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Genetic characterization of Brazilian annual *Arachis* species from sections *Arachis* and *Heteranthae* using RAPD markers

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Abstract

The genus *Arachis* is divided into nine taxonomic sections. Section *Arachis* is composed of annual and perennial species, while section *Heteranthae* has only annual species. The objective of this study was to investigate the genetic relationships among 15 Brazilian annual accessions from *Arachis* and *Heteranthae* using RAPD markers. Twenty-seven primers were tested, of which nine produced unique fingerprintings for all the accessions studied. A total of 88 polymorphic fragments were scored and the number of fragments per primer varied from 6 to 17 with a mean of 9.8. Two specific markers were identified for species with $2n = 18$ chromosomes. The phenogram derived from the RAPD data corroborated the morphological classification. The bootstrap analysis divided the genotypes into two significant clusters. The first cluster contained all the section *Arachis* species, and the accessions within it were grouped based upon the presence or absence of the 'A' pair and the number of chromosomes. The second cluster grouped all accessions belonging to section *Heteranthae*.

Abbreviations: AFLP – amplified fragment length polymorphism; bp – base pairs; EMBRAPA – Empresa Brasileira de Pesquisa Agropecuária (Brazilian Agriculture Research Corporation); PCR – polymerase chain reaction; RAPD – random amplified polymorphic DNA; RFLP – restriction fragment length polymorphism; UPGMA – unweighted pair-grouping with arithmetic average

Introduction

The genus *Arachis* L. is native to South America and its putative center of origin is the Central Brazilian region (Halward et al. 1994). There are approximately 80 species and *Arachis hypogaea* L. (cultivated peanut) is the best known due to its use for human direct consumption and as an oilseed crop (Valls 1996).

The systematic classification proposed by Krapovickas and Gregory (1994) divides genus *Arachis* into nine sections, based on morphology and cross-compatibility, associated to geographic distribution. Section *Heteranthae* contains four

annual diploid species and is mostly concentrated in Northeastern Brazil, whereas section *Arachis* has the largest number of species (27), which are distributed from the Atlantic Coast to the foothills of the Andes. Besides the two allotetraploid species, *Arachis hypogaea* and *A. monticola* Krapov. et Rigoni which are considered to have two distinct (A and B) genomes, section *Arachis* consists mostly of annual and perennial species with $2n = 20$, usually assigned to one of the genomes above, based on karyotype analyses and cross-compatibility with *A. hypogaea*. Diploids characterized by the presence of a small pair of chromosomes, known as 'A' pair, are listed as A genome species. An heterogeneous group of annual diploid species with $2n = 20$, but lacking the 'A' chromosome pair, are loosely gathered as B genome species, although not all of them are closely related to the B genome of *A. hypogaea* (Valls 2000). A single diploid with $2n = 20$, without the 'A' pair and characterized by the presence of several subtelocentric chromosomes is considered to have a D genome (Stalker 1991). Cytological studies conducted by Lavia (1998) and Peñaloza and Valls (1997) revealed the presence of three species within this section, *A. decora* Krapov., W.C. Gregory et Valls, *A. palustris* Krapov., W.C. Gregory et Valls, and *A. praecox* Krapov., W.C. Gregory et Valls, with $2n = 18$ and without the 'A' pair. These species have not been assigned to a specific genome yet, and were also included in our investigation.

Several studies conducted in the genus *Arachis* utilizing biochemical and molecular markers showed low levels of genetic variability in the cultivated peanut *A. hypogaea*, and abundant polymorphism in the wild species of this genus (Garcia et al. 1996; He and Prakash 1997; Galgaro et al. 1998; Gimenes et al. 2002a, b). These facts have led breeders to seek plants in the secondary genepool, some of which are known to possess agronomically useful characters, such as tolerance to biotic and abiotic factors (Subramanian et al. 2000). However, the appropriate use of available genetic resources of wild relatives for plant improvement requires a broad understanding of the genetic relationships among the accessions found in germplasm collections. In this context, molecular markers are powerful tools to reveal the genetic variability found between individuals and within populations. Recently, Galgaro et al. (1998) and Raina et al. (2001) demonstrated the utility of RAPD markers in the characterization of *Arachis* germplasm.

To complement the characterization of the *Arachis* germplasm, this study analysed 15 Brazilian annual accessions from sections *Arachis* and *Heteranthae* using RAPD markers.

Material and methods

Plant material

The 15 accessions of wild *Arachis* species used in this work were provided by the germplasm collections maintained at 'Embrapa Recursos Genéticos e Biotecnologia' (EMBRAPA/CENARGEN, DF, Brazil). The commercial cultivar 'Tatu' of the cultivated peanut *A. hypogaea* was included for comparison purposes (control). The species and germplasm accessions are listed in Table 1, and their range of distribution is shown in Figure 1. Two accessions of section *Heteranthae* represent still undescribed species. Two accessions of section *Arachis* (Sv 2411 and W 422) were initially included to represent what seemed to be a new species, closely related to *A. stenosperma* Krapov. et W.C. Gregory. Data from this study unveiled their conspecific status, so that *A. stenosperma* is here represented by three accessions from the North (W 422) and West Central (Sv 2411 and V12575) regions of Brazil and one from the Atlantic Coast (V 10229), allowing for the analysis of similarity of materials from very disjunct populations.

DNA extraction

Genomic DNA of each genotype was extracted from leaves using the CTAB method proposed by Doyle and Doyle (1990), quantified in a spectrophotometer at 260 nm (Beckman), and the quality estimated by the ratio A_{260}/A_{280}.

RAPD assay

Twenty-five random decamer primers from kit G (1–20) and kit AN (3, 8, 11, 14, 15) from Operon Technologies (Alameda, CA, USA), and two

Table 1. Brazilian germplasm of sections *Arachis* and *Heteranthae*, with information on the chromosome number, genome, access code, collection number, accession abbreviation and the site of collection of each accession.

Section	Species	Chromosome number	Genome	Accession Code	Collection No.[a]	Accession abbreviation	Origin
Arachis	*A. stenosperma* Krapov. et W.C. Gregory	20	A	BRA-023001	VMiSv 10229	10229	Cananéia, SP
Arachis	*A. stenosperma* Krapov. et W.C. Gregory	20	A	BRA-030767	VGaRoSv 12575	12575	General Carneiro, MT
Arachis	*A. stenosperma* Krapov. et W.C. Gregory	20	A	BRA-033367	SvSz 2411	2411	S. Félix do Araguaia, MT
Arachis	*A. stenosperma* Krapov. et W.C. Gregory	20	A	BRA-033529	WPz 422	422	Araguasu, TO
Arachis	*A. decora* Krapov., W.C. Gregory et Valls	18	unknown	BRA-022811	VSW 9955	9955	Campos Belos, GO
Arachis	*A. palustris* Krapov., W.C. Gregory et Valls	18	unknown	BRA-030058	VPmSv 13023	13023	Filadélfia, TO
Arachis	*A. praecox* Krapov., W.C. Gregory et Valls	18	unknown	BRA-012726	VSGr 6416	6416	Cáceres, MT
Arachis	*A. hoehnei* Krapov. et W.C. Gregory	20	B	BRA-022659	VPoBi 9146	9146	Corumbá, MS
Arachis	*A. valida* Krapov. et W.C. Gregory	20	B	BRA-022675	VPoBi 9157	9157	Corumbá, MS
Arachis	*A. hypogaea*	40	AB	cv. Tatu	–		–
Heteranthae	*A. dardani* Krapov. et W.C. Gregory	20	unknown	BRA-031739	VSgSv 13400	13400	Porto Real do Colégio, AL
Heteranthae	*A. giacomettii* Krapov., W.C. Gregory, Valls et C.E. Simpson	20	unknown	BRA-032115	VPzVaW 13202	13202	Montalvânia, MG
Heteranthae	*A. pusilla* Benth.	20	unknown	BRA-025763	VRSv 11022	11022	Piracuruca, CE
Heteranthae	*A. sylvestris* (A. Chev.) A. Chev.	20	unknown	BRA-030961	VSPmPzWiRs 13306	13306	Iaciara, GO
Heteranthae	*A.* spp.	20	unknown	BRA-030121	VFaPzSv 13082	13082	Monte Azul, MG
Heteranthae	*A.* spp.	20	unknown	BRA-025623	VRSv 10969	10969	Acari, RN

[a] *Abbreviations for collector's names*: Bi – L. Bianchetti; Fa – L.F. Freitas; Ga – M.L. Galgaro; Gr – A. Gripp; Mi – S. Miotto; Pm – R. Pittman, Po – A. Pott; Pz – E. Pizarro; R – V.R. Rao; Ro – D. Rocha; Rs – R. Santos; S – C. Simpson; Sg – A.K. Singh; V – J. Valls; Va – S. Valente; Sv – G. Silva; Sz – R. Schultze-Kraft; W – W. Werneck; Wi – D. Williams.

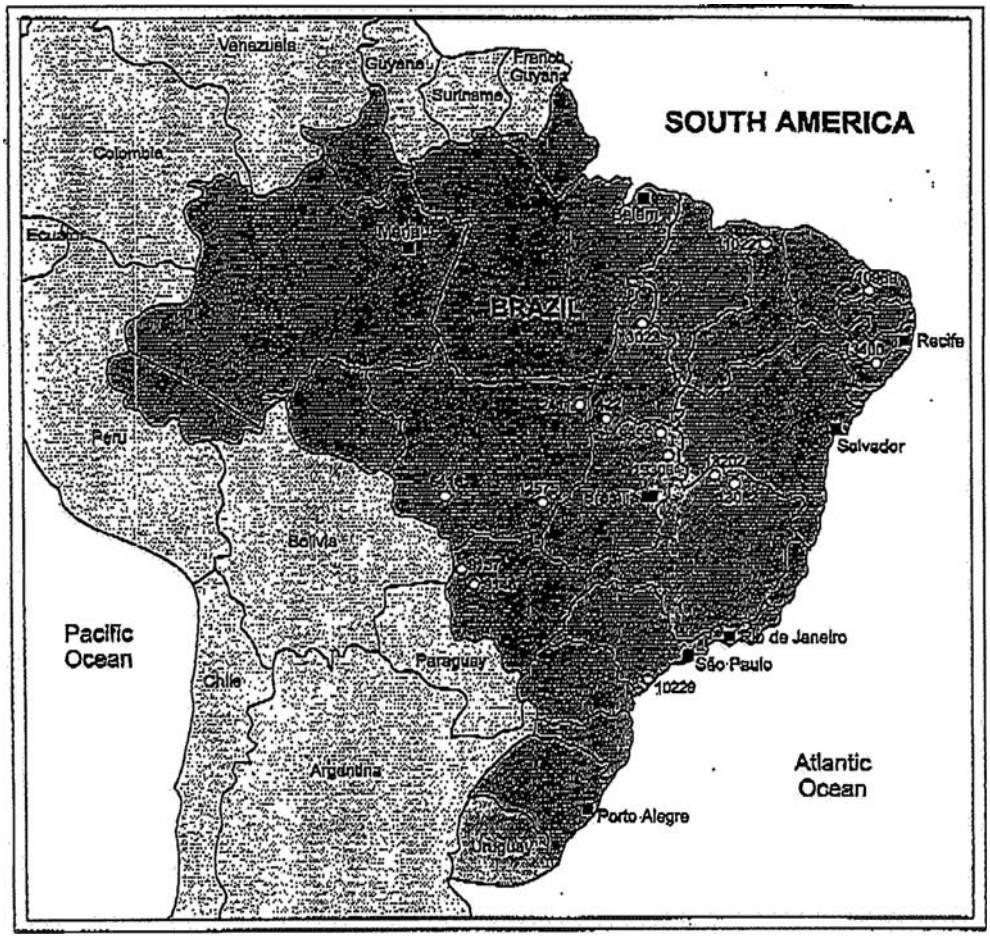

Figure 1. Map of Brazil showing the distribution of the accessions of sections *Arachis* and *Heteranthae*. See Table 1 for accession abbreviations.

primers from University of British Columbia (Vancouver, Canada), UBC (111 and 112), previously employed by Galgaro et al. (1998), were used for amplification. The reaction mixes were performed in total volume of 13 μL containing 2 mM of $MgCl_2$, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 100 μM of each nucleotide, 5 pmol of random decamer primer, 20 ng of genomic DNA, 1.0 U of *Taq* DNA polymerase (Life Technologies do Brasil) and overlaid with a drop of mineral oil. Amplifications were performed in a 480 Perkin Elmer DNA thermocycler (Perkin Elmer Corp., Norwalk, CT, USA). An initial denaturation temperature of 94 °C for 5 min was followed by 40 cycles each at 92 °C for 1 min, 37 °C for 1 min; 72 °C for 2 min, with a final extension at 72 °C for 7 min. The amplification products were analyzed by gel electrophoresis, in 1.2% agarose (Promega, Madison, WI, USA) with ethidium bromide (0.25 μg mL^{-1}) running in 1X TAE buffer, visualized under UV light, and photographed.

Data analysis

The presence or absence of the amplified fragments was scored in all 15 accessions and control for each primer. The genetic similarity between all accessions was calculated according to the Jaccard coefficient. Relationships among genotypes were evaluated with a phenetic cluster analysis using the unweighted pair-grouping with arithmetic average (UPGMA) clustering, and plotted in a phenogram using NTSYS-PC version 2.0j (Exeter

Table 2. RAPD primers that detected polymorphic fragments in *Arachis* species.

Primer code	Primer sequence (5′–3′)	Number of scored fragments
OPG-02	GGCACTGAGG	13
OPG-08	TCACGTCCAC	8
OPG-10	AGGGCCGTCT	8
0PG-14	CAGCTCACGA	6
OPG-15	ACTGGGACTC	9
OPG-16	AGCGTCCTCC	10
OPG-18	GGCTCATGTG	11
UBC-111	AGTAGACGGG	17
UBC-112	GCTTGTGAAC	6
Total		88
Mean		9.8

Software, Setauket, NY, USA). Bootstrap analysis was performed using the WinBoot program (Yap and Nelson 1996), with 1000 repetitive samplings of the RAPD data to compute bootstrap *P* values.

Results

Preliminary RAPD analyses conducted with three individuals of each accession revealed uniform banding patterns (data not shown). From these results, we chose to use only one individual to represent each accession to proceed with the analyses. Among 27 RAPD primers tested to characterize the *Arachis* germplasm, only nine (33%) were selected, because they produced well-defined amplification patterns. These nine primers produced 88 fragments with a mean of 9.8 fragments per primer, ranging from six (OPG 14 primer) to 17 bands (UBC 111 primer) (Table 2). No common fragments were found for all accessions; 20 fragments (22.7%) were exclusive to the section *Arachis* species, and 16 (18.2%) were exclusive to section *Heteranthae*. Sixteen fragments (18.2%) were found in species with $2n = 18$ chromosomes (*A. palustris*, *A. decora* and *A. praecox*) with two detected exclusively in these three species (OPG $18_{500\,bp}$ and $OPG8_{600\,bp}$, Figure 2). Thirteen fragments (14.8%) were common in A genome species, while nine (10.3%) were common to the group of species of B genome.

Bootstrap analysis divided the genotypes into two main clusters with significant *p values* above 75% (Figure 3). The first cluster ($p = 77.8\%$) contained all genotypes from section *Arachis*, and

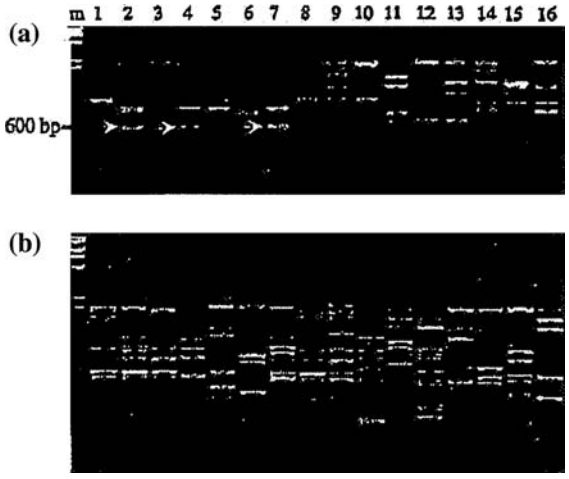

Figure 2. RAPD DNA polymorphism in *Arachis* germplasm with primers OPG 08 (a) and UBC 111 (b). m = λ *Hin*d III molecular marker; 1: *Arachis stenosperma* (422); 2: *A. palustris*; 3: *A. stenosperma* (12229); 4: *A. praecox*; 5: *A. hoehnei*; 6: *A. valida*; 7: *A. decora*; 8: *A. stenosperma* (12575); 9: *Arachis stenosperma* (2411), 10: *A. giacomettii*; 11: *A.* sp. (13082); 12: *A. sylvestris*; 13: *A. pusilla*; 14: *A. dardani*; 15: *Arachis* sp. (10969); 16: *A. hypogaea*. Arrows indicate the marker for species with $2n = 18$ chromosomes.

showed two distinct subgroups. The first subgroup ($p = 36.9\%$) gathered the four accessions of *A. stenosperma*, that possess the 'A' pair, and was linked to cultivar 'Tatu', the representative of *A. hypogaea*. The second subgroup ($p = 73.3\%$) clustered the three species with $2n = 18$ chromosomes (*A. palustris*, *A. decora* and *A. praecox*). The species *A. hoehnei* Krapov. et W.C. Gregory and *A. valida* Krapov. et W.C. Gregory showed the lowest similarity values with the other species from section *Arachis*, and therefore, formed an outgroup for this section. The second cluster ($p = 75.6\%$) contained all species from section *Heteranthae*.

Discussion

The characterization of accessions in this study using RAPD markers is in line with the classification established by morphological descriptors (Krapovickas and Gregory 1994; Veiga et al. 2001). Only nine primers were needed to generate a similarity matrix, of which a phenogram was derived that separated the species of the two sections evaluated. Likewise, Gimenes et al. (2002a) characterized, by AFLP, 20 genotypes representing

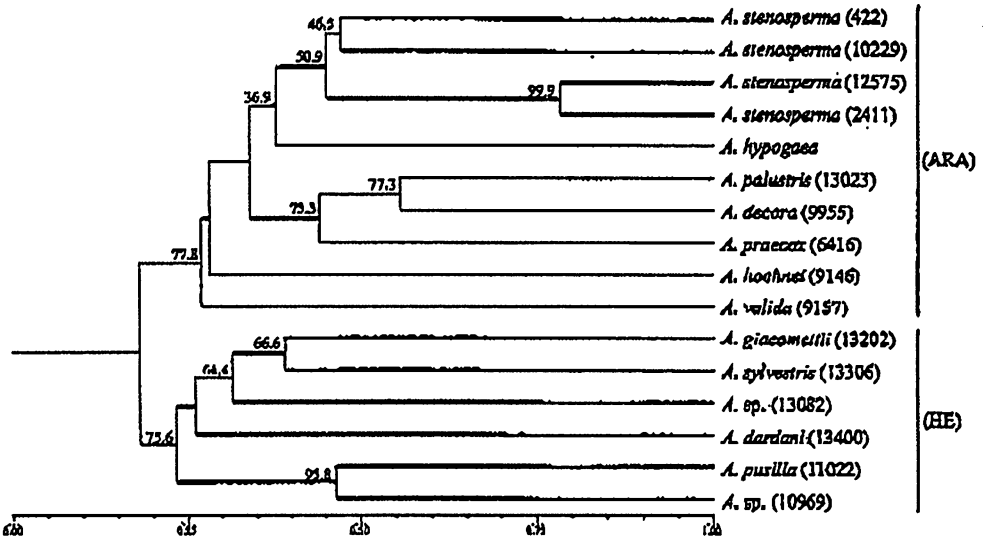

Figure 3. Phenogram demonstrating the genetic relationships among *Arachis* accessions based on RAPD markers, derived from Jaccard coefficient of similarity. Bootstrap *P* values are given at the corresponding node for each cluster. ARA – *Arachis*; HE – *Heteranthae*.

seven out of nine taxonomic sections of the genus *Arachis*. Only three combinations of primers were needed to differentiate all genotypes assessed. On the other hand, studies conducted by Galgaro et al. (1998) on species of sections *Heteranthae*, *Triseminatae* and *Extranervosae* concluded that, by RFLP markers, *A. dardani* Krapov. et W.C. Gregory was more closely related to the species of section *Triseminatae* than to *A. pusilla* Benth. (section *Heteranthae*), whereas by RAPDs, *A. dardani* was more closely related to species of section *Extranervosae*. Analogous results were found in the present study, when we used primers that showed absence of amplifications in some genotypes. In this case, the interpretation of these individuals as missing data established *A. dardani* as more closely related to the species of section *Arachis* than to those of *Heteranthae* (data not shown). The use of primers that produced polymorphic fragments in all accessions generated a phenogram that grouped all accessions according to their respective sections.

In section *Arachis*, the two accessions initially ascribed to *A. stenosperma* (10229 and 12575) had 45% genetic similarity. It became evident that accessions 422 and 2411 also correspond to *A. stenosperma*, since the level of genetic similarity between 12575 vs. 2411 and 10229 vs. 422 has been 78% and 47%, respectively.

The two species associated to the B genome, *A. hoehnei* and *A. valida*, were less related to each other than the remaining species of section *Arachis*. Similar results were found by Gimenes et al. (2002b), who investigated eight diploid species of section *Arachis* using RFLP markers. On the other hand, the clustering of *A. hoehnei* and *A. valida* as an outgroup in the phenogram suggests that these two species are not closely related to *A. hypogaea* (AABB genome). As suggested by Gimenes et al. (2002a), the absence of the small chromosome pair is not a good criterion for grouping species of the section *Arachis* as B genome species, since their genome might be distinct from the B genome of *A. hypogaea*.

The three species with $2n = 18$ (*A. palustris, A. praecox* and *A. decora*), whose chromosomic characterization was provided by Lavia (1998) and Peñaloza and Valls (1997), formed a robust group. According to Lavia (1998), *A. palustris* and *A. praecox* have karyotypes unlike those from other species of this genus and the most significant difference of $x = 9$ would probably be the result of aneuploidy of an ancestor with $x = 10$. It is unlikely that a chromosome pair was lost, since the loss of genetic material compromises the viability of a species more than the maintenance of the genome with rearrangements. The presence of these species within the section *Arachis* may suggest

that they represent a new branch with a recent origin. These species are annual plants and have distinctive, potentially useful traits. *Arachis palustris* is tolerant to flooding (Krapovickas and Gregory 1994), while *A. praecox* has an extremely short life cycle. The latter trait is very important from the standpoint of peanut breeding for cultivation in regions with a very low rainfall (Valls 1996). Crossings between species with different chromosome numbers usually produce non-viable embryos, but the percentage of occurrence of interspecific hybridization between species with $2n = 18$ and $2n = 20$ chromosomes has been high (C.E. Simpson, pers. commun.). This fact suggests that these species have similar genomes with apparently no chromosome loss. Therefore, it is possible that the three species with $2n = 18$ originated from a unique event that occurred in a common ancestor, which, after a chromosomal fusion, formed separate species such as *A. praecox*, *A. palustris* and *A. decora*. Moreover, even though these species may today represent a distinct genome, they probably originated within the *Arachis* section, because they share significant similarity with A genome species. However, cytogenetic studies confirmed that these three species do not have the pair 'A' (Lavia 1998; Peñaloza and Valls 1997). These species formed a well-defined group and are more closely related to the typical species of A genome than to those initially associated to the B genome. More studies should be conducted to better define the correct position of these three species within section *Arachis*, as well as to investigate their very close morphological relationship, which became apparent in a recent study (Veiga et al. 2001) involving nine accessions of *A. decora*, two of *A. palustris* and one of *A. praecox*, and including all three populations from where the type-specimens have been collected.

The species from section *Heteranthae* showed low genetic similarities between themselves, when compared to species from section *Arachis*. The low similarity value found for accession 13082 (*Arachis* sp.) helps to confirm it as a new species within section *Heteranthae*. On the other hand, the accession 10969 (*Arachis* sp.) showed 46% of genetic similarity with *A. pusilla*. If the RAPD data is taken alone, it appears that this accession is just a variant type of *A. pusilla*, since the similarity value is very close to that found between accessions of *A. stenosperma* (10229 vs. 12575). However, on morphological grounds, the two seem to be quite distinct, so that further studies, including more accessions of *A. pusilla*, are recommended.

The consistence of the data obtained in this study allows us to suggest that the RAPD technique should be widely used as a complementary technique to the morphological characterization of the germplasm of *Arachis*. RAPD markers are comparatively less expensive and faster to analyse, when compared to those generated by RFLPs, AFLPs, and microsatellites. Moreover, their analysis does not require sophisticated laboratory skills or equipments. However, the utilization of RAPDs has caused controversies due to results with low reproducibility. Our experience has shown that this problem can be minimized by improving the operator's ability and standardizing the working conditions.

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