Multilevel D-loop PCR identification of hunting game

V. Parkanyi *, L. Ondruska, D. Vasicek, J. Slamecka

Animal Production Research Centre Nitra, Hlohovecka 2, 951 41 Luzianky, Slovakia

ABSTRACT

The control region of mtDNA (D-loop) was used for hair samples of the five hunting game species identification: red deer *(Cervus elaphus)*, roe deer *(Capreolus capreolus)*, fallow deer *(Dama dama)*, mouflon *(Ovis aries musimon)*, and wild boar *(Sus scrofa)*. For D-loop multilevel PCR detection scheme was applied in six primers (CE CVZV 1 = 5′-GATCACGAGCTTGATCACCA-3′; CE CVZV 2 = 5′-AGGAGTGGGCGATTTTAGGT-3′; DD CVZV 3 = 5′-CGCGTGAAACCAACAACCCGC-3′; DD CVZV 4 = 5′-CCGGGTCGGGGCCTTAGACG-3′; SSW CVZV 5 = 5′-ACACGTGCGTACACGCGCATA-3′; SSW CVZV 6 = 5′-GGTGCCTGCT T TCGTAGCACG-3′) designed to identify unknown biological samples of the hunting game animals. The PCR reaction volume was 25 μl at conditions 95 °C for 2 min, 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, 35 cycles, with last extension at 72 °C for 10 min. D-loop mtDNA amplicons of the game animals are characterized with specific PCR product sizes depending on species: red deer = 163 bp and 140 bp, fallow deer = 280 bp and 138 bp, roe deer = 303 bp, 280 bp, 160 bp and 138 bp, mouflon = 299 bp and 178 bp, wild boar = 137 bp and 229 bp.

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1. Introduction

One area of conservation genetics that has long been recognized but is now receiving increasing attention is the development of analytical techniques capable of providing DNA evidence to assist in conservation law enforcement, commonly termed wildlife DNA forensics. Wildlife DNA forensics is essentially concerned with the identification of evidence items in order to determine the species, population, relationship or individual identity of a sample. Unjustified killing of wild game and protected animal species, generally called poaching, is considered the most important crimes in environmental crime (Frankham et al., 2002). Mitochondrial DNA (mtDNA) is often favoured as a genetic marker over nDNA for species identification of wildlife because mtDNA is easier to type from highly processed and degraded tissue (Randi, 2000). Universal mtDNA markers have been successfully applied in the identification of wildlife for forensic cases. The most commonly used universal markers for species identification are the mitochondrial cytochrome b (Cyt b) and cytochrome oxidase I (COI) genes (Alacs et al., 2010; Linacre et al., 2011). Besides these genes are used D-loops (control regions) of mtDNA, too. The use of non-human
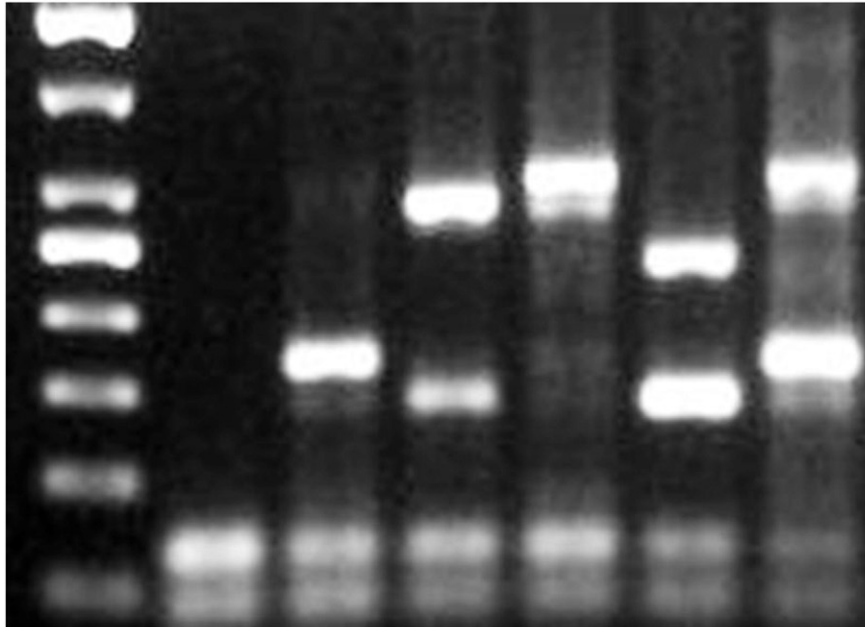
DNA typing in forensic science investigations, and specifically that from animal DNA, is ever increasing. The term animal DNA refers to animal species encountered in a forensic science examination but does not include human DNA. Non-human DNA may either be: the trade and possession of a species, or products derived from a species, which is contrary to legislation; as evidence where the crime is against a person or property; instances of animal cruelty; or where the animal is the offender (Linacre et al., 2011). DNA species characteristic and an international effort are underway to use this gene catalogue all vertebrate biodiversity on the earth (www.barcodinglife.org). Determining the species origin of meat is an integral part of food regulatory control with respect to economic fraudulence, too. For example, game meat products are often a target for fraudulent labelling, because of the different prices between game and other meat species (Brodmann et al., 2001; Wolf et al., 1999). In contrast, considerably fewer studies have been reported so far about game meat authentication employing PCR-based approaches. Among them, the use of the mitochondrial genome as marker for the development of techniques such as PCR-RFLP is a widely extended choice (Brodmann et al., 2001; Fajardo et al., 2006; Pfeiffer et al., 2004; Wolf et al., 1999). Polymerase chain reaction (PCR) based on oligonucleotide primers targeting the mitochondrial 12S rRNA gene was applied to the specific identification of meats from red deer *(Cervus elaphus)*, fallow deer *(Dama dama)*, and roe deer *(Capreolus capreolus)*. The use of a common reverse primer, together with forward specific primers for red deer, fallow deer, and roe deer, allowed the selective amplification of the desired cervid sequences (Fajardo et al., 2007a).

* Corresponding author. Tel.: +421 6546180; fax: +421 6546401.
 E-mail address: parkanyi@cvzv.sk (V. Parkanyi).
 URL: http://www.cvzv.sk (V. Parkanyi).

Table 1
Primers used for the hunting game D-loop mtDNA (red deer = CE, roe deer = CC, fallow deer = DD, mouflon = OM, wild boar = SSW) determination.

Primer name	Primer sequence from 5′ to 3′	GenBank Acc. No.	nt position at D-loop	PCR product = species amplicon
CE CVZV 1	GATCACGAGCTTGATCACCA	NC_007704	15776–15938	**163 bp = CE**
CE CVZV 2	AGGAGTGGGCGATTTTAGGT	NC_007704	15799–15938	**140 bp = CE**
DD CVZV 3	CGCGTGAAACCAACAACCCGC	JN632629	15793–16072	**280 bp = DD**
DD CVZV 4	CCGGGTCGGGGCCTTAGACG	JN632629	15793–15930	**138 bp = DD**
		JN632610	15777–16079	**303 bp = CC**
		JN632610	15800–16079	**280 bp = CC**
		JN632610	15777–15937	**160 bp = CC**
		JN632610	15800–15937	**138 bp = CC**
		HM236185	16051–16349	**299 bp = OM**
		HM236185	16074–16351	**278 bp = OM**
		FJ236998	15767–15903	**137 bp = SSW**
SSW CVZV 5	ACACGTGCGTACACGCGCATA			
SSW CVZV 6	GGTGCCTGCT T TCGTAGCACG	FJ236998	16593–16822	**229 bp = SSW**

	1	2	3	4	5	6	7
	M	**NC**	**CE**	**DD**	**OM**	**SSW**	**CC**
			163bp	280bp	299bp	229bp	303bp
			140bp	138bp	278bp	137bp	280bp
							160bp
							138bp
		+ primer dimers	**+ primer dimers**	**+ primer dimers**	**+ primer dimers**	**+ primer dimers**	**+ primer dimers**

Lane 1 = M 50 bp, GeneRuler™ 50 bp DNA Ladder-Fermentas

Lane 2 = NC = negative control = primers CE CVZV 1 + CE CVZV 2 + DD CVZV 3 + DD CVZV 4 + SSW CVZV 5 + SSW CVZV 6 + H$_2$O

Lane 3 = primers CE CVZV 1 + CE CVZV 2 + DD CVZV 3 + DD CVZV 4 + SSW CVZV 5 + SSW CVZV 6 + mtDNA CE (*Cervus elaphus*)

Lane 4 = primers CE CVZV 1 + CE CVZV 2 + DD CVZV 3 + DD CVZV 4 + SSW CVZV 5 + SSW CVZV 6 + mtDNA DD (*Dama dama*)

Lane 5 = primers CE CVZV 1 + CE CVZV 2 + DD CVZV 3 + DD CVZV 4 + SSW CVZV 5 + SSW CVZV 6 + mtDNA OM (*Ovis aries musimon*)

Lane 6 = primers CE CVZV 1 + CE CVZV 2 + DD CVZV 3 + DD CVZV 4 + SSW CVZV 5 + SSW CVZV 6 + mtDNA SSW (*Sus scrofa*)

Lane 7 = primers CE CVZV 1 + CE CVZV 2 + DD CVZV 3 + DD CVZV 4 + SSW CVZV 5 + SSW CVZV 6 + mtDNACC (*Capreolus capreolus*)

Fig. 1. Electrophoretogram of PCR fragments (in bp) from hunting game animal hair samples, 2% agarose gels containing ethidium bromide.

Tobe and Linacre (2008) identified 18 common European mammalian species (badger, cat, cow, dog, donkey, fox, goat, guinea pig, harvest mouse, hedgehog, horse, housemouse, human, pig, rabbit, rat, red deer and sheep) by species-specific multiplex, many of which are often associated with forensic investigations, has been developed. The assay is based on the mitochondrial cytochrome *b* gene, which is commonly used in species identification and phylogeny studies.

At present study the control region of mtDNA (D-loop) was used for identification of hair samples of the five hunting game species.

2. Methods

The 50 samples were collected from hunting game species hairs (10 from red deer—*C. elaphus* = CE, 10 from roe deer—*C. capreolus* = CC, 10 from fallow deer—*D. dama* = DD, 10 from mouflon—*Ovis aries musimon* = OM, and 10 from wild boar—*Sus scrofa* = SSW). The hair samples were dropped in a 1.5 ml microcentrifuge tube. Samples were processed immediately to prevent bacterial growth and preserve

the quality of genomic DNA and viral DNA. Two hundred microliters of $1 \times$ PCR buffer (50 mM KCl, 20 mM Tris–HCl pH 8.4), supplemented with 50 mM DTT, 1% Triton X-100 and 400 ng/µl proteinase K was added to the tubes containing several hair samples from each game species, separately. The hair samples were incubated overnight at 56 °C, 5 min at 96 °C after incubation and then cooled to room temperature. Samples were centrifuged for 1 min at 16,060 $\times g$. Total DNA was isolated from lysate by standard protocol Isolation kit II on MagNa Pure LC 2.0 Instrument (Roche). Total DNA concentrations were measured by UV/VIS spectrophotometer NanoPhotometer (Implen) from 1.0 ng/µl to 5.5 ng/µl range. PCR reactions were performed using primers amplifying species-specific D-loop mtDNA fragments of the game animals (Table 1).

The PCR conditions (PTC-200 DNA Engine; MJ Research) were 95 °C for 2 min, 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, 35 cycles, with last extension at 72 °C for 10 min. The reaction volume (25 µl) contained 10 mM Tris–HCl (pH 8.6 at 25 °C, 50 mM KCl, 1.5 mM MgCl$_2$, 25 units/ml *Taq* DNA polymerase, 0.2 mM dNTPs each, 5% glycerol, 0.08% IGEPAL® CA-630, 0.05% Tween-20)

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GelQuant Express Report

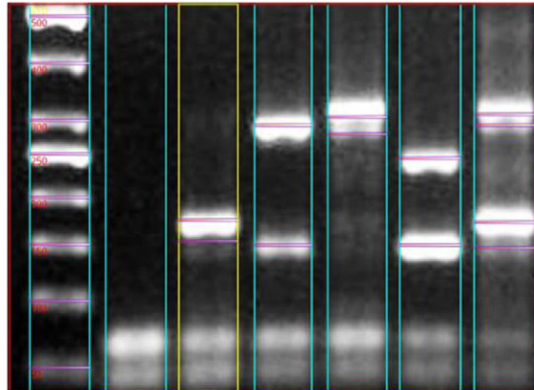Experiment P1CE-P1DD-P2SSW MULTILEVEL FOR GAME ANIMALS_25_02_2013 image

Image Parameters

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Band Detection Parameters

Background subtraction method	Rolling ball disk size :70
Band detection sensitivity	75
Molecular weight standard name	500bp
Molecular weight regression type	LinearLog
Mass regression type	Linear
Mass regression formula	N\A
Mass regression R square	N\A

Fig. 2. Analysis in situ (2% Agarose gel electrophoresis) by GelQuant Express.
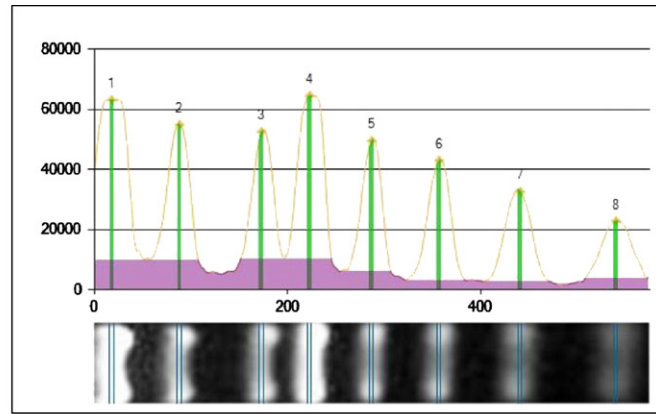
Lane 1 data table				
Number	MW (BasePairs)	RF	Volume plus Background	Net Volume
1	500,00	0,03	27794176	23410793
2	400,00	0,15	24068864	19685481
3	300,00	0,30	22870784	18281256
4	250,00	0,39	28344320	23754792
5	200,00	0,50	21676032	19002112
6	150,00	0,62	18806784	17403904
7	100,00	0,77	14345472	13125632
8	50,00	0,94	9999616	8317041

Standard Lane	Yes
Regression formula	$Log(Y) = -1,06X + 2,78$
R square	0,98

**M 50 bp, GeneRuler™ 50 bp DNA Ladder-Fermentas**
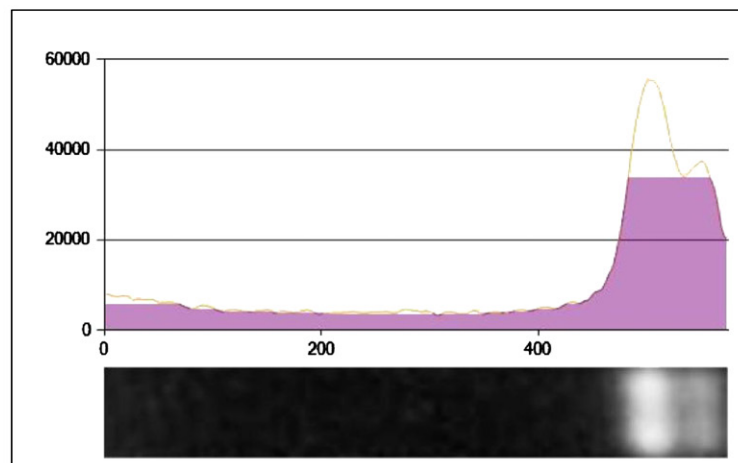
**NC = negative control +primer dimers**

Fig. 2 (*continued*).

(New England Biolabs), 10 pmol/µl each primers (CE CVZV 1, CE CVZV 2, DD CVZV 3, DD CVZV 4, SSW CVZV 5, SSW CVZV 6) and 1.0 ng/µl to 5.5 ng/µl each mtDNA sample. The amplified DNA was electrophoretically separated on 2% agarose gels containing ethidium bromide at 80 mA in 10 mM lithium borate buffer, pH 8.0 for 90 min. The products were visualized under UV light and photographed using a MiniBis Pro (Bio-Imaging Systems) (Fig. 1) with in situ analysis by GelQuant Express software (Fig. 2).
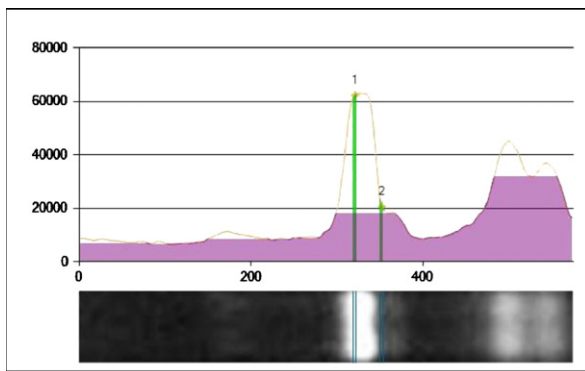
Lane 3 data table				
Number	MW (BasePairs)	RF	Volume plus Background	Net Volume
1	155,76	0,56	27418880	19517508
2	136,57	0,61	9202432	1301060

CE = *Cervus elaphus* D-loop mtDNA + primer dimers
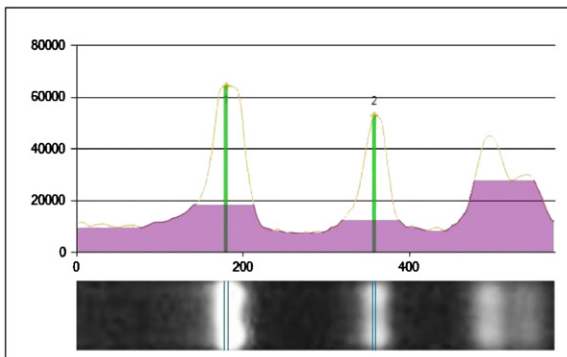
Lane 4 data table				
Number	MW (BasePairs)	RF	Volume plus Background	Net Volume
1	283,29	0,31	27343616	19433216
2	133,14	0,62	22344448	17083648

DD = *Dama dama* D-loop mtDNA + primer dimers

Fig. 2 (*continued*).

3. Results and Discussion

Complete D-loop mtDNA of the hunting game animals by species-specific multilevel PCR has not yet been provided. It was at the multiplexes hoofed, so only a maximum of three species within one PCR reaction-red deer, fallow deer and roe deer, or chamois, Pyrenean ibex and mouflon, respectively (Fajardo et al., 2007a, 2007b). Tobe and Linacre (2008) identified 18 common European mammalian species by species-specific multiplex, but with one hunting game animal-red deer, only. The D-loop multilevel PCR detection scheme using six primers (CE CVZV 1, CE CVZV 2, DD CVZV 3, DD CVZV 4, SSW CVZV 5, SSW CVZV 6) was designed to identify unknown biological samples of the game animals, first time. The primers sequences for the game animal detection (red deer, roe deer, fallow deer, mouflon, wild boar) were registered by authors Parkanyi V., Ondruska L., Vasicek D., Slamecka J. in BOLDSYSTEMS under items: DATABASES-PRIMER DATABASE-PRIMER SEARCH-D-LOOP or PRIMER NAME:

http://www.barcodinglife.org/index.php/ Public_Primer_PrimerSearch+ CVZV.

The D-loop mtDNA PCR products of the game and their specific position were detected by CLC Sequence Viewer 6.7.1 and BLAST (Table 1).

D-loop mtDNA amplicons of the game animals are characterized by specific molecular sizes depending on species and our technology is suitable for detection of unknown biological samples from wildlife. Two PCR fragments are typical for red deer: 163 bp and 140 bp. Fallow deer is identified by PCR products: 280 bp and 138 bp. Roe deer is specific with four PCR fragments: 303 bp, 280 bp, 160 bp and 138 bp. Mouflon has two PCR products: 299 bp and 278 bp. Wild boar is characterized with two amplicons of D-loop mtDNA: 229 bp and 137 bp (Fig. 2).

4. Conclusions

The oligonucleotides sequences (CE CVZV1, CE CVZV2, DD CVZV3, DD CVZV4, SSW CVZV5, SSW CVZV6) for multilevel PCR D-loop mtDNA identification of the hunting game species (red deer, roe deer, fallow deer, mouflon, wild boar) were registered in www. boldsystems.org and they are suitable for detection of unknown biological samples from wildlife.

Conflict of Interest Statement

None.

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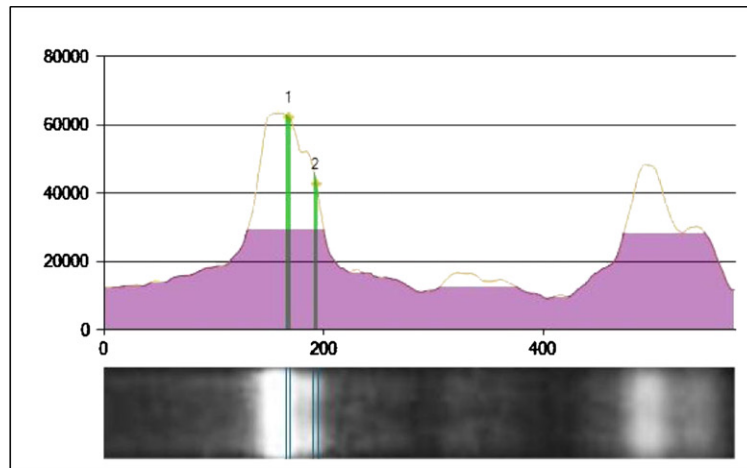
Lane 5 data table				
Number	MW (BasePairs)	RF	Volume plus Background	Net Volume
1	298,09	0,29	27370240	14471748
2	268,09	0,34	18784512	5886020

OM = *Ovis aries musimon* **D-loop mtDNA + primer dimers**
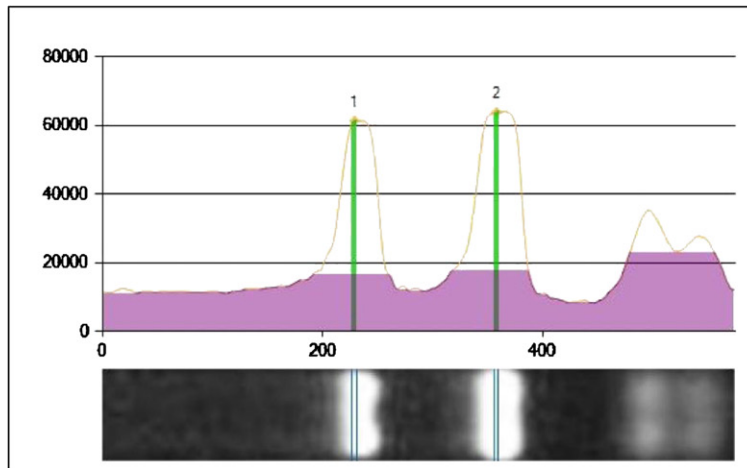
Lane 6 data table				
Number	MW (BasePairs)	RF	Volume plus Background	Net Volume
1	230,12	0,40	28148480	20573743
2	133,14	0,62	29382912	21293615

SSW = *Sus scrofa wild* **D-loop mtDNA + primer dimers**

Fig. 2 (*continued*).
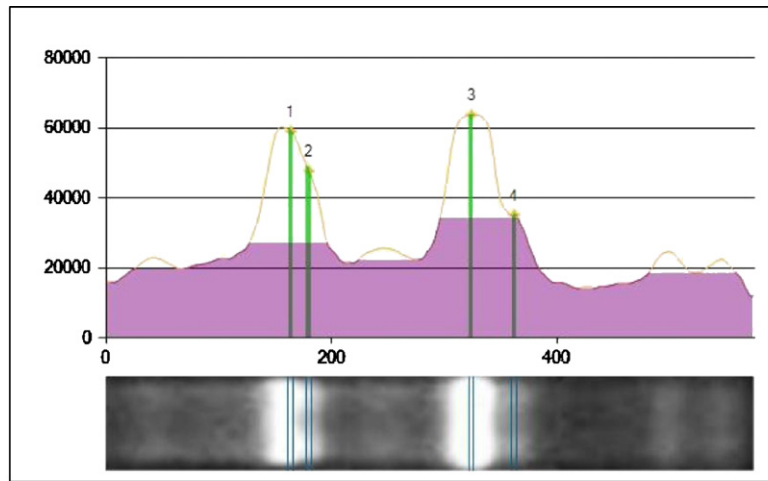
Lane 7 data table				
Number	MW (BasePairs)	RF	Volume plus Background	Net Volume
1	303,19	0,29	27455744	14850666
2	283,29	0,31	22132992	9527914
3	153,79	0,56	29633280	13748480
4	130,90	0,63	16298240	413440

CC = *Capreolus capreolus* D-loop mtDNA + primerdimers

Fig. 2 (*continued*).