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Detection of New Genomic Landmarks in the Maltese Goat Using Rapd PCR

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Abstract: Since no information of the Maltese goat genome is available, RAPD technique has been used to identify a number of DNA landmarks. Genome Landmarks have been obtained from the DNA of 66 Maltese goats which were studied with Random Amplification of Polymorphic DNA (RAPD). Eleven (11) reproducible RAPD polymorphic zones were identified. For sequencing, the RAPD zones were cloned into the Puc 18 vector utilising *E. coli* and then sequenced using both the forward (universal) and reverse primers specific for the Puc 18 vector. After sequencing a total of nine new markers (AF078170, AF078171, AF078172, AF078173, AF078174, AF078175, AF078176, AF078177, AF078178) amounting to approximately 5,500 bp of sequence from the goat genome were identified and their homologies with known nucleic acid and protein databases were described. The new sequences could provide useful anchors for more extensive mapping and sequencing and for genomics assisted breeding of the Maltese goat.

Key words: Maltese goat, DNA, rapd, landmark

INTRODUCTION

The Maltese goat (*Capra hircus hircus*) is considered to be of great socio-economic importance in the Mediterranean countries and South Africa[1]. Vaiman, *et al*., (1996) described the first genetic linkage map of the goat genome. A meitotic map covering 2300 cM, i.e. >80% of the total estimated length of the goat genome was contructed. Moreover, eight cosmids containing microsatellites were mapped by fluorescent *in situ* hybridisation in goat and sheep.

A significant advance in DNA fingerprinting technology was made in the early 1990s through the development of a PCR-based strategy which involved the use of single oligonucleotide primers of arbitrary sequence to amplify random genomic DNA fragments[2-4].

The random amplification strategy is shared by three methods called arbitrary primed PCR (AP-PCR;[2] random amplification of polymorphic DNA[3] and DNA amplification fingerprinting[4]. The methods differ in the length of primers used, the amplification conditions and in the analysis of the PCR products. Consequently, they generate markedly different fingerprint patterns varying from quite simple to complex.

Random amplification technology is an efficient tool to quickly and easily screen a very large number of possible DNA polymorphisms from loci and which are usually referred to as random amplified polymorphic DNA (RAPD). These polymorphisms have proved capable of discriminating between closely related individuals. This feature combined with their easy identification makes RAPDs a valid type of marker, potentially useful in many areas of genetic research such as genomic mapping, marker-assisted selection in breeding tasks and individual or strain identification.

In this study we describe the detection of anonymous fragments from the genome of the Maltese goat, employing RAPD PCR that may be suitable to anchor a more extensive genome map or for linkage analysis.

MATERIALS AND METHODS

Blood/DNA samples: Various blood samples were obtained from sixty-six (66) Maltese goats at the animal research farm in Potenza, Italy and shipped to our laboratory on wet ice. The genomic DNA was extracted from leucocytes by the phenol-chloroform method as described by Poncz *et al.*, 1982.

DNA amplification and genotyping analysis: RAPD PCR was conducted as described by Williams, *et al*,[3]with some modifications; 0.5 μM primer, 0.5 μg of genomic DNA, 10mM Tris Cl pH 8.8 @25°C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% (v/v) Triton X-100, 100 μM dNTPs, 0.6 units Tbr polymerase in 50 μL. The thermal cycling conditions were 1 min. at 94°C, 1 min. at 36°C and 4 min, at 72°C for 45 cycles. The amplified products were separated on a 4% polyacrylamide gel.

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The 10bp primer used ^{5}TAG CTG TAC G^{3} was selected with the following criteria: Exclusion of self homology, as these can lead to partial double-stranded (snap-back) cross structures which prevent annealing to template DNA and of nucleotide sequence repeats; a G+C ratio between 50-60% was chosen to direct specific binding and allow efficient melting during PCR;

Cloning and DNA sequencing: The RAPD PCR products were treated with Klenow polymerase (Boehringer Mannheim GmbH, Mannheim, Germany) for 30 minutes to ensure blunt ending of the DNA fragments Hunter and Hunter[5]. This mixture of RAPD PCR fragments was purified using Microcon centrifugal concentrators followed by ligation to Sma I digested Puc 18 (Boehringer Mannheim GmbH, Mannheim, Germany). *E. Coli* TGI, made competent by the calcium chloride method, were transformed with this ligation mixture. Efficiency of ligation was analysed by blue/white selection. Insert size was determined by restriction fragment analysis of mini-prep DNA. CsCl purified DNA from the positive clones was sequenced using dye-primer cycle sequencing.

The reactions were performed on the CATALST 800 workstation with universal forward and reverse primers and analysed on a 373A Applied Biosystems automated sequencer (Perkin-Elmer Applied Biosystems, Foster City, USA). The chosen colonies were grown in 50 mL cultures in Terrific Broth and the plasmid DNA was isolated and purified using the CsCl method Sambrook *et al.*[6]. The plasmid DNA was dialysed against three changes of TE buffer pH 8.0 and ethanol precipitated and checked on a 0.7% agarose gel for any contamination with RNA.

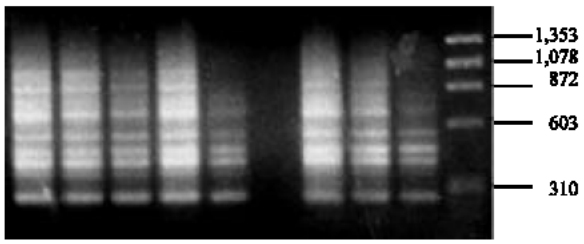
The plasmid DNA was sequenced using a CATALST 800 workstation with the forward and reverse primers for Puc 18 vector and a 373A DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, USA). The ABI dye-primer cycle sequencing and dye-deoxy termination cycle sequencing kits were used to label the sequencing products.

Analysis of sequencing results: Data from the sequencing gels were analysed and aligned using GeneJockey II (Biosoft, Cambridge, UK) and DNASIS 2.0 software (Hitachi Software, San Bruno, USA). The homologies of these sequences with known sequences obtained from various databases mainly Genbank, cDNA library and ESTs, were sought using the BLAST TN software obtained from internet site www.ncbi.nlm.nih.gov Warren and States[7]. Homologies with protein databases of the translated frameshifts (from +3 to –3) was sought using BEAUTY 1.4 and BLAST TN software from internet site www.ncbi.nlm.nih.gov Atschul, S.F., *et al.*[7,8]. The restriction maps were obtained using WebCutter 2 computer software from internet site www.firstmarket.com/cutter/cut2.html.

RESULTS

The appearance of discrete RAPD products depended on many factors which require standardisation. These include the quality of the genomic DNA and the molar ratios of templates, primers and polymerase.

The optimal ratios of genomic DNA to *Tbr* polymerase which gave discrete RAPD profiles are shown in Fig. 1.



Lane	1	2	3	4	5	6	7	8	9
DNA μg^{-1}	0.5	0.5	0.5	0.4	0.4	0.4	0.3	0.3	0.3
Tbr/U	1	0.8	0.6	1	0.8	0.6	1	0.8	0.6

Fig. 1: Agarose gel showing the effects of the variation of polymerase and DNA template on the RAPD profiles. Lanes 1,2 and 3, 0.5 μg of goat genomic DNA; lanes 4, 5 and 6, 0.4 μg of DNA; lanes 7, 8 and 9, 0.3 μg of DNA; lanes 1, 4 and 7, 1U of *Tbr* polymerase; lanes 2, 5 and 8, 0.8U; lanes 3, 6 and 9, 0.6U. Lane 10 is the bacteriophage ϕX174 digested with Hae III was used as a standard marker of DNA size (bp)

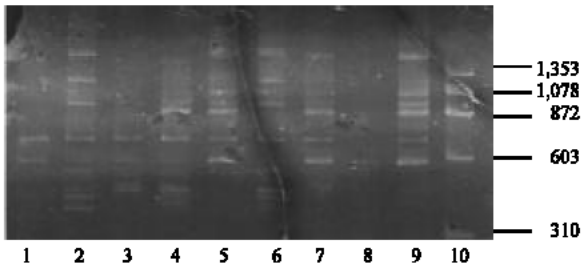


Fig. 2: Different genetic profiles on different DNA templates using the same RAPD R1 primer. Lane 1, sample no. 6; lane 2 sample no. 12; lane 3 sample no. 25; lane 4 sample no. 22; lane 5 sample no. 9; lane 6 sample no. 27; lane 7 sample no. 62; lane 8 no amplification product; lane 9 sample no 10. Lane 10 is a standard marker of DNA size (bp): bacteriophage ϕX174 digested with Hae III

Table 1: The genetic profile of each animal and the total number of RAPD zones (markers) present. 1 = presence of band

	RAPD zones (markers)										
DNA NO.	1	2	3	4	5	6	7	8	9	10	11
1	1				1						1
2	1		1	1	1		1				1
3	1	1	1		1		1	1	1		
4						1	1	1			
5			1	1						1	
6			1	1	1		1				
7	1	1	1		1						
8	1	1			1	1					
9	1	1	1	1	1		1				
10	1	1	1	1	1		1				
11	1		1		1		1				1
12	1	1	1		1		1	1	1	1	
13		1	1		1		1		1	1	
14	1	1	1		1		1		1		
15	1	1	1	1	1		1	1	1	1	
16					1					1	
17					1				1		
18		1	1		1		1		1	1	
19	1		1	1	1		1		1	1	
18		1	1		1		1		1	1	
19	1		1	1	1		1		1	1	
20	1	1	1	1	1		1	1	1	1	
21			1	1	1		1				1
22		1		1	1		1			1	
23	1	1	1		1	1	1	1	1		
24		1	1	1	1	1			1	1	
25					1		1		1	1	
26	1	1		1	1		1				
27	1	1	1			1					
28	1		1		1		1				
29	1	1	1	1	1			1	1	1	
30		1	1	1	1		1		1	1	
31	1		1	1	1						
32				1							1
35	1		1			1	1				
36	1		1	1	1						1
37			1		1		1				
38	1	1	1	1	1		1				
39		1	1	1	1			1	1		
40	1		1		1						1
41	1				1		1				
42			1		1		1				
43	1	1			1	1	1				
44	1		1					1			1
45	1	1	1		1		1		1	1	
46		1	1	1	1	1		1			
47		1	1		1		1		1	1	
48	1		1							1	
49			1		1		1				
50	1	1			1	1	1				
51	1		1					1			1
52	1		1		1		1		1	1	
53		1	1	1	1	1		1			
54		1	1		1		1		1	1	
55	1		1				1			1	
56	1	1	1		1			1		1	
57	1	1	1		1		1		1	1	
58		1		1	1						1
59		1	1	1			1		1	1	
60	1		1		1		1				
61	1		1	1	1			1		1	
62	1		1		1		1		1		
63		1		1	1	1					
64		1	1		1		1			1	1
65	1		1	1	1			1	1		
66	1	1		1	1		1			1	
Totals	40	35	51	28	56	11	42	15	25	27	11

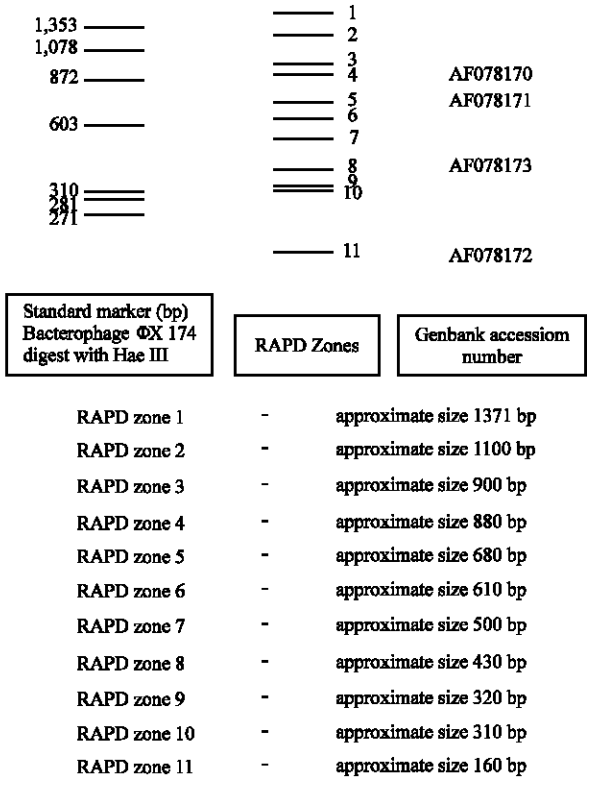


Fig. 3: Rapd bands obtained from the whole goat population and their respective sizes

Table 2: Percentage of the RAPD zones

RAPD band	Percentage RAPD zones
1	60.6
2	53.0
3	77.3
4	42.4
5	84.9
6	16.7
7	63.6
8	22.7
9	37.9
10	40.9
11	16.7

The RAPD PCR reaction for each animal was repeated at least three times and the same number and sizes of RAPD zones were obtained. The typical profiles obtained from the genomic DNA of the 66 goats with the single RAPD primer are shown in Fig. 2.

A total of 11 discrete RAPD zones were obtained from the 66 goat genomes studied. The approximate sizes of the designated RAPD products are given in Fig. 3. The genetic profile based on the RAPD zones present of each of the 66 individual animals is shown in Table 1. The percentage presence of each RAPD band was calculated as shown in Table 2.

The plasmid DNA from 11 different clones containing the different RAPD zones (corresponding to RAPD zones 4, 5, 8 and 11 as shown in Fig. 3) were sequenced and submitted to Genbank. Four different complete sequences were obtained from four clones (Genbank accession

Table 3: The homology search with nucleic acids databases and homology search of frameshift (from +3 to -3) compared to protein databases using Beauty 1.4 and Blast TN of the new genomic landmarks obtained from the Maltese goat

Genbank Accession No.	Size /bp	Repeats	Homology search with nucleic acid database	Homology search, frameshifts (from +3 to -3) compared to protein database
AF078170	877	No repeats	Low statistical significance homology	Low homology and low statistical significance homology
AF078171	681	MIR repeat, 174 bp long	84% homology, high statistical significance ($p = 1.2 \times 10^{-7}$) with human sequence from PAC 302C05 from Xq 23 (from 307 to 366 bp)	Low homology and low statistical significance homology
AF078172	164	No repeats	Low homology and low statistical significance homology	Low homology and low statistical significance homology
AF078173	430	No repeats	81% homology, high statistically significance (p = 0.095) with *Bos taurus* (cow) clone bm4005 microsatellite and a -2b repeats	Low homology and low statistical significance homology
AF078174	666	MIR repeat, 179 bp long	75% homology, high statically significance (p = 0.0002) with human sequence PAC181NI on chromosome X containing ESTs, STS polymorphic C.A. repeats	Low homology and low statistical significance homology
AF078175	675	No repeats	Low homology and low statistical significance homology	Low homology with high significance (p = 0.043) with PPE family protein of *Mycobacterium tubercolosis*
AF078176	696	No repeats	Low homology and low statistical significance homology	Low homology and low statistical significance homology
AF078177	670	No repeats	High homology 78%, 80% & 79%, high statistical significance ($p = 1.4 \times 10^{-18}$, 4.5×10^{-16}, 3.4×10^{-36}, respectively) with bovine thyroglobulin gene 5' end.	High homology (73%) and high statistical significant p = 0.057 with T-cell receptor gamme chain V-J-C region (clone 197G1) from sheep (*Ovis aries*) fragment gi/1394(Z12964).
AF087178	707	Simple repeat, 53 bp long	High homology 86%, 83%, 83%, high statistical significance ($p = 0.0075$, 3.3×10^{-10}, 1.6×10^{-17} with *Mus musculus* cDNA	Low homology (41%) and high statistical significance (p = 0.045) with 61K protein from *Autographia california* nuclear polyhedrosis virus gi/58673 (Z11662) fragment

numbers: AF07170, AF07171, AF07172, AF07173) and five incomplete sequences from three clones (Genbank accession numbers: AF07174, AF07175, AF07176, AF07177, AF07178).

The homology search with nucleic acids databases and homology search of frameshift (from +3 to -3) compared to protein databases using BEAUTY 1.4 and BLAST TN of each fragment is shown in Table 3.

DISCUSSION

The main disadvantage of RAPD PCR is the low stringency of amplification that directly influences profiles of the products and their reproducibility. Micheli *et al.*[9], reported that DNA contaminants precipitated by ethanol were the major factor influencing the reproducibility. In this project the phenol/chloroform method of DNA extraction was utilised and the final DNA was precipitated in ethanol. High quality reagents were used and special care was taken to avoid chemical carry over especially phenol as this could affect the final quality of the DNA. Another factor affecting the RAPD profiles and not previously reported was the fragmentation of DNA especially during centrifugation. Thus, prior to amplification, the DNA was checked on a 0.5% agarose gel to confirm that it was intact.

The template/primer/polymerase ratio was also reported to affect reproducibility of RAPD amplification. Schierwater, B[10]. Williams *et al.* [3]indicated that by decreasing the concentration of either the polymerase or genomic DNA, a non-discrete range of amplification products, appearing as a 'smear', can be converted to discretely sized bands by reducing the concentration of either the polymerase or the genomic DNA. In this study, various combinations of primer/template/polymerase ratios were applied until discrete sized bands were obtained (Fig. 2). This combination of primer/template/ polymerase ratio was utilised to amplify different goat samples.

The same brand of *Tbr* polymerase and the same thermal cycler was utilised to amplify all goat samples since these have also been reported to influence reproducibility[11].

Although the RAPD PCR reaction for each animal was repeated at least three times the same numbers and sizes of RAPD zones were obtained. Thus by using constant PCR conditions including primer/template/polymerase ratios as well as same polymerase and thermal cycler a reproducible RAPD fingerprint was obtained.

RAPD polymorphisms are typically detected through the presence or absence of particular fragments after gel electrophoresis. However, other types of polymorphisms (e.g. length and brightness) have been identified[3,12,13,11]. The brightness polymorphisms may be the result of amplification of a tandem repeat locus which is polymorphic for copy number or of differential amplification due to sequence differences in the priming site(s)[4,13].

Bielowski, J.P.[14] grouped these variations of RAPD intensities into four categories i.e. strong, faint, fuzzy and sharp. Three explanations were offered that could account for these mixed-intensity bands: (i) the target DNAs are undefined, (ii) one or more copies of the target DNA may exist per genome and (iii) the percentage of hybridisation of primer to target may vary. Although some goat's RAPD zones were noted to be more intense than others, the degree of intensity of these bands were not assessed as the different intensities could have been the result of variation in the concentrations of reagents and template, the pH and other reaction parameters.

CONCLUSION

RAPD, although not a straight forward procedure, was found to be a relatively simple, robust and an efficient way of identifying new genomic landmarks from a genome whereby there are no information available. Due to the fact that the RAPD PCR is based on low stringency conditions and in order to get reproducible RAPD profile the same conditions have to be kept constant, sequencing of the RAPD zones was carried out to allow the synthesis of highly stringent oligonucleotides. Thus these new genome landmarks that were produced using RAPD PCR can now be amplified by PCR in a more straight forward procedure. These new genome landmarks produced in this study can possibly be used in the Maltese goats to establish pedigrees or to select animals with a particular economic trait e.g., high milk production, high prolificacy and adaptation to heat stress, traits that characterise the Maltese goat.

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