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Genetic resources of *Centrosema* spp.: genetic changes associated to the handling of an active collection

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Summary

The potentials of the species of *Centrosema* as pasture and fodder crops in Tropical and Subtropical areas have promoted their germplasm collection and evaluation in Brazil and other countries of Central and South America. The species *C. acutifolium*, *C. pubescens* and *C. brasilianum* are of particular interest. Samples of the same accessions from wild materials collected in Brazil have been agronomically evaluated in Brazil and Colombia, and handled as self-pollinating species. Pairs of samples (one from Brazil and the other from Colombia) of four accessions have been genetically analyzed using isozyme markers at 16 loci. A noticeable genetic differentiation has occurred between members of each pair in few generations. Experimental evidences indicate that frequency of outcrossing are relatively high in these species. It is proposed that outcrossing between non-isolated neighboring accessions and genetic drift in small size plantings are the causes of the genetic differentiation between Brazilian and Colombian samples. Guides to evaluate and multiply *Centrosema* germplasm are suggested.

Introduction

The family Leguminosae includes a large number of species, many of which have a relevant economic and/or scientific interest. The genus *Centrosema* (DC.) Benth. belongs to the tribe Phaseolae which, with approximately 84 genera and 1500 species, represents the largest and economically most important tribe of the subfamily Papilionoideae.

The species of *Centrosema* are native to Central and South America. Brazil is one of the most important centers of genetic diversity. Among the nearly 35 described species of the genus, 26 are indigenous to Brazil (Barbosa-Fevereiro, 1977; Schultze-Kraft & Clements, 1990). These species show a large morphologic diversity and exhibit a great potential for being utilized as fodder plants (For revisions on taxonomy and utilization see Clements et al., 1983; Schultze-Kraft & Clements, 1990; Thomas & Schultze-Kraft, 1992).

Beef cattle in Brazil are mainly raised in the Central West Region, in extensive systems, where animal feeding is based on grazing. The weather conditions in this region show a very drastic dry season, and in general soils are acid and poor in phosphorus. Grasses, the most important fodders in that region, are characterized by a low protein content and show a large decrease in production during the dry season. Therefore, under these conditions, the possibility of using legumes as forage represents an important alternative. Legumes bring to the system a major source of protein, improve the physico-chemical conditions of soils and also increase the productivity and nutritive value of grasses.

The genus *Centrosema* has been agronomically evaluated for many years and its potential as forage has been studied extensively (Clements et al., 1983; Schultze-Kraft & Clements, 1990). Three species are particularly interesting: *C. acutifolium* Benth., *C. pubescens* Benth. and *C. brasilianum* (L.) Benth.. These species are short-lived perennials and, as oth-

er species from the same taxonomic group, have been considered as self-pollinating (Hutton, 1960). Although some natural outcrossing was suspected to occur by the observation of a high phenotypic variability within accessions in field-trial evaluations (Penteado, 1986; Penteado et al., 1990) and although evidences of outcrossing in some species of this genus were reported (Battistin, 1983; Schultze-Kraft & Belalcazar, 1988), *Centrosema* germplasm has been and still is handled as if self-pollinated. Recently, outcrossing estimates of 31.2% and 53.3% were reported in two accessions of *C. brasilianum* (Maass & Torres, 1992).

Although *Centrosema* has probably received more attention from plant breeders than other tropical legume forages, basic genetic knowledge on its species are particularly scarce. Aspects such as floral biology, mating system and genetic variability are not well studied and very few papers are available on these subjects. Even chromosome numbers are controversial, thus 2n numbers of 18, 20 and 22 have been reported in these and other *Centrosema* species (Clements et al., 1983; Battistin & Vargas, 1989; Miles et al., 1990). The number of all species and accessions used in this work was observed to be 2n = 22 (Novaes & Penteado, 1993).

The object of this work is to evaluate the genetic structure of different accessions of three species of *Centrosema*, selected for its agronomic importance, and to study genetic implications for germplasm conservation.

Materials and methods

The materials analyzed in this experiment consist of four *Centrosema* accessions, belonging to the species *C. acutifolium* (2n = 22), *C. brasilianum* (2n = 22) and *C. pubescens* (2n = 22). The registered numbers of accessions at the Brazilian Germplasm Bank are: *C. acutifolium* (BRA-013501 and BRA-004990), *C. pubescens* (BRA-017764) and *C. brasilianum* (BRA-007382), but they will be designated throughout this work respectively as GC 350, GC 351, GC 495 and GC 489, which are their numbers in the active collection under agronomical evaluation.

All of these accessions were collected in Brazil at the end of the 70's and multiplied in two different sites. Some of the original seeds were grown by the 'Empresa Brasileira de Pesquisa Agropecuaria' (EMBRAPA) at the Centro Nacional de Pesquisa de Gado de Corte (CNPGC) in Campo Grande, Mato Grosso do Sul (Brazil), and part by 'Centro Internacional de Agricultura Tropical' (CIAT) at the Experimental Station of Quilichao in Cali (Colombia). Two samples of each accession were studied, one grown in Brazil and the other in Colombia and designated with the letters B and C, respectively. Accessions under field evaluation were planted in small plots with between 20 and 40 adult plants.

Seeds of each accession collected in 1989 were germinated under controlled conditions. Crude extracts from leaf tissue of two-week-old seedlings were analyzed by standard starch gel electrophoresis, in two discontinuous buffer systems, Tris-Citric Acid pH 7.0 and Tris-Boric Acid pH 8.5 (Penteado, 1994).

The isozymatic systems analyzed were glutamate oxaloacetate transaminase (GOT, EC 2.6.1.1), glucose phosphate isomerase (GPI, EC 5.3.1.9), phosphoglucose mutase (PGM, EC 2.7.5.1), peroxidase (PRX, EC 1.11.1.7), malate dehydrogenase (MDH, EC 1.1.1.37), 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44) and isocitrate dehydrogenase (IDH, EC 1.1.1.42). The staining procedures were, with minor modifications, those described by Vallejos (1983). The genetic control of these isozymatic systems was studied by means of segregating progenies (Penteado, 1994). The estimation of mating system parameters was performed by a multilocus method (Shaw et al., 1981) from family array data of seeds sampled in larger Brazilian populations. Several polymorphism parameters were estimated from genotypic and allelic data.

Results

Allelic frequencies estimated from genotypic frequencies (not shown) of the analyzed samples are shown in Table 1. Three additional monomorphic loci (*Got2*, *Mdh3* and *Mdh4*) fixed for a single allele at the three species have not been included in Table 1, but parameters of genetic variability have been estimated from the total sixteen loci. It is clear from the data in Table 1 that, in general, samples grown in Colombia have a greater genetic variability than the Brazilian samples: the former show a higher number of alleles with less different frequencies. These results can be observed in Table 2 which summarizes the values observed for some polymorphism parameters. With the exception of accessions GC 350 of *C. acutifolium* the average number of alleles per locus (n), number of polymorphic loci (P), mean heterozygosity (H) and polymorphic index (P_s) are equal or higher in the samples grown in Colombia than in Brazil. Figure 1 shows the number of alleles

Table 1. Estimated allelic frequencies

		C. brasilianum		*C. pubescens*		*C. acutifolium*			
		489 B	489 C	495 B	495 C	350 B	350 C	351 B	351 C
N[1]		112	95	57	33	96	110	46	70
Got1 -	*1*	–	–	–	.182	.411	.620	.848	.685
	2	.964	.879	–	–	–	–	–	–
	3	.036	.121	1.000	.818	.589	.379	.152	.315
Got3 -	*1*	–	–	.188	–	.260	.108	.141	.178
	2	–	–	.733	.954	.557	.820	.750	.693
	3	–	–	.078	.045	.182	.072	.109	.129
	4	1.000	1.000	–	–	–	–	–	–
Gpi1-	*1*	–	.005	.815	.667	.833	.645	.881	.700
	2	1.000	.994	.184	.333	.166	.350	.120	.286
	3	–	–	–	–	–	.004	–	.014
Gpi2 -	*1*	–	.021	–	–	.984	.963	.891	.900
	2	–	.005	1.000	1.000	.005	.036	–	.071
	3	.018	.602	–	–	–	–	–	–
	4	–	.116	–	–	–	–	–	–
	5	–	.127	–	–	–	–	.043	.029
	6	–	.064	–	–	.010	–	–	–
	7	.982	.064	–	–	–	–	.065	–
Gpi3 -	*1*	–	.042	1.000	.969	.984	.995	1.000	1.000
	2	1.000	.829	–	–	.015	.005	–	–
	3	–	–	–	.030	–	–	–	–
	4	–	.085	–	–	–	–	–	–
	5	–	.043	–	–	–	–	–	–
Prx1 -	*1*	.906	.354	.017	.060	.425	.331	1.000	.542
	2	.094	.645	.983	.940	.575	.669	–	.457
Pgm1 -	*1*	–	–	1.000	1.000	.979	.995	1.000	.978
	2	.982	.289	–	–	.021	.005	–	.022
	3	.018	.432	–	–	–	–	–	–
	4	–	.279	–	–	–	–	–	–
Mdh1 -	*1*	1.000	1.000	.035	.364	–	.027	–	.435
	2	–	–	–	–	1.000	.949	1.000	.450
	3	–	–	.965	.636	–	.023	–	.115
Mdh2 -	*1*	1.000	.994	1.000	1.000	.990	1.000	1.000	1.000
	2	–	.006	–	–	.010	–	–	–
6Pgd1 -	*1*	–	–	–	–	1.000	1.000	1.000	.801
	2	1.000	1.000	1.000	1.000	–	–	–	.125
	3	–	–	–	–	–	–	–	.051
	4	–	–	–	–	–	–	–	.022
6Pgd2 -	*1*	–	–	–	–	1.000	1.000	1.000	.448
	2	1.000	1.000	–	–	–	–	–	.044
	3	–	–	1.000	1.000	–	–	–	.029
	4	–	–	–	–	–	–	–	.183
	5	–	–	–	–	–	–	–	.299
6Pgd3 -	*1*	–	–	.938	1.000	.692	.100	.891	.993
	2	–	–	.061	–	.308	.900	.109	.007
	3	1.000	1.000	–	–	–	–	–	–
	4	–	–	–	–	–	–	–	–
Idh1 -	*1*	.013	.963	.956	.985	.969	.959	.946	.828
	2	–	.037	.044	.015	.031	.041	.054	.172
	3	.987	–	–	–	–	–	–	–

[1]Number of seedlings analyzed

Table 2. Number of polymorphic loci (P), mean observed heterozygosity (H), mean polymorphic index[1] (Ps), and mean number of alleles per locus (n) in the 16 loci scored

	P	H	Ps	n
C. brasilianum				
GC 489 B	5	0.014	0.021	1.31
GC 489 C	6	0.070	0.142	2.00
C. pubescens				
GC 495 B	6	0.037	0.064	1.44
GC 495 C	7	0.064	0.094	1.44
C. acutifolium				
GC 350 B	10	0.115	0.153	1.75
GC 350 C	8	0.082	0.129	1.81
GC 351 B	6	0.039	0.079	1.50
GC 351 C	10	0.092	0.248	2.25

[1]The polymorphic index is equal to the expected heterozygosity under random mating

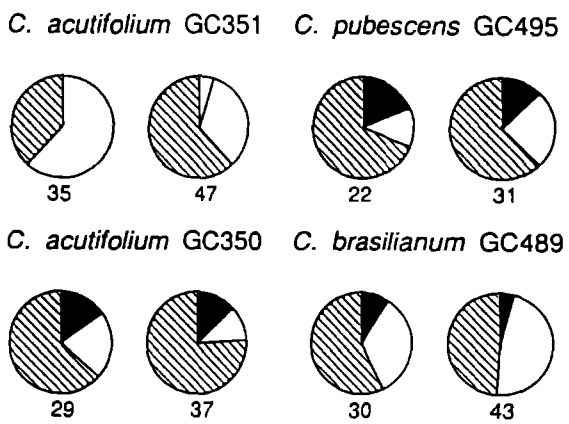

Fig. 1. Number of alleles (left) and genotypes (right) observed in the total of isozymatic systems. Striped sectors indicate alleles and genotypes shared by Brazilian and Colombian samples; black, observed in Brazil, white; observed in Colombia.

and genotypes in common or exclusive to each locality for the four accessions.

Of the 33 alleles identified in the 16 loci (the 13 loci of Table 1 plus *Got2*, *Mdh3* and *Mdh4*) of *C. brasilianum*, 32 were observed in Colombian seedlings, while only 22 were detected in the Brazilian ones, among which *Idh1 3* was specific of this sample. The same situation occurs for genotypes, among a total of 47 genotypes 45 occur in the material from Colombia while only 24 were observed in the Brazilian GC 489. A similar result was observed for the two accessions *C. acutifolium* GC 350 and GC 351. With regard to GC 351 which showed the highest difference, it was observed that all the 37 alleles except *Gpi2 7* and all the 49 genotypes except two of them were present in the Colombian material while 24 alleles and 30 genotypes were present in the Brazilian one. For *C. pubescens* GC 495 variability is similar in the two sites with some alleles and genotypes exclusively present in each locality, although genetic polymorphism parameters are higher in the Colombian samples (Table 2). It is also clear from the data in Table 1 that some very significant changes in allelic frequencies have occurred between samples of the same accession. For instance, in *C. brasilianum* GC 489 highly significant changes can be observed in loci *Gpi2*, *Gpi3*, *Prx1*, *Pgm1* and *Idh1*. The same is true for several loci of *C. acutifolium*, but significance is different between the two accessions, thus, for instance, there are highly significant differences in *Got3* between samples of the accession 350 but not in 351, or in *Mdh1* in the accession 351 but not in 350. In *C. pubescens* only *Mdh1* locus showed a highly significant difference.

Several parameters were calculated to estimate the distribution of genetic variability in and among samples of each accession (Table 3). Variability within accessions is estimated by the mean value of P_s, while the total variability as the polymorphic index (P_T) was estimated for each accession pooling the data from the two sites in a synthetic sample. Finally, the coefficient of gene differentiation G_{ST} (Nei, 1987) was estimated from these two previous parameters. The value of G_{ST} ranges from 0, all the gene diversity is due to variability within population, to 1, in which gene diversity is due exclusively to variability between population, and in which 0.5 means that gene diversity is equally distributed within and between populations. The G_{ST} value of *C. brasilianum* is very close to this value (0.529), while in the other accessions their values indicated that gene diversity within samples is more important than between samples in each accession.

Discussion

The first noticeable result is the relatively high frequency of observed heterozygosity in these samples and accessions of *Centrosema* species generally described and handled as self-pollinating species. The heterozygosity observed ranged between 1% and 11% with an average for all samples of about 6% (Table 2). Although evidences of outcrossing existed in some

Table 3. Variability indices estimated based on data of two different sites of seed multiplication

	C. brasilianum	*C. pubescens*	*C. acutifolium*	
	GC 489	GC 495	GC 350	GC 351
$\bar{P}_S$	0.081	0.079	0.141	0.163
P_T	0.172	0.108	0.209	0.266
G_{ST}	0.529	0.268	0.325	0.387

species of this genus, not a single measure of outcrossing rate in any *Centrosema* species has been recorded (Schultze-Kraft & Clements, 1990), until Maass & Torres (1992) using flower color as marker determined outcrossing rates of 32.2% and 53.3% in two accessions of *C. brasilianum*. Recently (Penteado, 1994) the outcrossing rates of three (*C. acutifolium*) populations were estimated to range from 26% to 40% using isozymatic markers and the Shaw et al. (1981) multilocus estimator. Outcrossing values estimated by an indirect method from the fixation index ranged from 0.33 to 0.59 in this species, from 0.15 to 0.47 in *C. brasilianum*, and from 0.30 to 0.56 in *C. pubescens* (Penteado, 1994).

Therefore, it can be concluded that these species of *Centrosema* are not true inbreeding species but they should be included in the mixed mating group of species in which self-pollination and outcrossing occur at measurable rates (Brown, 1990). The presence of relatively high frequencies of outcrossing can explain the high heterozygosity observed in these materials and that the gene diversity was generally more important within than between samples or accessions. Differences in outcrossing frequencies are expected since mating system parameters vary depending upon both genetic (i.e., selection) and environmental factors (i.e. plant density, pollinating insects, temperature) (see Brown, 1990; Vaquero et al., 1989; and references cited in these papers). This mixed mating system in *Centrosema* is very relevant and should be taken into consideration when collecting, multiplying and conserving genetic resources of these species.

The second relevant result is the genetic differences between Brazilian and Colombian grown samples. A very likely explanation of allelic differences is genetic drift due to the small sample size of both evaluation plots and some samples when divided between Brazilian and Colombian collections. Although it can not be completely excluded, it is unlikely that directional selection has been the cause because selection would have to be very intense to produce such marked changes in few generations (i.e., in GB 489 *Idh1 1* showed a frequency of 0.013 in Brazil and 0.963 in Colombia, or in GC 351 *6Pgd2 1* changes from 1.00 to 0.448), and because changes are not consistent in the two accessions of *C. acutifolium*, for instance, while the change in allele *Mdh1 2* was highly significant (1.00 vs. 0.45) in accession GC 351 it was not significant (1.00 vs. 0.949) in GC 350, or while allele *6Pgd3 1* increased significantly (0.891 vs. 0.993) in GC 351 C in relation to GC 351 B it decreased highly significantly in GC 350 (0.692 vs 0.100). Sampling errors can also be the cause of the higher allelic richness in the relatively larger samples grown in Colombia.

The general high heterozygosity level observed in Colombian samples could also be the result of high outcrossing frequencies in Colombia due to different environmental conditions, although this hypothesis must be confirmed. Lower outcrossing rates in Brazil are probably due to climatic conditions in Campo Grande. Flowering time coincides with winter and the dry season, and low temperatures at night (below 11°C) interferes with flower development. These circumstances may have a clear effect on self versus insect-mediated cross pollination decreasing outcrossing rates.

Since isolation among accessions was not enforced and, in addition to the four accessions studied here, many other accessions of the three species active collections are under evaluation and have been planted in the experimental field, accession allelic richness can be increased by genetic flow among closely planted accessions.

To sum up, we postulate that at least these three species of *Centrosema* should no longer be considered as self-pollinating species and that this fact should be taken into consideration in conserving and multiplying their germplasm. Outcrossing rates maintain a relatively high heterozygosity in *Centrosema* populations

determining a genetic structure different from the structure of true inbreeding species. This means that in evaluating and multiplying their germplasm the sample of adult plants of each accession should be large enough in order to avoid a significant genetic drift causing a loss of heterozygosity and allelic richness, and also that isolation among accessions should be considered to avoid genetic flow among them. The violation of these rules is probably the main cause of the great genetic differentiation between Brazilian and Colombian samples of this active collection. The study of original material from the base collection will allow for a better assessment of these changes.

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