotype	I	II	III	I	II	I	III	III	III	III	II	I	I	III	III	II	II	III	II	III	II
Species Name	Av	Pg1	Pg2	Av	Pg1	Av	Pg2	Pg2	Pg2	Pg2	Pg1	Av	Av	Pg2	Pg2	Pg1	Pg1	Pg2	Pg1	Pg2	Pg1

*) samples from previous experiment
Av : *Alopias vulpinus*
Pg 1 : *Prionace glauca* (Ionian Sea)
Pg 2 : *Prionace glauca* (South Adriatic)
Note : sample number of 2, 3, 5, 6, 7, 8, 9, 11, 12, 14, 18, and 31 were not successfully amplified their D-loop region