DETERMINATION OF TAXONOMIC RELATIONSHIPS AMONG BRAZILIAN TAXA OF *STYLOSANTHES* SW., LEGUMINOSAE, USING RAPD MARKERS[1]

MARIA LUCIA CARNEIRO VIEIRA[2], MARIA HELENA PELEGRINELLI FUNGARO[3], MARIE-FRANCE JUBIER[4] and BERNARD LEJEUNE[5]

ABSTRACT - The potential use of RAPDs for taxonomic studies was investigated in six *Stylosanthes* Sw. taxa. Based on the presence/absence of RAPDs bands, genetic similarities were evaluated and a dendrogram was built according to the UPGMA procedure. Two groups were clearly differentiated, one represented by an annual species (*S. humilis*) and another by the controversial taxa belonging to the *S. guianensis* complex. Among taxa of the latter group RAPDs have proven useful for discriminating *S. gracilis*, that should be under a process of evolutionary divergence.

Index terms: molecular taxonomy.

DETERMINAÇÃO DAS RELAÇÕES TAXONÔMICAS ENTRE TÁXONS BRASILEIROS DE *STYLOSANTHES* SW., LEGUMINOSAE, USANDO MARCADORES RAPD

RESUMO - A potencialidade dos RAPDs para estudos taxonômicos foi investigada em seis táxons de *Stylosanthes* Sw. Baseando-se na presença/ausência das bandas de RAPD, similaridades genéticas foram calculadas e um dendrograma construído de acordo com o método UPGMA. Dois grupos foram claramente diferenciados: um, representado por uma espécie anual (*S. humilis*), e outro, pelos táxons pertencentes ao complexo *S. guianensis*. Entre os táxons deste último grupo, os RAPDs foram capazes de discriminar *S. gracilis*, a qual estaria sob processo de divergência evolutiva.

Termos para indexação: taxonomia molecular.

INTRODUCTION

The American species of *Stylosanthes* Sw. genus are native to tropical and subtropical regions of the continent where they are important for forage and valuable for their ability to colonize poor, infertile and acid soils. The geographic range of *Stylosanthes* includes species and ecotypes adapted to the Amazonian region. Some species are preferentially found along the Brazilian coast. In addition, central areas of South America represent the centre of origin of the genus, where extensive morphological and ecological variation exists.

In the last decades considerable progress has been made towards the identification and selection of promising species for pasture. In consequence, some cultivars were released by national institutions in Brazil and Colombia (Thomas & Grof, 1986). *S. guianensis* (Aubl.) Sw. has been of particular interest. It is distributed from Mexico through Central and South America down to Northern Argentina (Mohlenbrock, 1958) and its natural populations are diverse in many respects.

[1] Accepted for publication on September 26, 1996.

[2] Bióloga, Drª, Dep. de Genética, Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, Caixa Postal 83, CEP 13418-900 Piracicaba, SP, Brasil.

[3] Biomédica, Drª, Profª. Adjunta, Dep. de Biologia Geral, Universidade Estadual de Londrina, Caixa Postal 6001, CEP 86055-900 Londrina, PR, Brasil.

[4] Chercheuse CNRS, Institut Biotechnologie des Plantes, Bât. 630, Université de Paris XI, 91405 Orsay, France.

[5] Professeur de 1ère Classe, Institut de Biotechnologie des Plantes.

The present taxonomic framework shows the *Stylosanthes* genus divided in two sections: *Styposanthes* and *Stylosanthes* (Kirkbride Junior & Kirkbride, 1987). The classification system is based primarily on the fruit morphology. There are about 44 species described, most of them diploid and perennial. The *Stylosanthes guianensis* species itself shows very wide morphological variation and a number of botanical variants are recognized. When consulting the latest studies (Mannetje, 1977, 1984; Costa & Ferreira, 1984) it is apparent that there is disagreement in the classification of these variants. Mannetje divided *S. guianensis* in seven varieties (Mannetje, 1977, 1984). Moreover, analysing and collecting plants in their area of dispersion, Costa & Ferreira (1984) described four new varieties and have treated the other seven, recognized by Mannetje (1977, 1984), as different species. This controversy persists in the literature and reflects the great variability among the botanical varieties and related species of *S. guianensis*.

Cytological studies have also contributed to the understanding of the taxonomy of this genus (Stace & Cameron, 1984). The karyological comparison carried out by Vieira et al. (1993) among taxa belonging to the controversial *S. guianensis* species complex showed clear differences in size of their chromosome complements and morphology. Because *Stylosanthes* relationships have been well documented by cytogenetic studies, comparisons could be readily made between these karyological studies and the results obtained by using molecular techniques such as RFLPs and RAPDs. An earlier use of RAPDs for an estimation at the molecular level of genetic distances allowed Kazan et al. (1993) to establish or clarify relationships between different Australian accessions of the *S. guianensis* complex that were introduced in Australia in the mid-century. The prospect in the present work was somewhat different: authentic collection taxa of *Stylosanthes* from Brazilian origin were chosen in order to assess their genetical distance and even their possible phylogenetical relationships by introducing the distantly-related species *S. humilis* as an outgroup. As in Kazan et al. (1993), RAPD markers were chosen for the estimation of genetical distances.

MATERIAL AND METHODS

Sources of genomic DNA

RAPD analysis were carried out using total genomic DNA from six taxa of *Stylosanthes*, all obtained from a Brazilian collection maintained at the Escola Superior de Agricultura Luiz de Queiroz at Piracicaba (SP), Brazil. The taxa studied are given in Table 1 in which the classification proposed by Costa & Ferreira (1984) is maintained. Three seeds of each taxon were taken at random from the population to constitute the samples from which DNA was extracted.

In order to avoid the high content in polysaccharides and phenolic compounds when plants are grown in greenhouses, surface-sterilized seeds were germinated on agar-solified MS medium (Murashige & Skoog, 1962) free of hormones and containing 20 $g.L^{-1}$ of sucrose. After 30 days, DNA was extracted from whole seedlings using a CTAB procedure (Doyle & Doyle, 1987). DNA concentrations in the extracts were estimated on ethidium bromide-containing agarose gels using standards of known concentration.

TABLE 1. *Stylosanthes* taxa used for RAPDs analysis.

Taxa	Special features	Codes and abbreviations
S. guianensis (Aubl.) Sw. var. *canescens* Ferr. et Costa[1]	Cultivar IRI 1022	(1) iri
S. guianensis (Aubl.) Sw. var. *pauciflora* Ferr. et Costa[1]	Tropical fine stem or tardio type	(4) pauci
S. guianensis (Aubl.) Sw. var. *vulgaris* Ferr. et Costa[1]		(5) vulg
S. gracilis HBK[1]	= *S. guianensis* (Aubl.) Sw. var. *gracilis* (HBK) Vog.	(2) gra
S. grandifolia Ferr. et Costa[1]	= *S. guianensis* (Aubl.) Sw. var. *robusta* Mann.	(3) gran
S. humilis	Annual species	(6) hum

[1] Perennial species and varieties belonging to *S. guianensis* alliance.

RAPD materials

The 10-mers used as random primers in the RAPD were purchased from Operon Technologies, Alameda, CA, USA. Fourty decamers were assayed and 12 were selected. According to the manufacturer's denominations, the selected primers used in this work are: D11, D12, E01, F01, F02, F03, F05, F06, F12, G02, G03, H01. Their base sequences are listed in Table 2. Taq DNA polymerase and DNA polymerization mix (dNTP) were supplied by Pharmacia. The 10x concentrated buffer contained 100 mM TRIS-HCl pH 8.3, 500 mM KCl, 30 mM $MgCl_2$ and 0,01% gelatin. Amplifications were carried out in a BRAUN Thermal Cycler.

Amplification conditions

After optimization of the amplification conditions, reactions were conducted as follows: the mixture, made up to 25 µl with freshly autoclaved water, contained 1µl of DNA solution (about 25 ng), 2.5 µl of 10x buffer, 4 µl of dNTP (200 µM for each), 1 µl of primer (0,2 µM), 0.4 µl of enzyme (2.0 units) and 5 µl of 5 mM $MgCl_2$ to bring magnesium final concentration to 4 mM. The mixtures were overlaid with mineral oil and subjected to the following amplification program: 92°C for 3 min, followed by 40 cycles of 92°C for 45 s, 40°C for 1.5 min, 72°C for 1.5 min and ending with 5 min at 72°C. Samples of 22 µl of the RAPD products were analyzed by electrophoresis (3 V/cm) in 1.4% agarose gels run with 1x TBE buffer. Gels were stained with ethidium bromide and photographed under UV light.

TABLE 2. Nucleotide sequences of the 12 primers used for RAPD analysis.

Primer denomination	Nucleotide sequence
D11	AGCGCCATTG
D12	CACCGTATCC
E01	CCCAAGGTCC
F01	ACGGATCCTG
F02	GAGGATCCCT
F03	CCTGATCACC
F05	CCGAATTCCC
F06	GGGAATTCGG
F12	ACGGTACCAG
G02	GGCACTGAGG
G03	GAGCCCTCCA
H01	GGTCGGAGAA

Data analysis

Data were scored for the presence or absence of amplification products by means of indicator variables. This variable was taken as 1 if a product was present in a taxon, and as 0 when no shared product was present in other taxa. This type of scoring was done for each amplification product across all taxa.

Pair-wise comparisons of genotypes based on both unique and shared products were used to generate similarity coefficients (Jaccard, 1908). Jaccard's coefficient is a/(n-d) where a = positive matches, n = sample size (total number of bands) and d = negative matches. The coefficient excludes negative matches. Since there are several ways in which a genotype may loose a product, it may be argued that basing similarity on mutual absence of a character is improper (Vierling & Nguyen, 1992). These data were used to construct dendrograms based on the unweighted pair-group method with arithmetical averages (UPGMA). The Numerical Taxonomy and Multivariate Analysis System (NTSYS) version 1.70 (Applied Biostatistics Inc.) was used for computations.

RESULTS AND DISCUSSION

Twelve of the forty Operon decamers assayed were selected because they showed good reproducibility and informativity to discriminate the taxa. Fig. 1 displays the results obtained with two of them. The total number of bands revealed by each of these primers was generally over 20, the largest score being obtained with primer OP D12 that generated as many as 38 different bands. Band sizes ranged from about 300bp to 2.5 kb.

The number of seeds taken for each taxon was based on preliminary studies revealing a high homogeneity among individuals per taxon (Fig. 2). For instance, a similarity coefficient of 0.9395 was calculated for *S. guianensis canescens* using 8 primers and 32 progenies (3 plants per progeny).

Altogether the selected primers generated 308 bands that were used to construct the data matrix. Three amplification products only were shared by all taxa. The greatest number of unique products, 78, appeared in *S. humilis*. This was expectable since this annual species does not belong to the perennial *S. guianensis* alliance. *S. gracilis* displayed less original patterns that revealed 26 unique products. On the other hand, the *S. guianensis* alliance was

characterized by many common bands; for instance, 20 amplification products were present in all taxa of this group.

According to the statements of other previous studies a minimal of 10 primers was needed to adequately establish distance in *Brassica* (Demeke et al., 1992) and in *Azola* (Van Coppenolle et al., 1993). In the present work it was estimated that the 308 RAPD products generated by the 12 primers provided a reliable data set that could be used for further distance calculation.

The calculated coefficients of similarities and the corresponding dendrogram are given in Table 3 and Fig. 3. Jaccard's coefficient of similarity gave a tree in which relationships among taxa were identical to those determined using simple matching coefficients of similarity. The robustness of these results was tested using samples of decreasing number of bands (240, 200, 160, 120, 80 and 40 bands). The minimal number of bands requested to obtain stable trees was 160, i.e. about half of the total bands actually used.

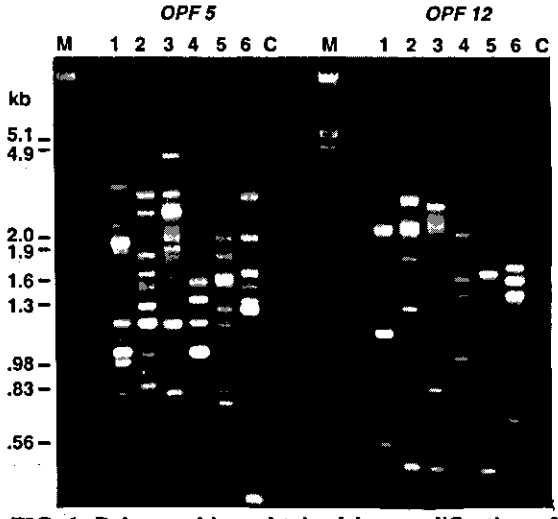

FIG. 1. Polymorphism obtained by amplification of the DNAs of six taxa of *Stylosanthes* using the primers OPF05 (left) and OPF12 (right). Lane 1: *S. guianensis* var. *canescens;* lane 2: *S. gracilis;* lane 3: *S. grandifolia;* lane 4: *S. guianensis* var. *pauciflora;* lane 5: *S. guianensis* var. *vulgaris;* lane 6: *S. humilis.* M: size marker (Hind III/Eco R1 digested Lambda DNA); C: control reaction without DNA template.

Results indicated a clear distinction between two groups, one consisting of the annual species *S. humilis* and the other of the perennial taxa. Inside the *S. guianensis* var. *alliance*, *S. guianensis* var. *vulgaris*, *S. guianiensis* var. *pauciflora*, *S. guianensis* var. *canescens* and *S. grandifolia* appeared grouped together and relatively separated from *S. gracilis*.

The dendrogram clustering of *Stylosanthes* taxa supports the expected grouping from cytogenetics

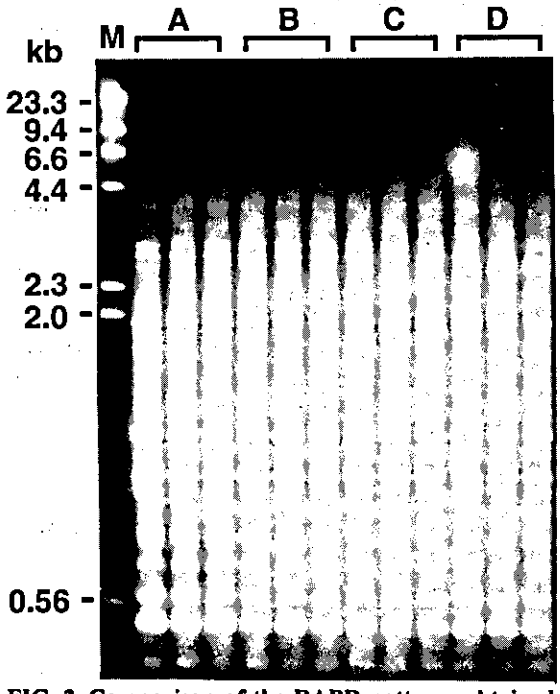

FIG. 2. Comparison of the RAPD patterns obtained in four different accessions (A,B,C,D) of *S. guianensis* var. *canescens* using primer OPF05. The DNAs of three seeds were individually tested for each accession. M: size marker (Hind III digested Lambda DNA).

TABLE 3. Jaccard's coefficients of similarities among six taxa of *Stylosanthes*.

Taxa	gra	iri	gran	pauci	vulg	hum
gra	-					
iri	0.301	-				
gran	0.243	0.354	-			
pauci	0.321	0.363	0.355	-		
vulg	0.310	0.322	0.325	0.449	-	
hum	0.040	0.066	0.063	0.055	0.045	-

data of these plants. *S. humilis*, for example, differentiates from the other taxa by its smallest total chromosomal length (Cameron, 1967; Vieira et al., 1993), which is in agreement with its external position on the tree. Within the *S. guianensis* group the most divergent taxon is *S. gracilis*. Its karyotype is accordingly distinct from those of the *S. guianensis* varieties by the morphology of chromosomes 3, 6 and 8 (Vieira et al., 1993). *S. gracilis* could therefore be considered a different species, as proposed by Ferreira & Costa (1979). On the other hand, the highest similarity coefficient found for *S. guianensis* var. *vulgaris* and *S. guianensis* var. *pauciflora*, agrees with the almost identical karyological features in both taxa (Vieira et al., 1993). When associated with the karyological similarity between *S. guianensis* var. *vulgaris* and *S. grandifolia*, molecular data support the hypothesis that *S. guianensis* var. *canescens*, *S. guianensis* var. *pauciflora*, *S. grandifolia* and *S. guianensis* var. *vulgaris* are evolutionary derivations from a common taxon showing great variability. The values of pollen stainability and fertility of the hybrids between *S. guianensis* accessions reported by Kazan et al. (1993) confirm their clustering on the RAPD--based-phenogram obtained in the same study.

The RAPD approach used here has proven to be a valuable tool to estimate variability and genetical distance in many instances. Using well-defined type--taxa of the genus *Stylosanthes* it was shown here the consistency of taxonomic data obtained independently by the molecular results reported here and by previously established cytogenetics.

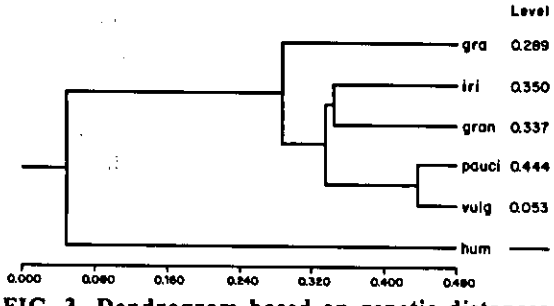

FIG. 3. Dendrogram based on genetic distances demonstrating relationships among six *Stylosanthes* taxa. Abbreviations are listed in Table 1. Abscissa scale: Jaccard's coefficient of similarity.

Although the distant species *S. humilis* has been included the phenetic analysis performed here with the genus *Stylosanthes* should have its phylogenetic implications examined with a more appropriate method, such as molecular phylogenies using nucleotide sequence comparison.

CONCLUSION

The RAPD technique resulted in a more definitive separation of the taxa belonging to the controversial group named *Stylosanthes guianensis* complex. The most significant contribution of this DNA-based marker analysis is the more accurate determination of *Stylosanthes gracilis* as a differente species.

ACKNOWLEDGEMENTS

This publication results from a study started in ORSAY in Pr. F. Quetier laboratory to whom Maria Lúcia Carneiro Vieira (MLCV) wishes to express gratitude, as well as to Evelyne Besin for her excellent technical assistance. MLCV was supported by the Programme de Bourses D'Excellence (AUPELF/UREF). Maria Helena Pelegrinelli Fungaro is a Visitor Researcher at ESALQ/USP on leave from Universidade Estadual de Londrina, Brazil. We are grateful to Pr. Roland Vencovsky for reading the manuscript. This project was supported in part by CAPES/COFECUB Agreement 136/93.

REFERENCES

CAMERON, D.F. Chromosome number and morphology of some introduced *Stylosanthes* species. **Australian Journal of Agricultural Research**, Melbourne, v.18, p.375-379, 1967.

COSTA, N.M.S.; FERREIRA, M.B. Some Brazilian species of *Stylosanthes*, In: STACE, H.M.; EDYE, L.A. (Eds.). **The biology and agronomy of *Stylosanthes*.** Sidney: Academic Press, 1984. p.23-48.

DEMEKE, T.; ADAMS, R.P.; CHIBBAR, R. Potential taxonomic use of random amplified polymorphic DNA (RAPD): a case study in *Brassica*. **Theoretical and Applied Genetics**, New York, v.84, p.990-994, 1992.

DOYLE, J.J.; DOYLE, J.L. A rapid DNA isolation procedure to small amounts of fresh leaf tissue. **Phytochemical Bulletin**, Athens, v.19, p.11-15, 1987.

FERREIRA, M.B.; COSTA, N.M.S. **O gênero *Stylosanthes* Sw. no Brasil.** Belo Horizonte: EPAMIG, 1979. p.1-107.

JACCARD, P. Nouvelles recherches sur la distribution florale. **Bulletin de la Societé Vaudoise des Sciences Naturelles**, Lausanne, v.44, p.223-270, 1908.

KAZAN, K.; MANNERS, J.M.; CAMERON, D.F. Genetic relationships and variation in the *Stylosanthes guianensis* complex assessed by random amplified polymorphic DNA. **Génome**, Ottawa, v.36, p.43-49, 1993.

KIRKBRIDE JUNIOR, J.H.; KIRKBRIDE, M.C.G. Typification of *Stylosanthes* (Leguminosae) and its sections. **Taxon**, Berlin, v.36, p.455-458, 1987.

MANNETJE, L. t'. A revision of varieties of *Stylosanthes guianensis* (Aubl.) Sw. **Australian Journal of Botany**, Melbourne, v.25, p.347-362, 1977.

MANNETJE, L. t'. Considerations on the taxonomy of the genus *Stylosanthes*, In: STACE, H.M.; EDYE, L.A. (Eds.). **The biology and agronomy of *Stylosanthes***. Sidney: Academic Press, 1984. p.1-21.

MOHLENBROCK, R.H. A revision of the genus *Stylosanthes*. **Annals of the Missouri Botanical Garden**, Saint Louis, v.44, p.299-355, 1958.

MURASHIGE, T.E.; SKOOG, F. A revised medium for rapid grouth and bioassays with tobacco tissue cultures. **Physiologia Plantarum**, Copenhagen, v.15, p.473-497, 1962.

STACE, H.M.; CAMERON, D.F. Cytogenetics and evolution of *Stylosanthes*, In: STACE, H.M.; EDYE, L.A. (Eds.). **The biology and agronomy of *Stylosanthes***. Sidney: Academic Press, 1984. p.49-71.

THOMAS, D.E.; GROF, B. Some pastures species for the tropical savannas of South America. I. Species of *Stylosanthes*. **Herbage Abstracts**, Farnham Royal, v.55, p.446-453, 1986.

VAN COPPENOLLE, B.; WATANABE, I.; VAN HOVE, C.; SECOND, G.; HUANG, N.; McCOUCH, S.R. Genetic diversity and phylogeny analysis of *Azolla* based on DNA amplification by arbitrary primers. **Génome**, Ottawa, v.36 p.686-693, 1993.

VIEIRA, M.L.C.; AGUIAR-PERECIN, M.L.R.; MARTINS, P.S. A cytotaxonomic study in twelve Brazilian taxa of *Stylosanthes* Sw., Leguminosae. **Cytologia**, Tokyo, v.58, p.305-311, 1993.

VIERLING, R.A.; NGUYEN, H.T. Use of RAPD marker to determine the genetic diversity of diploid, wheat genotypes. **Theoretical and Applied Genetics**, New York, v.84, p.835-838, 1992.