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Development of eight-plex microsatellite PCR for parentage control in deer

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Abstract

Nine loci have been compiled into two multiplex microsatellite polymerase chain reaction (PCR) sets (four and five loci) and used as a tool to determine the most probable hind for each calf. The two sets were suitable to combine them in an eight-plex reaction. The exclusion probabilities of the eight-plex reaction and the nine loci were 99.3 and 99.6% respectively, which allows the routine application of eight loci in wildlife management – as a first attempt to use molecular genetic information for such a task and it eliminates multiple sample handling in consecutive PCRs. Two loci out of the nine were never been used in deer previously.

Keywords: parentage, microsatellite, deer

Zusammenfassung

Entwicklung eines achtfach mulitplexen Microsatelliten PCR Verfahrens zum Abstammungsnachweis beim Hirsch

Neun Loci wurden in zwei multiplex Microsatelliten Polymerase Kettenreaktion (PCR) Sets (vier und fünf Genorte) kombiniert und genutzt, um die wahrscheinlichste Mutterkuh für jedes Kalb zu bestimmen. Beide Ansätze waren geeignet, um sie in einer achtfach multiplexen Reaktion zu kombinieren. Die Ausschlusswahrscheinlichkeiten der achtfach multiplexen Reaktion der neun Genorte waren jeweils 99,3 und 99,6%. Dies erlaubt den routinemäßigen Einsatz der acht Genorte in der Wildhege als einen ersten Versuch, molekulargenetische Informationen für eine solche Aufgabe in Ungarn zu nutzen und beseitigt die Notwendigkeit einer mehrfachen Probenhandhabung in fortlaufenden PCRs. Drei der neun Genorte wurden nie zuvor beim Hirsch eingesetzt.

Schlüsselwörter: Abstammung, Microsatellite, Hirsch

Introduction

Molecular genetic information was already gained successfully in different species e.g. for determination of origin of a modern cattle (BEJA-PEREIRA *et al.* 2006), for analysis of genetic diversities in donkey (JORDANA *et al.* 2001) or horse (ABERLE and DISTL 2004) and for classification purposes in pig (ZSOLNAI *et al.* 2006).

In deer, comprehensive studies have been conducted in searching for quantitative trait loci (SLATE *et al.* 2002), and for determination of inbreeding effect on breeding success (SLATE *et al.* 2000). Molecular data have helped to confirm genetic integrity of Charpatian red deer (FEULNER *et al.* 2004) and were used to seek for key genes in antler development (MOLNÁR *et al.* 2007) which might lead to useful markers for breeders.

One aspect of the methodologies is the application of paternity test which is already routinely implemented for example in cattle and sheep (GLOWATZKI-MULLIS *et al.* 2005, 2007) in many countries. There is an obvious demand from the wildlife managements including deer breeders, to follow-up the lineage, especially when important traits (antler characteristics, body weight, etc.) are logged in the herdbook. Molecular genetic approach has already been implemented for completing this task previously. BONNET *et al.* (2002) applied eleven microsatellites in 3 multiplex reactions and HAANES *et al.* (2005) used 25 loci in 6 reactions for genotyping in deer.

The farms sampled in this study are using single mating system, where few stags are in use. Hinds are monitored visually for pregnancy, but after parturition the fate of a calf can not be followed easily by observation, because the calf sometimes chooses other lactating animal than its own mother.

Here we aimed to test and select markers suitable in both fallow and red deer, to incorporate them in one reaction to reduce time and costs in laboratory and to characterise its efficiency in parentage assignment.

Material and methods

In the summer and fall of 2007, 119 red deers from two farms were selected for parentage analysis. Farm A had 25 hinds including 4 empty ones and 46 calves. Farm B presented 29 hinds including 3 empty ones and 26 calves. Additional 47 fallow-deer samples from two farms were also available. Blood samples were collected into EDTA coated tubes and stored frozen until DNA preparation (Zsolnai *et al.* 2003). Muscle pieces were stored in Eppendorf tubes under 96% ethanol on room temperature. Microsatellite loci used for analysis were;

- Set1: BM4208, NVHRT 21, RT 1, NVHRT 73;
- Set2: CSSM066, DIK082, IDVGA59, NVHRT48, OARCP26.

DNA amplification was performed in a Hybaid Thermal Cycler in a total volume of 10 µl. Parameters of Set1 were: 3 mM $MgCl_2$, 200 µM dNTPs, 0.66 U DyNAzyme, and the corresponding primer pairs in concentration of 0.30, 0.15, 1.00 and 1.00 mM, respectively (Table 1). PCR conditions included: a denaturation step of 4 min at 94°C; 30 cycles of 40 s at 94°C, 45 s at 54°C, 45 s at 72°C and a final extension of 10 min at 72°C. Parameters of Set2 were: 3.5 mM $MgCl_2$, 200 µM dNTPs, 0.66 U DyNAzyme, and the corresponding primer pairs in concentration of 0.20, 0.70, 1.20, 2.00 and 0.70 mM, respectively (Table 1). PCR conditions included: a denaturation step of 4 min at 94°C, 2 cycles of 30 s at 94°C, 30 s at 58°C, 1 min at 72°C; 3 cycles of 30 s at 94°C, 30 s at 57°C, 1 min at 72°C; 3 cycles of 30 s at 94°C, 30 s at 56°C, 1 min at 72°C; 11 cycles of 30 s at 94°C, 30 s at 54°C, 1 min at 72°C; 16 cycles of 20 s at 94°C, 30 s at 52°C, 1 min at 72°C; and a final extension of 10 min at 72°C.

The eight-plex reaction amplifies all loci listed under Set1 and Set2 except OARCP26. Primer concentrations are the same as in Set1 and Set2. $MgCl_2$ concentration is 4.1 mM. PCR cycling conditions is the same as for Set2.

Table 1
PCR primers and their concentrations in the Set1 and Set2 reactions
PCR Primer und ihre Konzentration in den Reaktionen von Set1 und Set2

Multiplex	Locus	FL	PC (mM)	Source	Primers
Set1	BM4208	NED	0.15 0.15	BISHOP *et al.* 1994	TCA GTA CAC TGG CCA CCA TG CAC TGC ATG CTT TTC CAA AC
	NVHRT21	VIC	0.075 0.075	RØED and MIDTHJELL 1998	GCA GCG GAG AGG AAC AAA AG GGG GAG GAG CAG GGA AAT C
	RT1	PET	0.50 0.50	WILSON and STROBECK 1997	TGC CTT CTT TCA TCC AAC AA CAT CTT CCC ATC CTC TTT AC
	NVHRT73	6FAM	0.50 0.50	RØED and MIDTHJELL 1998	CTT GCC CAT TTA GTG TTT TCT TGC GTG TCA TTG AAT AGG AG
Set2	CSSM066	VIC	0.10 0.10	MOORE *et al.* 1994	ACA CAA ATC CTT TCT GCC AGC TGA AAT TTA ATG CAC TGA GGA GCT TGG
	DIK082	6FAM	0.35 0.35	HIRANO *et al.* 1996	CCC ACT CTG TCT CCA GTT TG TAT CCT GAG AAA AGC TGC TAG A
	IDVGA59	6FAM	0.60 0.60	MEZZELANI *et al.* 1995	CAG TCC CTC AAC CCT CTT TTC AAC CCA AAT ATC CAT CAA TAG
	NVHRT48	PET	1.00 1.00	RØED and MIDTHJELL 1998	CGT GAA TCT TAA CCA GGT CT GGT CAG CTT CAT TTA GAA AC
	OarCP26	NED	0.35 0.35	EDE *et al.* 1995	GGC CTA ACA GAA TTC AGA TGA TGT TGC GTC ACC ATA CTG ACG GCT GGT TCC

FL fluorescent label, PC primer concentrations

Amplified fragments were resolved on ABI310 Automatic Fragment Analyzer. LIZ600 was used as internal standard. Fragment length allocation was accomplished by Genescan and Genotyper programs.

The following softwares were used for data processing and crosschecking the results.: Microsatellite Toolkit (PARK 2001) has been used for data formatting in order to meet the input format of GeneClass (PIRY *et al.* 2004), WhichRun (BANKS and EICHERT 2000), PAPA (DUCHESNE *et al.* 2002) and CERVUS (MARSHALL *et al.* 1998).

Results and discussion

Altogether 41 microsatellite loci were tested.

Twenty-two of them were checked in simplex reactions using Taq and DyNAzyme polymerases with different PCR conditions. Eight and eleven loci out of the forty-one used by others for parentage tests of sheep (advised by the International Society for Animal Genetics) and bovine (GLOWATZKI-MULLIS *et al.* 1995), respectively. These sets have also been investigated on deer samples.

Finally nine loci were selected from the 41, which displayed clear electrophoretic patterns on agarose gel or in capillary electrophoretic separation in case of both red and fallow-deer as well.

Based on the observed lengths of the fragments the primer labelling colours were devised to facilitate multiplexing.

In case of red deer the combined exclusion probabilities for the first parents (CEP) of 4- and 5-plex PCR were 92 and 95%, respectively. If the two reactions were used consecutively, the previous values were changing to 99.6% (Table 2). Heterozygozity deficits were observable on most loci (Table 3), but deviation from Hardy-Weinberg equilibrium was significant only in one case (FarmB, locus IDVGA59, data not shown).

Table 2
Characterisation of the multiplex sets used in two farms
Beschreibung der multiplex Sets aus zwei Farmen

	Farm A (n=67)	Farm B (n=52)	Farm A+B (n=119)	Farm A+B (n=119)	Farm A+B (n=119)	Farm A+B (n=119)
Locus number	9	9	9	8	5	4
Mean H_o	0.6892	0.7392	0.7121	0.6950	0.7524	0.6618
Mean H_e	0.7790	0.7991	0.8017	0.7979	0.08110	0.7910
Mean *PIC*	0.7466	0.7699	0.7761	0.7738	0.7841	0.7694
F_{is}	0.156	0.153				
CnEP (first parent)	0.0080	0.0038	0.0037	0.0071	0.04613	0.0777

n number of sampled animals, H_o observed heterozigosity, H_e expected heterozigosity, *PIC* polymorphic information content, *CnEP* combined non-exclusion probability, F_{is} inbreeding coefficient

Table 3
Observed and expected heterozygosity, number and size range of alleles, *PIC*, F_{is} in different loci and farms
Erfasste und erwartete Heterozygotie, Anzahl und Größe der Allele, PIC und F_{is}-Werte bei unterschiedlichen Loci und Farmen

Locus	H_o	H_e	H_o	H_e	No. of alleles		Size range		*PIC*		F_{is}	
	A		B		A	B	A	B	A	B	A	B
NVHRT73	0.791	0.806	0.788	0.839	13	17	202-234	202-238	0.776	0.815	0.019	0.061
NVHRT21	0.500	0.799	0.596	0.854	13	10	142-193	142-168	0.767	0.827	0.376	0.304
BM4208	0.687	0.860	0.615	0.866	10	10	144-182	144-182	0.838	0.842	0.203	0.291
RT1	0.642	0.654	0.635	0.566	8	10	208-232	208-232	0.625	0.540	0.019	−0.122
DIK82	0.627	0.767	0.769	0.849	8	9	122-137	122-137	0.727	0.821	0.184	0.095
IDVGA59	0.806	0.766	0.962	0.846	12	11	254-275	254-274	0.728	0.822	−0.053	−0.139
CSSM66	0.657	0.756	0.750	0.760	8	10	161-187	161-187	0.718	0.716	0.132	0.013
CP26	0.851	0.837	0.846	0.811	10	11	125-162	123-162	0.809	0.778	−0.017	−0.044
NVHRT48	0.642	0.767	0.692	0.802	11	10	81-107	90-107	0.733	0.768	0.164	0.138

H_o observed heterozigosity, H_e expected heterozigosity, *PIC* polymorphic information content, F_{is} inbreeding coefficient, A, B farm

PAPA, Whichrun, GeneClass and CERVUS programs have been compared for the ability to point on possible hinds. Animals selected as parents by the programs were the same in 93% of the cases. However the genotype assignment was clear, and repeated PCRs gave the same genotypes, different programs displayed different suggestions for a most probable mother in the remaining cases (7%). In order to resolve these contradictions, genotypes of the animals in question were aligned and checked visually.

Since the output interpretation was mostly convenient in case of GeneClass and CERVUS, we suggest using them for parentage control if many samples have to be compared. Additionally CERVUS can correct genotyping errors increasing the success of

paternity assignment (KALINOWSKI *et al.* 2007) and proved to be useful for forensic applications (CASTAGNASSO *et al.* 2007).

The two PCR sets are suitable for parentage control in red deer and might be used for other species and farms as well. The combined 8-plex PCR is still retaining high, 99.3% CEP value for the first parent (Table 3), and can be used alone for the same purpose.

Redesign of primer labelling colours might help in creating the 9-plex PCR, which includes the OaerCP26 locus as well. In this study seven loci are identical with those described in the paper of HAANES *et al.* (2005), however

- BM4208, NVHRT48 and OarCP26 were used in simplex,
- NVRT73, CSSM066 and NVHRT21 were used in duplex,
- RT1 was used in a triplex reaction by them.

These seven loci were in our initially tested primer-pool and proved to be suitable for multiplexing. The remaining two loci (DIK082, IDVGA59) were developed by independent authors for cattle (Table 1).

In case of fallow-deer (47 animals from two farms), the investigated populations were quite homogeneous, the inbreeding coefficient (F_{is}) were 61 and 43%. In these populations more microsatellite loci should be involved for useful parentage test.

The described microsatellite reactions (especially the 8-plex) support the wildlife management's decisions, help to minimise labour and running costs and minimise the possibility of cross contamination or sample swapping. Since there is a chance that the multiplex reaction works on other deer species (BONNET *et al.* 2002), it might be interesting for the community working in this field.

The gained genotype data

- will be applied to assign lost or strayed individuals to their subpopulation,
- hopefully serve as an essential basis of the efforts of trait mapping,
- helps to select breeding individuals for maintaining diversity in the red deer populations,
- provide basis for the search of populations capable to decrease inbreeding status of the fallow-deer.

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