RAPD analysis of systematic relationships among the Cervidae

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We investigated the possible application of RAPD (Random Amplified Polymorphic DNA) analysis to the study of the systematic relationships of five cervid taxa. Amplifications with eight different primers gave reproducible electrophoretic patterns which could be regarded as a data-set consisting of monomorphic and polymorphic characters. Some of these characters are species- and subspecies-specific. Band-sharing analysis and numerical taxonomy methods allowed us to generate a phenetic tree. Our results point out new possible systematic considerations within the examined taxa.

Keywords: Cervidae, deer, molecular systematics, RAPD.

Introduction

Despite the continuous accumulation of palaeontological, morphological and behavioural data, systematic and phylogenetic relationships among the most important Cervidae subfamilies are not yet completely understood (Harrington, 1985; Groves & Grubb, 1987). Recently several models of phylogenetic relationships, based on different criteria, have been reported. These criteria include: (i) karyotypic analysis (Neitzel, 1987; Wang & Du, 1988; Fontana & Rubini, 1990); (ii) studies on constitutive heterochromatin localization (Wessman & Gripenberger, 1993); (iii) Southern blot hybridization of repetitive DNA (Scherthan *et al.*, 1987); (iv) the distribution of repetitive sequences of IA satellite DNA (Bogenberger *et al.*, 1987); (v) comparative studies of mitochondrial DNA sequences (Miyamoto *et al.*, 1990) and mitochondrial DNA restriction site maps (Cronin, 1991); and (vi) allozyme studies (Emerson & Tate, 1993).

A molecular technique based on the polymerase chain reaction (PCR) provides an effective tool for the rapid identification of genetic markers known as Random Amplified Polymorphic DNA (RAPD) (Williams *et al.*, 1990; Welsh & McClelland, 1990). Such markers, derived from priming sites randomly distributed throughout the genome, are inherited in a Mendelian fashion (Rothuizen & Van Wolferen,

1994). These polymorphisms allow the analysis of complex genomes, like those of eukaryotes, without prior knowledge of DNA sequence (Hadrys *et al.*, 1992). The potential use of RAPD in phylogenetic studies (Chalmers *et al.*, 1992; Demeke *et al.*, 1992; Castiglione *et al.*, 1993; Fani *et al.*, 1993; Tibayrenc *et al.*, 1993; Gwakisa *et al.*, 1994; Kaukas *et al.*, 1994; Bandi *et al.*, 1995) and population genetics (Lynch & Milligan, 1994; Baruffi *et al.*, 1995) has been widely documented in a large variety of organisms. The goal of this work is to study the systematic relationships among the Cervidae by means of RAPD analysis.

Materials and methods

Specimens and sources of genomic DNA

The study has been conducted on different species of the Cervidae family: *Cervus elaphus* (red deer) and *Dama dama* (fallow deer), from the Cervinae subfamily; *Capreolus capreolus* (roe deer) and *Odocoileus hemionus hemionus* (mule deer) from the Odocoileinae; *Muntjac muntjac vaginalis* (Indian muntjac) from the Muntjaginae. One bovid species, *Bos taurus* (Bovidae), was employed as an outgroup. Whole blood samples were obtained from the following species and locations: *C. elaphus* and *D. dama* from Bosco della Mesola (Ferrara, Italy), *C. capreolus* from Bosco di Carrega (Parma, Italy). DNAs of *M. m. vaginalis* and *O. h. hemionus* were

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extracted from ATCC (American Type Culture Collection) cell lines (one sample for each species). Bovine DNA was derived from a representative of Italian Fresian breed. DNAs of the other species were extracted by the standard phenol-chloroform procedure from peripheral blood leucocytes (four samples each for *C. elaphus*, *D. dama* and *C. capreolus*).

Amplification conditions

Amplification reactions were performed in volumes of 10 μ L containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 0.01 per cent gelatin (w/v), 0.1 per cent Triton X-100, 0.2 mM of each of the dNTPs, 250 ng of each single primer, 10 ng of purified DNA and 0.5 units of Taq DNA polymerase (Promega, USA). The reactions were overlaid with one drop of mineral oil. We report results obtained with the MJ PT-100 Thermal Cycler (Watertown, USA) programmed with a first denaturation of 1 min and 30 s at 94°C, followed by 45 cycles of 30 s at 94°C, 1 min at 45°C and 2 min at 75°C. Two final steps of 10 min at 75°C and 10 min at 60°C were made to extend further the amplification products.

Selection of the primers

Purified primers were supplied by Genenco (Firenze, Italy) and analysed to determine their annealing temperatures and possible secondary structure formation (OLIGO 4.0, Medprobe). We used primers ranging in size from 19 to 26 bases, with different nucleotide-proportion (G + C content) and designed for other purposes. From a previous screening of a wide set of primer-template combinations, we chose eight primers that gave a high number of polymorphic markers and a high statistically significant percentage of comigrating bands, assumed to be monomorphic characters. Sequences of the selected primers and their G + C content (in percentage) are as follows:

P1: 5'-CGAAACAAGCGTGCATGAGCCCGAA (56)

P2: 5'-CTAATGCAGGAGTCGCATAAGGGAGA (46)

P3: 5'-GATGAACTCCAGGATAAAATC (38)

P4: 5'-CTTCAGTGACAAAATAGCCAAA (36)

P5: 5'-CCTGACCAGGCACTGGCAGA (65)

P6: 5'-CTCAATAACTTCTGGAGAAAG (38)

P7: 5'-GAAGCAGCCCCGGTAGTAGGTTGAGG (60)

P8: 5'-TTTGATGTCTCCTTAGAGT (37)

RAPD detection

Five μ L of each amplification reaction were loaded onto 3 per cent (w/v) agarose gels (1.5 per cent NuSieve-GTG; 1.5 per cent SeaKem-GTG) with TBE buffer (0.89 M Tris-OH, 0.89 M boric acid and 0.11 M EDTA) containing 0.5 μ g mL⁻¹ (w/v) ethidium bromide (Sambrook *et al.*, 1989) and electrophoresed at 10 V cm⁻¹. Because of the complexity of the patterns, detection of RAPDs was performed using a computer-assisted imaging system (BIOPROFIL-2D, Vilber-Lourmat).

Statistical analysis

RAPD markers were scored as present or absent and coded as two state characters to calculate a matrix based on the Dice similarity index (Sneath & Sokal, 1973). Similarity measure computations, principal coordinate (PCO) and cluster analysis (according to the unweighted pair-group method with arithmetic averages, UPGMA) were effected using NTSYS (Exeter Software). Data resampling and matrix calculations for bootstrap analysis were performed using a specifically developed software (available on request). The consensus tree construction was performed using PHYLIP 3.5c (Felsenstein, 1993). The statistical analysis was performed for each primer separately and for the combined data of all the primers.

Results

The amplifications were carried out twice independently in two different laboratories, using the same thermal cycler model, obtaining reproducible results. The electrophoretic patterns from amplifications with eight different primers (P1–P8) were generally complex: 5–10 monomorphic bands common to all samples and many polymorphic markers, ranging from 50 to 2000 bp in size. The primers gave different species-specific markers in terms of length and intensity of the amplification fragments (Figs 1 and 2 show, as examples, RAPDs obtained with primers P1 and P2). In addition, all the individuals tested within each group showed a different pattern. The matrix of the estimated values of similarity, including the amplification data, is reported (Table 1). The derived phenetic tree (Fig. 3) clearly

discriminates the systematic groups, giving different values of similarity between and within taxa. In particular, the dendrogram shows three main hypothetical taxonomic units (HTU): two HTUs including *C. elaphus* and *D. dama* are grouped together in

a single cluster. The third contains the *C. capreolus*. The remaining terminal nodes are the *O. h. hemious* and *M. m. vaginalis*. As expected from an outgroup, *B. taurus* stems at the deepest position of the tree. The dendrogram calculated on eight primers is similar to those obtained analysing each single primer (data not shown). The topology of the dendrogram is congruent with the distribution of the groups revealed by the PCO (Fig. 4).

Discussion

We have applied the RAPD analysis to the study of the systematic relationships in the deer family. This technique is rapid and useful to detect large amounts of polymorphism. Considerable advantages of the technique are that no prior knowledge of the molecular biology of the investigated organisms is required and it is very cost-effective. Theoretically, the number of DNA polymorphisms that can be detected with RAPD analysis is higher than with traditional methods. RAPD data have been computed using the model of fingerprinting analysis, making the assumption that RAPD markers are in Hardy-Weinberg equilibrium within and between loci (Gilbert *et al.*, 1990; Lynch, 1990). This assumption obeys the criteria proposed by Clark & Lanigan (1993) for estimating population genetic parameters using RAPD data. From a technical point of view, a

Fig. 1 RAPD patterns obtained with primer P1. The marker (MW) is 100-bp ladder (Pharmacia, Wisconsin). Lanes 13 (O), 14 (M) and 15 (B) are, respectively, *Odocoileus hemious hemious*, *Muntjac muntjac vaginalis* and *Bos taurus*.

Fig. 2 RAPD patterns obtained with primer P2. The marker (MW) is 100-bp ladder. Lanes 13 (O), 14 (M) and 15 (B) are, respectively, *Odocoileus hemious hemious*, *Muntjac muntjac vaginalis* and *Bos taurus*.

Table 1 Matrix of the estimated values of Dice similarity index computed through amplifications with eight primers. C (numbered from 1 to 4) are *Cervus elaphus*; Cp (1–4) *Capreolus capreolus*; D (1–4) *Dama dama*. O, M, B represent, respectively, *Odocoileus hemious hemious*, *Muntjac muntjac vaginalis* and *Bos taurus*

	C1	C2	C3	C4	Cp1	Cp2	Cp3	Cp4	D1	D2	D3	D4	O	M	B
C1	1.000														
C2	0.969	1.000													
C3	0.961	0.976	1.000												
C4	0.947	0.961	0.954	1.000											
Cp1	0.492	0.500	0.496	0.519	1.000										
Cp2	0.492	0.500	0.496	0.519	0.985	1.000									
Cp3	0.496	0.504	0.500	0.523	0.977	0.977	1.000								
Cp4	0.481	0.488	0.485	0.507	0.977	0.962	0.955	1.000							
D1	0.476	0.484	0.480	0.472	0.413	0.413	0.416	0.403	1.000						
D2	0.500	0.508	0.504	0.496	0.438	0.438	0.441	0.428	0.968	1.000					
D3	0.508	0.516	0.512	0.504	0.431	0.431	0.450	0.421	0.921	0.953	1.000				
D4	0.532	0.541	0.537	0.544	0.484	0.484	0.488	0.472	0.833	0.846	0.855	1.000			
O	0.432	0.440	0.436	0.446	0.432	0.432	0.436	0.421	0.467	0.495	0.486	0.476	1.000		
M	0.382	0.389	0.386	0.379	0.365	0.365	0.368	0.359	0.414	0.407	0.400	0.422	0.375	1.000	
B	0.330	0.336	0.333	0.345	0.348	0.348	0.351	0.356	0.234	0.248	0.226	0.239	0.250	0.220	1.000

Fig. 3 UPGMA dendrogram based on Dice similarity index calculated from data of all primers (left). On the right is reported the taxonomy of the investigated organisms (species, subfamily and family names). The numbers at the nodes are the bootstrap confidence values obtained after 100 replicates.

high magnesium chloride concentration (5 mM) combined with a low annealing temperature (45°C) were selected to enhance the stability of primer/template interactions, resulting in reproducible fingerprinting-like patterns. According to our experience the protocol does not appear to be strongly dependent on the quality/quantity of target DNA:

this fact makes it extremely flexible, applicable without modification to a large variety of organisms, using both purified DNAs and crude extractions (Fani *et al.*, 1994; Bandi *et al.*, 1995; Baruffi *et al.*, 1995). Preliminary primers selection is critical to evaluate those that produce a high statistically significant ratio of mono/polymorphic characters, thus

Fig. 4 Principal coordinate analysis of the RAPD data obtained with eight primers. The main components (x, y, z) explain 82 per cent of the variance (56 + 14 + 12 per cent). C (from 1 to 4) are *Cervus elaphus*; Cp (1–4) *Capreolus capreolus*; D (1–4) *Dama dama*. O, M, B represent, respectively, *Odocoileus hemionus hemionus*, *Muntjac muntjac vaginalis* and *Bos taurus*.

providing an internal control of the reliability of the entire process of amplification. each electrophoretic pattern is primer-specific. Additional polymorphisms can be detected with endonuclease digestions of the amplification products (data not shown).

The method has been demonstrated to be valid, being able to distinguish correctly the different species, even within the same subfamily (Cervinae). The similarity between *C. elaphus* and *D. dama* revealed by our results is in total agreement with the data derived from morphological analysis, palaeontological (Groves & Grubb, 1987), molecular (Scherthan *et al.*, 1987) and karyological (Rubini *et al.*,

1990) studies. On the contrary, *C. capreolus* and *O. h. hemionus*, although belonging to the same subfamily, show a low degree of similarity. As a whole, our data suggest a separation of *C. capreolus* from both the Cervinae and the Odocoleinae. This separation has previously been pointed out first by Brooke (1878) and more recently by Groves & Grubb (1990). Our data are in agreement with these authors who consider these species as belonging to distinct tribes: respectively, Capreolini and Odocoleini. The result that most disagrees with the earlier classification methods, based on anatomical features, is the systematic position of *M. m. vaginalis*. This

species is considered to be closer to Cervinae because it belongs to Plesiometacarpalia rather than to the more primitive Telometacarpalia, which include all Odocoileinae (Brooke, 1878). A similar conclusion has also been derived from molecular data (Bogengerger *et al.*, 1987; Scherthan *et al.*, 1987; Miyamoto *et al.*, 1990). However, cytogenetic data show that the *M. m. vaginalis* karyotype is extremely different from that of the other species (Wurster & Benirschke, 1970); this could indicate a long independent evolutionary history for *M. m. vaginalis*. Because for this species and for the *O. h. hemious*, only one cell line sample was available, no genetic variation within the species was observed.

In conclusion, RAPD analysis provides an efficient and sensitive method to estimate the status of organisms of controversial systematics. Further extension of the analysis could better define the phylogenetic relationships among the Cervidae.

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