INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 13, No. 2, p. 255-262, 2018

RESEARCH PAPER OPEN ACCESS

Optimization of DNA from Musk Deer (*Moschus chrysogaster*) Hair Follicle: extraction, PCR Amplification for discriminatory analysis of RAPD markers

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Key words: Musk Deer, *Moschus chrysogaster*, Genetic Marker, RAPD, Discriminatory power.

http://dx.doi.org/10.12692/ijb/13.2.255-262 Article published on August 30, 2018

Abstract

Of 62 hair follicle samples of Himalayan musk deer ((*Moschus chrysogaster)* collected by hair-snares appreciable quantity of DNA was isolated from 60.70%. Largest quantity of DNA (220.11±15.09ng/µL) was obtained by modified phenol-chloroform method, followed by phenol-chloroform (199.13±11.09ng/µL) and Qiagen kit (146.34 ±20.42ng/µL) methods. The qualities of extracted DNA by Qiagen kit and modified phenol-chloroform method were not significantly different within (paired t =1.68, df =33, $P \geq 0.05$; paired t=1.75, df=33, $P \geq 0.05$) but significantly higher (paired t=2.56, df=33, $P \leq 0.05$) than phenol-chloroform. Of 35 RAPD markers tested, 29 amplified. Optimal conditions varied for responding for PCR-amplification, as optimum concentration of: $MgCl_2$; ranged minimum 1.5mm, 4 (13.79%) to maximum 2.5mm (6.89%), template DNA; ranged 30-50ng/µL, primer; majority,0.25 (16, 55.17%) to 0.15 (6, 20.68%), by using of A PCR profiles (Ta=26°C -37°C) for better and consistent amplification. Polymorphic information content (PIC) found higher (FA-18; 0.54) and lower (FA-9; 0.07) with resolution power (Rp) ranged 13.14-32.22. RAPD marker loci and molecular indices suggested significant correlation between PIC and Rp; (r^2=0.95; $P \leq 0.01$), followed MI and PIC; (r^2=0.67; $P \leq 0.05$) and MI and Rp (r^2=0.63: $P \leq 0.05$). The probability of identical by chance ranged between 0.0012 (OPA-20) and 0.0357 (FA-14), and total probability by multiplying of 4.234×10^{-61}. However, discriminatory indices of RAPD markers are indicative of high resolution and have potentials of significance, for using in DNA finger printing which can be employed for efficient identification, conservation and breeding approaches of musk deer.

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Introduction

Himalayan musk deer (*Moschus chrysogaster*, Janis and Scott, 1987) is a small human shy deer species distributed in western Himalayan range in unapproachable mountains and associated valleys (Roberts, 1997; Qamar *et al.*, 2008). Himalayan musk deer is on a rapid decline and has been listed as critically endangered in Pakistan (Sheikh and Molur, 2005) and globally (CITES, 2008).

Future conservation requires information on population biology, including isolations existing between sub-population and possible genetic fixation through inbreeding depression. Genetic analysis using DNA derived through non-invasive sampling of hair follicle or faeces provides opportunities to study the rare and elusive species (Sloane *et al.*, 2000; Eggert *et al.*, 2005), like Himalayan musk deer, where using blood and tissue (having higher yield rates) for extraction and analysis of DNA is difficult or rather implacable; field trapping of this rare and human-shy animals being unsafe.

Molecular markers are used to define groups of genetically similar populations and to assess relationships among demes within a population and play an important role in estimating the genetic diversity among individuals by comparing the genotypes at a number of polymorphic loci (Wilson and Strobeck, 1999; Avise, 2004). Random Amplified Polymorphic DNA markers are frequently used because of their simple genotyping method and ability to test almost unlimited number of primers at relatively low cost and without previously knowledge of genome in studies on polymorphism (Klinbunga *et al.*, 2000). RAPD markers have several interesting characteristics that make these very useful for genetic diversity survey (Welsh and McClelland, 1990; Williams *et al.*, 1990). Shed or plucked hair roots have proved as an excellent DNA source for genetic analysis in many mammal species (Allen *et al.*, 1998; Taberlet *et al.*, 1997; Goossens *et al.*, 1998).

Hair-snare stations can be deployed over large areas at minimal costs. Collection, transportation and storage of hair bulbs do not require special conditions/ financial implications in contrast to those required for other tissues/ blood, previously used for population genetic structure analysis (Foran *et al.*, 1997).

Discriminatory power of RAPD markers is an important and reliable tool for basic analysis of genome in different species (Dhikari *et al.* 2017). However, the difficulties to achieve high pattern reproducibility represent a major drawback for the routine implementation of conventional RAPD-PCR and comparison of results among research groups working in a similar species. It can be applied to check the reproducible, well resolved and unambiguous fragments for discrimination and exploring the uses of DNA markers (Zargar *et al.* 2016). Before the conservation program of such a declining species, musk deer (*M.chrysogaster)*, it is necessary to harvest the basic information attributing the level of genetic structure for desirable future of species survival. Genetic characterization can be analyzed using morphological, biochemical markers and genetic markers. RAPD markers have a genetic advantage that is suitable for optimization and amplification of small quantity of isolated genome in endangered species of animals (Emara and Kim, 2003). Several types of molecular markers, including mitochondrial DNA (mtDNA) and nuclear DNA markers (RAPD and ISSR), are available but none of them can be regarded as optimal for all applications (Sunnucks, 2000). Hair follicles coming from hair snares set in an area have very small quantities of extractable DNA. Successful exploitation of such samples for any genetic study requires optimization of a protocol for DNA extraction in sufficient quantities and of good quality, which can be subjected to PCR amplification. The present study has been devoted to optimize isolation/extraction conditions for DNA from the hair follicle samples of Himalayan musk deer collected through hair-snares set in different parts of Pakistan. Three different extraction protocols have been tested for quantity and quality of extracted DNA. Moreover we have considered the studying the optimal conditions of PCR amplification and to evaluate the efficiency with discriminatory powers of these RAPD markers that can measure the reliability scale of markers for further analysis.

Materials and methods

Sample Collection

Hair samples of the Himalayan musk deer were obtained from hair snares (Roon *et al.*, 2003; Waits and Paetkau, 2005) set in distribution range in 7 broad areas falling in Gilgit-Baltistan (Ghizer, Ghangche, Gilgit), Khyber Pakhtunkhwa (Swat Kohistan) and Azad Jammu and Kashmir (Pirchinnasi, Shahrda, Gurase) in Pakistan. Snares were maintained in a place/locality for 2-3 weeks, and checked after every 2-3 days for the presence of hair. The snares were supported by camera traps (Wildview xtreme 4) recording for possible confirmation of hair samples. Individual hair of the sample was handled with forceps and examined under a dissecting microscope (magnifier X, NOIFXSZ-107BN) for the presence of intact follicle. All handling gadgets and hair samples were washed with 95 per cent ethanol then rinsed with double deionized water for sterilization according to Gagneux *et al.* (1997) from different contaminations. The collected hair samples were washed and stored at 4°C until further processing.

DNA extraction

Follicles from collected hair samples were separated, washed and sterilized following Gagneux *et al.*, (1997) and Zou *et al.* (2005). Three methods were employed for DNA extraction:

Phenol-Chloroform

Standard Sambrook and Russell, 1989 protocol was followed, with modifications as manipulated by Zou *et al.*, 2005, for DNA extraction from sterilized hair follicles.

Modified Phenol-Chloroform

Some modifications were introduced in standard phenol-chloroform (Sambrook *et al.*, 1989) extraction protocol. For single follicle 300µL of the lysis solution containing; 200 mM NaCl, 10 mm Tris-HCl, pH 8.0, 10mm EDTA, pH 8.0 and 0.1% Triton-100 was used for lysis of follicles along with 1M DTT (37µL) and 37µL ProteinaseeK (2mg/mL). After overnight incubation at 45°C DNA was extracted phenol-chloroform and precipitated with an equal volume of isopropanol and 0.3 M of sodium acetate.

Qiagen Kit

We used Qiagen kit (DNeasy Blood & Tissue Kit, Cat# 69504) for isolation of DNA from hair follicle, as per instructions of the manufacturer.

Quantity and quality analysis

The extracted DNA was confirmed by electrophoresis on 1.0% agarose gel. The confirmed DNA was assessed for quality and quantity using spectrophotometric method (Linacero *et al.,* 1998).

PCR Optimization

35 RAPD markers (Table 1), 20 of FA series, and 15 of operon series (Huifang *et al.*, 1999; Exadactylos *et al.*, 2003), were employed for RAPD analysis. The PCR amplifications was carried out in 25µL reaction mixture containing DNA template (20-100ng), 1X reaction buffer (50mm KCl, 1.5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9, and 0.1 per cent Triton X-100), 1U of Taq DNA polymerase (Fermentas, USA), while varying concentrations of dNTPs (0.10, 0.15 and 0.20mm), $MgCl_2$ (1.0mm, 1.5mm, 2mm, 2.5mm) and primers (0.10, 0.15, 0.20, 0.25µM), to obtain optimal concentrations for reproducible PCR product with high consistency. The PCR was carried out in Sprint Thermal Cycler (PCR Sprint; SPR 220362) with initial denaturation at 94-95°C for 5 min then followed by 35 cycles of 95 °C for 30 sec, annealing at 26–45°C (depending upon the RAPD marker) for 45 sec and extension at 72°C for 50 sec. Final extension was carried out at 72°C for 10 min (Guan *et al.*, 2009).

Discriminatory power

The RAPD gel were scored for each individual as presence "1" and absence "0" of band to constructed bivariate (1-0) data matrix in rows and columns using excel sheet for discriminatory analysis. Three parameters were used to determine the discriminatory power of each RAPD marker: (a) polymorphic information content (PIC = $2f_i$ ($1-f_i$): where f_i = frequency of marker band: Roldan-Ruiz*jet al.*, 2000), (b) resolving power (Rp = ∑ IB, where IB = band information: Prevost and Wilkinson, 1999) and (c) marker index proposed (Varshney *et al.* 2007) (MI = PIC . EMR, where PIC = polymorphic diversity index and EMR = effective multiple ratios: Powel *et al.*, 1996; Milbourne *et al.*, 1997).

The probability of identical by chance (Pi) using degrees of similarities and dissimilarities of RAPD fragments between individuals and populations was also estimated. The linear correlation coefficient (Pearson's r) between the marker efficiency parameters was performed using the XLSTAT Pro 7.5, Microsoft Office Excel-add in.

Results

Sample Collection and Dna Extraction

Sixty two hair samples from 137 snares set in different musk deer tracts of Pakistan were obtained. The details of collected sample along with percent success of DNA extraction is given in Table 1. The overall success rate of DNA extraction is 60.70%, ranging between 80% (Gurase sample) and 40% (Ghangche sample). The three methods used for DNA extraction are compared in Fig.1, depicting the modified phenol chloroform method as best among three.

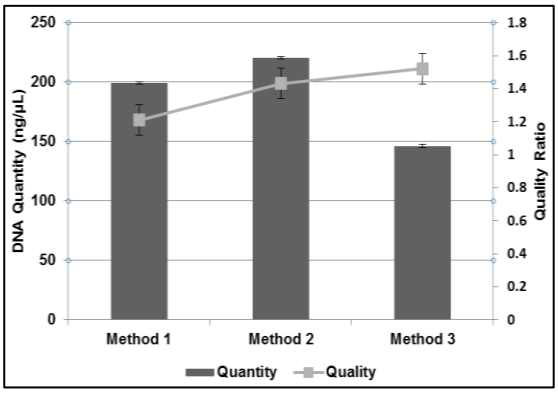

Fig. 1. The quality and quantity of DNA extracted by using three different methods (Method 1: Phenol-Chloroform, Method 2: Modified Phenol-Chloroform and Method 3: Qiagen Kit).

Table 1. The hair samples obtained from different location of Pakistan and success rate of DNA extraction.

Locality	(n)	Unsuccessful extracts (#)	DNA Extraction		
			Low extraction (#)	Successful extracts (#)	Success rate (%)
Pirchannasi	7	1	2	4	66.66
Guraze	5	0	1	4	80.00
Shahdra	6	2	2	2	50.00
Swat Kohistan	8	0	4	4	50.00
Gangche	10	0	6	4	40.00
Baltistan	10	1	3	6	60.00
Ghizer	16	2	4	11	68.81
Total	62	6	22	34	60.71

PCR Optimization

Approximately 83% of RAPD markers gave the successful amplification; the optimized PCR condition for each primer is given in Table 2.

Table 2. The optimized PCR Conditions for each RAPD marker used in the study for amplification of *Moschus chrysogaster*'s DNA.

Primer	$MgCl_2$ (mM)	Template (ng/µL)	Primer (pmol)	Ta (°C)	dNTPs (mM)	Taq Polymerase (U)
FA-1	1.5	50	0.25	34	0.15	0.2
FA-2	2.5	50	0.25	33	0.15	0.2
FA-3	2.0	50	0.25	32	0.15	0.2
FA-4	1.5	50	0.25	33	0.15	0.2
FA-5	1.5	40	0.25	37	0.15	0.2
FA-6	1.5	40	0.25	33	0.15	0.2
FA-7	1.5	40	0.25	37	0.15	0.2
FA-8	1.5	40	0.25	32	0.15	0.2
FA-9	1.5	50	0.25	32	0.15	0.2
FA-10	1.5	50	0.25	33	0.15	0.2
FA-11	1.5	50	0.20	34	0.15	0.2
FA-14	1.5	50	0.15	34	0.15	0.2
FA-15	2.5	50	0.15	32	0.15	0.2
FA-18	1.5	50	0.15	33	0.15	0.2
OPA-4	1.5	30	0.25	26	0.15	0.2
OPA-11	1.5	30	0.15	26	0.15	0.2

Primer	$MgCl_2$ (mM)	Template (ng/µL)	Primer (pmol)	Ta (°C)	dNTPs (mM)	Taq Polymerase (U)
OPA-17	2.0	30	0.15	26	0.15	0.2
OPA-19	1.5	30	0.15	26	0.15	0.2
OPA-20	1.5	30	0.20	26	0.15	0.2
OPN-04	1.5	30	0.20	29	0.15	0.2
OPN-11	1.5	30	0.20	26	0.15	0.2
OPMN-13	1.5	30	0.25	29	0.15	0.2
OPN-19	1.5	30	0.25	26	0.15	0.2
OPN-20	1.5	30	0.25	26	0.15	0.2
OPB-11	2.0	30	0.25	29	0.15	0.2
OPB-15	2.0	30	0.25	29	0.15	0.2
OPB-18	1.5	30	0.20	26	0.15	0.2
OPF15	1.5	30	0.20	26	0.15	0.2
OPF17	1.5	40	0.20	26	0.15	0.2

Discriminatory power

Table 3 represents the different statistics of discriminatory power of 29 RAPD markers. Values of polymorphic information content (PIC) were higher for FA-18 (0.54), FA-2/FA-11 (0.45) and FA-2 (0.45) and the lowest FA-9 (0.07).

Values of resolution power (Rp) ranged between 13.14 and 32.22, higher values for FA-18, FA-11 and FA-15/OPA-17 and lower FA-9. Marker indices (MI) were higher for OPN-11 (6.03), FA-3 (5.18), OPN-04 (4.83) and FA-9 (4.52), while lower for OPA-11 (1.23) and lowest in FA-7 (0.02). The significant positive correlation for all RAPD marker loci between PIC and Rp (r^2=0.95; df=28, $P \leq 0.01$), between MI and PIC (r^2=0.67, df=28, $P \leq 0.05$), and between MI and Rp (r^2=0.63, df=28, $P \leq 0.05$) was established. The probability of identical by chance ranged between 0.0012 (OPA-20) and 0.0514 (OPA-4), with total probability of 4.234×10^{-61}.

Table 3. Description of discriminatory power for RAPD markers used in the study.

Markers	T.A.F.P	PIC	Rp	MI	Pi
FA-1	2.85	0.22	16.86	1.65	0.0067
FA-2	3.50	0.45	32.22	3.29	0.0053
FA-3	3.26	0.14	18.00	5.18	0.0025
FA-4	3.99	0.28	25.38	2.23	0.0054
FA-5	3.72	0.18	20.53	2.48	0.0212
FA-6	3.26	0.14	18.00	3.45	0.0025
FA-7	3.96	0.18	21.87	0.02	0.0168
FA-8	2.88	0.14	18.31	1.51	0.0049
FA-9	2.22	0.07	13.14	4.52	0.0418
FA-10	3.38	0.20	24.27	2.94	0.0013
FA-11	3.50	0.45	32.22	3.22	0.0370
FA-14	3.58	0.17	19.73	1.56	0.0357
FA-15	3.45	0.35	28.60	3.61	0.0099
FA-18	3.41	0.54	36.25	3.21	0.0041
OPA-4	4.40	0.25	24.27	3.84	0.0514
OPA-11	3.53	0.26	24.27	1.23	0.0196
OPA-17	3.45	0.35	28.60	3.17	0.0101
OPA-19	2.20	0.14	18.20	2.09	0.0173
OPA-20	3.24	0.22	22.29	2.03	0.0012
OPN-04	3.58	0.19	21.14	4.83	0.0285
OPN-11	4.45	0.30	26.29	6.03	0.0234
OPMN-13	4.06	0.25	24.00	4.54	0.0062
OPN-19	3.53	0.22	22.46	2.74	0.0250
OPN-20	3.00	0.16	19.08	2.98	0.0034
OPB-11	3.96	0.24	23.43	3.45	0.0073
OPB-15	3.67	0.40	30.40	2.32	0.0039
OPB-18	3.02	0.14	17.86	3.18	0.0038
OPF15	3.96	0.32	27.33	3.66	0.0041
OPF17	3.00	0.42	31.00	3.46	0.0211
Mean	100	0.25±0.02	23.66±1.02	3.00±0.23	4.234×10^{-61}

T.A.F.P, % of total amplified fragment pool; PIC: Polymorphic information index; Rp, Resolution power; MI, Marker index; Pi, probability of identical by chance.

Discussion

Himalayan musk deer is rare, human shy, nocturnal and living in inaccessible terrain (Roberts, 1997), therefore safe live trapping for collection of tissues or blood for the DNA analysis is rather impossible. However, hair follicles and faeces give lower yield of extractable DNA compared with tissue or blood samples (Foran *et al.*, 1997). Standardization of extraction procedure and Purity of extracted DNA plays key role in PCR optimization and analysis of genetic diversity (Staub *et al.*, 1996).

We obtained significant higher quantity of DNA with modified phenol-chloroform extraction method than that obtained through chloroform-phenol or Qiagen kit methods. We attribute higher DNA yield totriton-100 regent, which required longer time for lysis and have been reported as effective detergent for cell lysis (Merante *et al.* 1994; Waldschmidt *et al.*, 1997). We obtained highest quality of DNA from Himalayan musk deer hair follicles extracted by Qiagen kit. The quantity of DNA extracted through Qiagen kit was though lower yet was sufficient for further PCR amplification. We regard Qiagen kit as optimal for future use for DNA extraction and its use for PCR amplification using RAPD markers. Qiagen kit method produced high quality of extractable DNA from hair follicle of brown (*Ursus arctos*) and black (*Ursus americanus*) bears (Akram, 2011). We suggests that DNA obtained from shed Himalayan musk deer hair can be used in research on genetic analysis, as has been previously used in a variety of animal species (Allen *et al.*, 1998), including forest musk deer (*Moschus berezovski*) (Zou *et al.*, 2005).

We achieved PCR amplification of Himalayan musk deer genome for 29 RAPD markers while 6 others did not respond. For reproducible amplification of genome, $MgCl_2$ concentration is considered as key factor because it affects the fidelity of PCR for amplification, ranging from 0.1 to 2.5mm generally used in genome amplification in different species (Bartlett and Stirling, 2003). Calculated values of different statistics of discriminatory power of markers showed significant discrimination for level of amplification, combinations and highly correlated is indicative of high resolution for these markers. Discriminatory power of RAPD primers attributes potentials of their use in DNA finger printing and estimation of variation in genome for Himalayan musk deer. Resolving power is a potential attribute of markers and a basic feature for primer combination having discriminatory potentials. Using PIC and MI values, Prevost and Wilkinson (1999) proposed another attributes of judging the resolving power of RAPD markers. Strong correlation has been reported between primer combinations of genotypes and resolving power (Powel *et al.* 1996) and PIC can be used to analyzed the level of utility of each marker (Femandez *et al.* 2002). MI and PIC values calculated for present markers remained close to those suggested by Roldan–Ruiz *et al.* (2000) and Powell *et al.* (1996). Higher rate of successful amplification of RAPD markers (29 out of 35) selected under the present study compared with that in previous studies [25 out of 42 primers selected for forest musk deer (*M berezovskii*) and Alpine musk deer (*M.sifanicus*)] can partly be ascribed to coincidence (Huifang *et al.* ,1999). Hence, all marker indices (Rp, PIC and MI) of discrimination showed significant correlated with each other, which can be used to collect information on polymorphism in primer, therefore markers have potentials of being used in DNA finger printing and estimation of variation in genome which can be used in efficient identification, conservation for breeding approaches of this endangered species of musk deer.

Conclusion

Although DNA optimization is the basis requirement for the approaches of advance DNA fingerprinting. DNA was successfully extracted and isolated DNA, so proved amenable to PCR amplification. The proposed study is rarely available and provided us for non-invasive (hair-follicle) techniques for such ecologically and economically endangered wild cryptic species that play important role in natural ecosystem of Pakistan. These resulted loci will be used to detect linkage between different traits of musk deer species. And such a potential and highly resolved polymorphic RAPD will be helpful and effectively used to study the genetic status for efficient identification and future conservation of dwindling species.

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