

Sources of genetic variability in a neotropical cleistogamous species, *Relbunium hypocarpium*, Rubiaceae

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ABSTRACT

Twenty-two strains of *Relbunium hypocarpium* collected from four localities in Rio Grande do Sul (Brazil) and one in Argentina were studied for glutamate oxaloacetate transaminase (GOT, EC 2.6.1.1) isoenzyme patterns by horizontal gel electrophoresis in order to obtain more precise data on their reproductive system and on the origin of the variability observed in this cleistogamous species. The study was conducted on adult F₁ and F₂ of plants from nature, and seedlings of their progenies. When some variation was observed in the progeny, the same or sib seedlings were also analyzed for isoesterases (EST, EC 3.1.1). The results showed that *R. hypocarpium*, a cleistogamous species, and reproduces mostly by self-fertilization, although at least one event of cross-fertilization that occurred in nature, was detected in one strain out of 22. An additional source of variability observed in 14% to 18% of the strains, in the GOT system, was found to be the occurrence of isoenzyme modifiers, possibly involving post-synthesis modifications, the adaptive significance of which we are not yet able to explain.

INTRODUCTION

In cleistogamy, which may be considered the extreme stage of self-fertilization, fecundation occurs before the flower opens or even without its opening. The ensuing homozygosis causes the sib plants to be genetically identical in both their structural and regulatory genes.

Mixed reproductive systems have been observed in all self-fertilizing species submitted to intensive study throughout their distribution (Stebbins, 1957). Total cleistogamy is rare, if it exists at all, and cleistogamous species may eventually show genuine explosions of variability in some populations (see Richards, 1986). Many researchers are trying to estimate the exact amount of self-fertilization in natural

populations of autogamous plants due to its importance for studies on plant biology and evolution, since this would permit an understanding of the evolutionary forces acting on reproductive systems (Barrett and Eckert, 1990; Motten and Antonovics, 1992).

It is generally accepted that self-fertilization is a highly advantageous reproductive system under certain ecological conditions such as temporary or pioneering habitats. One or few individuals can colonize a new region without depending on cross-pollination, thus warranting the maintenance of locally adapted gene combinations and favoring the fixation of advantageous new mutations.

Relbunium hypocarpium (Rubiaceae) is a perennial, herbaceous neotropical species (Ehrendorfer, 1955) presenting self-fertilization and cleistogamy (Mariath *et al.*, 1987; Mariath, 1990). Analysis of the morphoanatomy of its flowers, especially at the scanning electron microscope level, has shown that in

this species pollen germination and ovule fertilization occur before flower opening, proving the existence of cleistogamy in this species. Cavalli-Molina *et al.* (1989), in an electrophoretic study of esterases and/or peroxidases of the progeny of nine *R. hypocarpium* plants from various locations found that sib plants at the same stage of development and tissue maturation presented identical isoenzyme patterns in all cases. The results showed the absence of segregation of structural and regulatory genes, confirming self-fertilization.

In the present work, we extended the studies of Cavalli-Molina *et al.* (1989) using mainly the glutamate oxaloacetate transaminase system, since it is important to obtain more precise information about the reproductive system and gene variability in this cleistogamous species.

MATERIAL AND METHODS

We analyzed the isoenzyme patterns of glutamate oxaloacetate transaminases (GOT, EC 2.6.1.1) of 22 strains originated from seeds collected at four localities in Rio Grande do Sul (Brazil) and at one site in Argentina (Figure 1 and Table I). Further information on the collecting sites has been reported by Cavalli-Molina and Winge (1988). Each strain was established from seeds of a single adult plant from nature (P₁) and included all subsequent generations grown in the greenhouse.

The 22 strains analyzed are listed in Table I. Twenty of these were studied in the F₁ generation at the adult stage and their progeny (F₂) at the seedling stage. Strain HW 874 was analyzed in the F₂ generation as an adult plant and its progeny (F₃) at the seedling stage. Strain JEAM 525 was only studied as F₁ seedlings.

From at least one adult F₁ plant of each strain 1 cm of mature stem, close to the base of a branch, was analyzed as were five of its ripe fruits (Table I). When any variation was observed in the fruit isoenzyme patterns, the sample size was enlarged.

Cotyledonary leaves from ten sib seedlings of each of the 22 strains were analyzed for the study of the reproductive system. The cotyledonary leaves of these seedlings were newly freed from the seed integument which characterizes the first developmental stage according to Motta (1981).

The choice of tissues to be analyzed was based on the results of preliminary tests, considering the number, intensity and resolution of their GOT bands.

For some strains, especially when the sib seedlings showed differences in their GOT patterns, the

Table I - Strains of *Relbunium hypocarpium* analyzed for GOT and EST patterns. Number of individuals at the seedling (S) or adult (A) stage are indicated for each generation. Capital letters, A and B, assigned to strain JEAM 546 distinguish the two adult F₁ plants and their respective progenies. Lowercase letters are part of the collectors numbers.

| Localities of origin Collector no. | Generation | No. of individuals | | | |
|--|----------------|--------------------|---|-----------|----|
| | | GOT | | EST | |
| | | S | A | S | A |
| <i>Itaimbezinho</i> | | | | | |
| JEAM 520 | F ₁ | - | 1 | - | - |
| | F ₂ | 10 | - | - | - |
| JEAM 521 | F ₁ | - | 1 | - | - |
| | F ₂ | 15 | - | - | - |
| JEAM 525 | F ₁ | 10 | - | 10 | - |
| JEAM 544 | F ₁ | - | 2 | - | - |
| | F ₂ | 10 | - | - | - |
| JEAM 546 A,B | F ₁ | - | 2 | - | 1* |
| | F ₂ | 25(6A+19B) | - | 10(5A+5B) | - |
| JEAM 556 | F ₁ | - | 1 | - | - |
| | F ₂ | 10 | - | - | - |
| <i>S. Pinto</i> | | | | | |
| HW 874 | F ₂ | - | 2 | - | - |
| | F ₃ | 10 | - | - | - |
| <i>Restinga</i> | | | | | |
| HW 811b | F ₁ | - | 1 | - | - |
| | F ₂ | 10 | - | - | - |
| HW 1468 | F ₁ | - | 2 | - | - |
| | F ₂ | 10 | - | - | - |
| HW 1470 | F ₁ | - | 1 | - | - |
| | F ₂ | 10 | - | - | - |
| HW 1471 | F ₁ | 10 | 2 | 10 | - |
| | F ₂ | 10 | - | - | - |
| HW 1472 | F ₁ | - | 1 | - | - |
| | F ₂ | 10 | - | - | - |
| <i>Jarau</i> | | | | | |
| SCM 172 | F ₁ | - | 1 | - | 1 |
| | F ₂ | 21 | - | 18 | - |
| SCM 188 | F ₁ | - | 1 | - | - |
| | F ₂ | 10 | - | - | - |
| SCM 196 | F ₁ | - | 2 | - | - |
| | F ₂ | 10 | - | - | - |
| SCM 200 | F ₁ | - | 2 | - | - |
| | F ₂ | 10 | - | - | - |
| SCM 204b | F ₁ | - | 1 | - | - |
| | F ₂ | 10 | - | - | - |
| <i>Argentina</i> | | | | | |
| HW 1384 | F ₁ | - | 2 | - | - |
| | F ₂ | 10 | - | - | - |
| HW 1388 | F ₁ | - | 1 | - | - |
| | F ₂ | 15 | - | - | - |
| HW 1457a | F ₁ | - | 1 | - | - |
| | F ₂ | 10 | - | - | - |
| HW 1459 | F ₁ | - | 2 | - | - |
| | F ₂ | 10 | - | - | - |
| HW 1460 | F ₁ | - | 1 | - | - |
| | F ₂ | 10 | - | - | - |

*One adult F₁ plant was analyzed by Schiengold (1985).

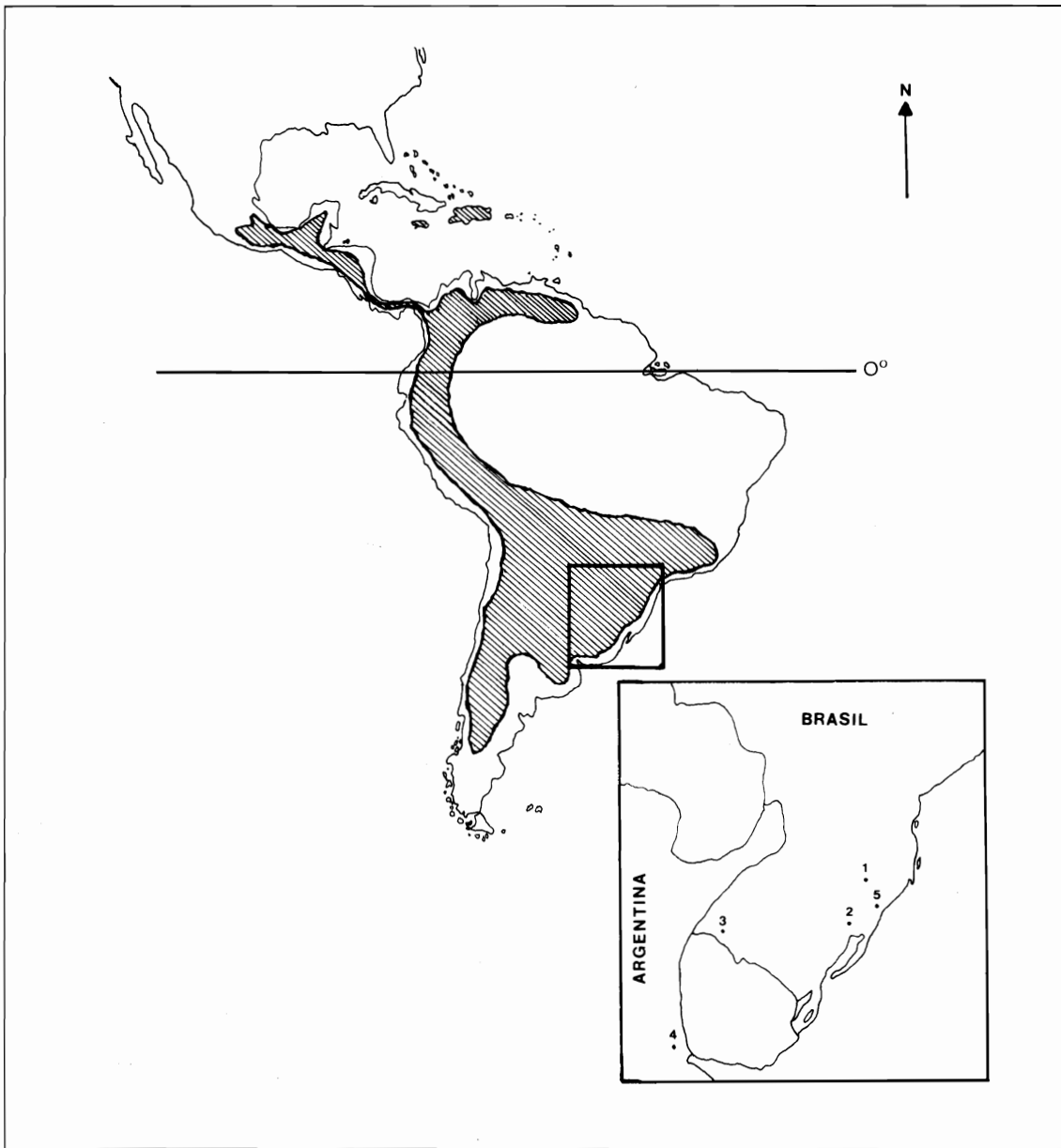


Figure 1 - Geographic distribution of *Relbunium hypocarpium* and sites of origin of the studied strains: 1 - Itaimbezinho, Aparados da Serra National Park, Cambará do Sul County, Rio Grande do Sul State (RS), Brazil ($29^{\circ}15'S$; $50^{\circ}15'W$); 2 - Restinga, Porto Alegre, RS ($30^{\circ}10'S$; $51^{\circ}15'W$); 3 - Cerro do Jarau, Quaraí, RS ($30^{\circ}23'S$; $56^{\circ}27'W$); 4 - Braço Largo Station, Province of Entre Rios, Argentina ($33^{\circ}55'S$; $58^{\circ}50'W$); 5 - Serra do Pinto, Osório, RS ($29^{\circ}53'S$; $50^{\circ}16'W$).

progeny sample was enlarged, being also analyzed for esterases (EST, EC 3.1.1) see Table I. Esterases were chosen since they proved to be a multilocus and highly polymorphic system (see Motta, 1981; Schiengold, 1985; Cavalli-Molina and Winge, 1988).

GOT analysis was performed by sequential horizontal 11% starch gel electrophoresis using the buffer systems of Scandalios (1969) and Poulik (1957), with a field intensity of 10 V/cm. The staining method of Harris and Hopkinson (1976) was used. For EST sequential electrophoresis was performed using 6% and 8% polyacrylamide gel and Scandalios' buffers (1969);

staining procedures were those of Scandalios (1969), with modifications: 4ml α -naphthylacetate and 80 mg fast blue BB salt were used.

Every gel included three or more applications of cotyledonary leaves from three plants of the HW 874 strain, as a migration control.

RESULTS

No variation in GOT pattern was observed among fruits of the same plant when five ripe fruits

collected at random along different branches of each adult plant were analyzed in a search for eventual somatic mutations.

With the single exception of strain JEAM 546, no difference between sib adult plants was detected in their stem or fruit GOT patterns.

Progeny analysis using 10 sib seedlings were performed for the 22 strains; 17 of them with no indication of any variation in their GOT patterns. Among these, strain JEAM 525 was also studied for EST pattern and no difference was observed among the sib seedlings. Figure 2 illustrates an example of progeny analysis carried out on three of these uniform strains.

The progenies of strain HW 1471, analyzed in the F₁ generation, and of strains JEAM 521, JEAM 546, SCM 172 and HW 1388, analyzed in the F₂ generation, showed variation in their GOT patterns. To investigate whether this was due to allozyme or to regulatory gene segregation and in order to obtain information on the possible origin of the observed variation in the autogamous strains, we also analyzed the same progenies, and eventually the same seedlings, for their esterases.

One of the ten F₁ seedlings of strain HW 1471, from Restinga, RS, presented a GOT band with lower relative mobility (RM = 0.76) than the remaining sib seedlings, which presented a band of RM = 0.78. No other variation in GOT pattern was detected in these seedlings, nor was any variation detected in the EST pattern analyzed in 10 other sib seedlings. Besides, two adult F₁ plants and the progeny of one of them (10 F₂ seedlings) were also analyzed, but only the faster migrating band (RM = 0.78) was observed. On the other hand, another strain, HW 1388, from Argentina, presented the same variant GOT pattern detected in line HW 1471, in three of its 15 F₂ seedlings (Figure 3). Based on the results for HW 1388, in which the adult

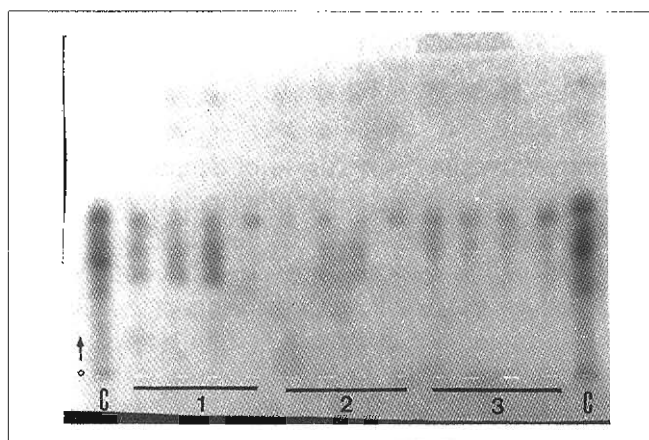


Figure 2 - GOT patterns indicating absence of segregation. Three applications of cotyledonary leaves and one of hypocotyls were made per strain: 1 - JEAM 520, 2 - JEAM 556 and 3 - SCM 200; C = control (strain HW 874).

plant presented only the fast band (RM = 0.78) and its progeny segregated at an approximate 3:1 ratio for RM = 0.78: RM = 0.76 ($P > 0.05$), without the simultaneous occurrence of the two bands (without heterozygotes), we suggest that this variation could be due to a modifier gene with epigenetic effects changing the mobility of the band from RM = 0.78 to 0.76 and that this modifier gene may be recessive. In this case the adult plant (F₁) would be heterozygous for this gene. This hypothesis would also apply to the segregation observed in strain HW 1471 (not statistically rejected) thus permitting us to suggest that the plant from nature (P₁) was heterozygous for this modifier gene. An alternative hypothesis, of a dominant modifier gene causing the slower relative migration, cannot be completely rejected. Results obtained for strain HW 1471, could be explained supposing a recent mutation in a single F₁ individual. The three F₂ individuals of strain HW 1388, which carry the slower band (RM = 0.76), might have originated from the same branch, mutant for the dominant modifier gene.

In strain JEAM 521, two of the 15 F₂ seedlings differed from their sibs by presenting a slower band of RM = 0.93, instead of RM = 0.96 (Figure 3). Since their mother presented only the band of RM = 0.96, we suggest that the F₂ sib seedlings were segregating for a recessive modifier gene that arose by mutation and that the adult plant is heterozygous for it, since its progeny segregated at the approximate 3:1 ratio ($P > 0.05$).

When the progenies of two adult sib plants from strain JEAM 546 A and B were analyzed, the same type

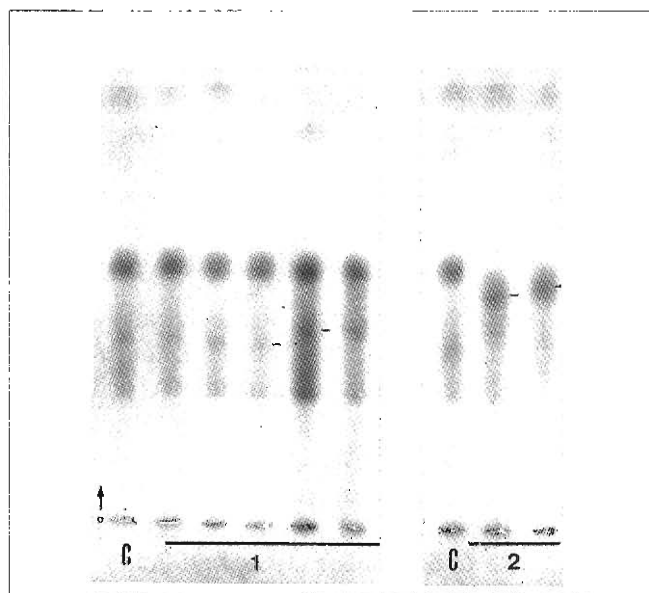


Figure 3 - GOT pattern variation among sib seedlings of two strains: 1 = HW 1388 (bands of RM = 0.76 and RM = 0.78) and 2 = JEAM 521 (bands of RM = 0.93 and RM = 0.96). C = control (strain HW 874).

of variation as in line JEAM 521 was observed, i.e. part of the sib seedlings of each progeny presented the slower band with $RM = 0.93$, whereas the remaining sibs exhibited the band 0.96. No heterozygous plant, with both bands, was detected. It is interesting to notice that the band of $RM = 0.93$ was only observed in these two strains (JEAM 521 and 546) from Itaimbezinho, the Aparados da Serra National Park. The esterase patterns of the progeny of each F_1 plant, JEAM 546A and B, did not differ either within or between the progenies. As can be seen in Figure 4, half the seedlings of the offspring of plant JEAM 546A presented the slower band ($RM = 0.93$), while that of JEAM 546B presented 11 seedlings with the faster and eight with the slower GOT band. Nevertheless, both adult F_1 plants, A and B, showed only the 0.96 band in stem and fruits. The hypothesis proposed to explain the occurrence of these bands in strain JEAM 521 could be also applied to this strain, though an excess of seedlings with the 0.93 band were obtained in JEAM 546. According to that hypothesis a recessive mutation could have occurred in the P_1 plant from nature in a modifier gene with an epigenetic effect on migration rate. The two adult F_1 plants grown in the greenhouse would be heterozygous for this gene. The observed segregation in the F_2 seedlings does not support the alternative hypothesis of a dominant modifier gene.

On the other hand, the two adult sib plants from the greenhouse differed in two other GOT bands. Fruits

of JEAM 546A presented a band with $RM = 0.39$, also found in all seedlings of its progeny, but not the $RM = 0.51$ band that was observed in JEAM 546B fruits and progeny (Figure 4). A possible explanation for these results, supposing bands 0.39 and 0.51 are alloenzymes, could be that the P_1 plant from nature was heterozygous and the two F_1 adult plants from the greenhouse were homozygous for each allele, as were their respective progenies. The heterozygosity of the P_1 plant could be originated by mutation or by a cross that occurred in nature, involving the P_1 plant or one of its ancestrals. The identical isoesterase pattern of the analyzed F_2 seedlings does not favor the crossing hypothesis. An alternative hypothesis to alloenzyme segregation would be to suppose the P_1 plant from nature to be heterozygous for yet another modifier gene, that may change the relative migration of the enzyme. Then the two F_1 adult plants, A and B, would be homozygous for different alleles. If this hypothesis is correct, one would expect the original GOT band to be the 0.51 since the band of $RM = 0.39$ was only detected in this strain while the 0.51 is a common band also found in 14 of the other strains.

Strain SCM 172, from Quaraí County, presented variation in both GOT and EST patterns of its progeny. The main variation in the F_2 seedlings' isoesterases were observed in the more anodal region of the gels (Figure 5B), which showed the expected pattern for monomeric alloenzyme segregation in an F_2 generation.

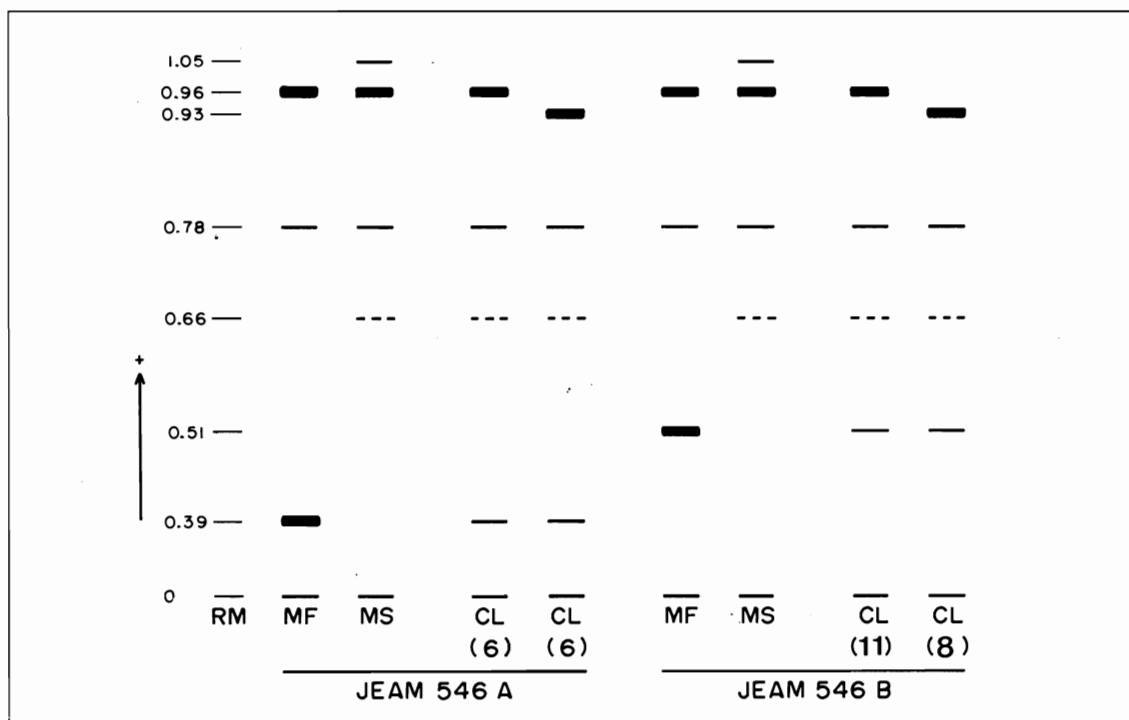


Figure 4 - GOT patterns for strain JEAM 546: MF = mature fruits and MS = mature stems of the two sib adult F_1 plants, A and B; CL = cotyledonary leaves of first stage F_2 seedlings. The number of F_2 individuals, of each progeny, with the same GOT pattern is given in parentheses. RM = relative migration.

Seedlings homozygous for each allele as well as heterozygotes were observed in a phenotypic proportion that did not differ from the theoretical 1:2:1 ratio ($P > 0.05$). As expected, the adult (F_1) plant proved to be heterozygous for this locus. The GOT pattern of the F_2 seedlings was more complex (Figure 5A). The more anodal region, involving bands of $RM = 1.00, 0.95$ and 0.90 , presented the expected segregation for dimeric alloenzymes, with a heterodimeric band, segregating at the 1:2:1 ratio ($P > 0.70$). A weak band of $RM = 0.82$ was detected in the next region in nine of the 21 seedlings studied. Since we cannot rule out the possibility that all seedlings of this strain present the same band but in part of them its intensity is below the detection threshold, the band 0.82 was disregarded in the analysis. The bands of $RM = 0.76, 0.71$ and 0.66 , as seen in Figure 5A, present patterns suggesting segregation of dimeric alloenzymes. This hypothesis can be ruled out for the following reasons: 1. the adult plant is

homozygous for the band of $RM = 0.76$, thus it would be very difficult to explain the origin of nine "heterozygous" seedlings of its progeny by mutation; 2. assuming the occurrence of cross-fertilization in the greenhouse, a very high rate of allogamy would be needed to explain the nine "heterozygotes" among the 21 seedlings analyzed, an unexpected fact in this clearly cleistogamous species; 3. the hypothesis of alloenzymes coding for bands 0.76 and 0.66 would require a *cis* regulator to explain the absence of activity of the allele *Got*^{0.66} in tissues of the adult plant, supposed to be heterozygous, and the observed segregation in its progeny (F_2 seedlings). This hypothesis is weakened by the presence of a band of $RM = 0.66$ in adult tissues of eight strains from the three populations in which this band was detected. Thus, we suggest that band 0.76 and 0.66 are coded by two different loci. The variation among seedlings could be explained hypothesizing the segregation of a regulatory

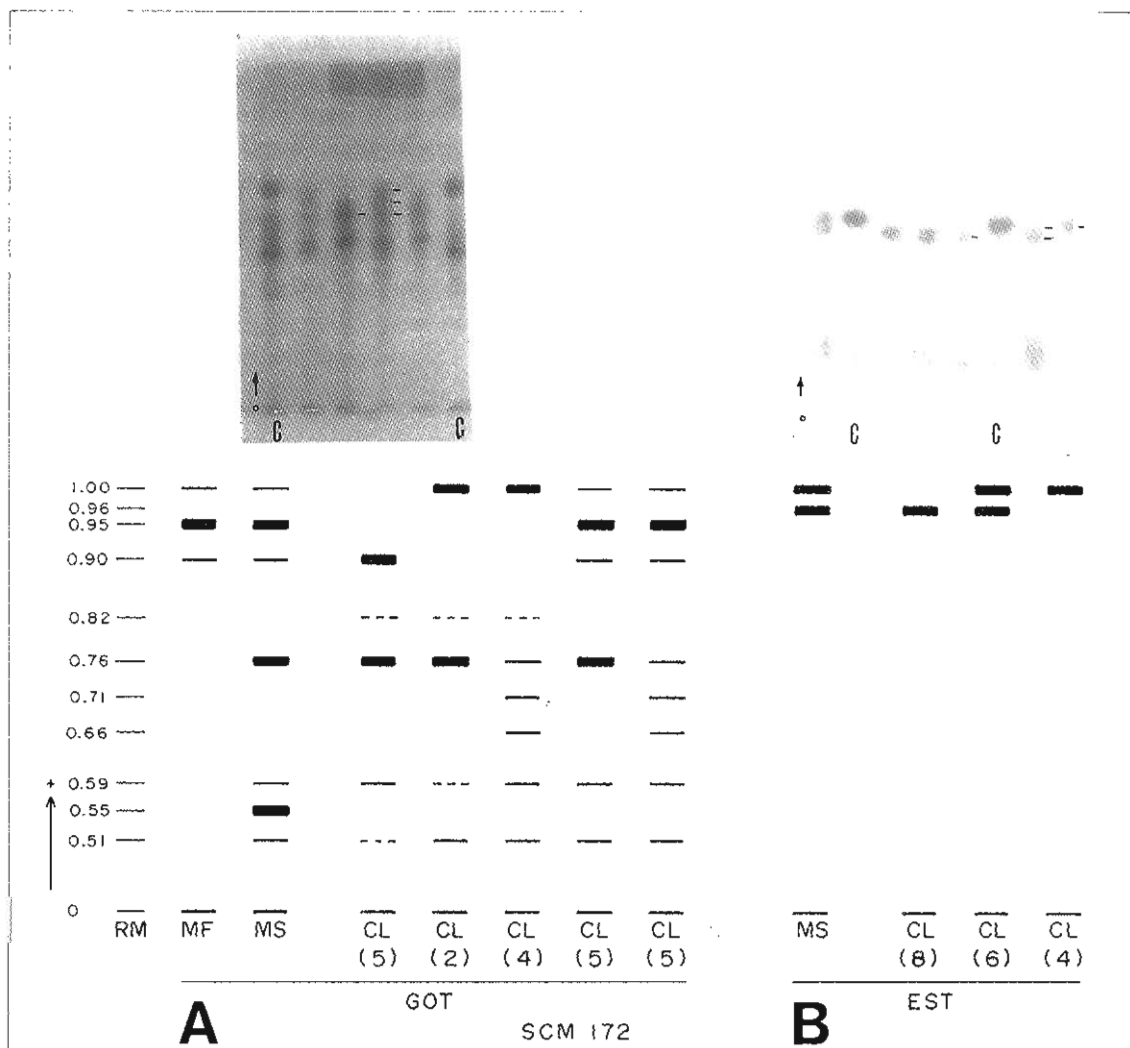


Figure 5 - GOT (A) and EST (B) patterns for strain SCM 172. Explanations as in Figure 4. Only the most anodal EST bands are shown.

gene controlling the (temporal?) activation of band 0.66, and present in heterozygosis in the adult mother plant. Since the RM = 0.71 band was only detected in this strain and only in the nine F₂ seedlings that simultaneously presented the 0.76 and 0.66 bands, we suggest it be an interloci heterodimer. The least anodal region of the gels involves bands of RM = 0.59, 0.55 and 0.51. Comparing the GOT patterns of the adult F₁ plant with those of its progeny we notice the absence of band 0.55 in the F₂ seedlings, which could be attributed to a regulatory gene that does not activate the respective locus in seedlings and in fruits. Alternately, we may assume the formation of an interloci heterodimer tissue-specific for mature stems. This hypothesis receives support from the fact that the only other case in which bands 0.51 and 0.59 occurred simultaneously in mature stems was in strain SCM 204b, where 0.55 also appeared.

Since it appears that the adult F₁ plant of strain SCM 172 is heterozygous for several loci and considering the extensive segregation in its progeny both of GOT and of EST isoenzymes, we suggest that this adult F₁ plant was originated from a cross possibly involving the P₁ plant from nature.

DISCUSSION

The present results confirm that *R. hypocarpium* reproduces mostly by self-fertilization, thus endorsing and extending the data reported by Cavalli-Molina *et al.* (1989). Pooling the results of these two studies, a total of 26 different progenies of plants from nature were analyzed. These plants belonged to six natural populations separated by considerable geographic distances, with localities ranging from humid and mountainous forests in the Itatiaia National Park, State of Rio de Janeiro, Brazil (22°20' S/44°35' W) to the Brazo Largo Station, Province of Entre Rios, Argentina (33°55' S/58°50' W), a field region, approximately at sea level. Progenies of the 26 plants were analyzed for one, two or four different enzymatic systems, totaling 22 strains studied for GOT, 10 for EST, three for peroxidase and one for acid phosphatase patterns.

Only one of the 26 strains (SCM 172) did present allozyme segregation in both the enzymatic systems, GOT and EST, with clear indications that its progeny was the result of a cross that occurred in nature, probably involving the plant (P₁), the seeds of which we collected at Cerro do Jarau. The variation observed in the progeny of another strain, JEAM 546, from Aparados da Serra National Park, could eventually be the result of another cross, that also occurred in nature, but involving the parent or the grandparent of the P₁

plant from nature. Nevertheless, in this case the hypothesis of a cross is quite fragile since the progeny presented variation only in GOT and were fully uniform in their EST pattern, an enzymatic system with high interindividual variation in this species, especially in temporal regulatory genes (Motta, 1981; Schiengold, 1985; Schiengold and Winge, 1990). Thus, considering the total sample, there would have occurred at least one allogamy event in 4% to 8% of the strains, probably closer to the lower limit. This frequency of allogamy levels is similar to that of other self-fertilizing species (see Richards, 1986).

The potential advantages of such a mixed reproductive system, i.e. self-fertilization with rare outcrossing events, raises interesting theoretical problems. Richards (1986) suggests that heterozygote advantage, and thus presumably heterosis, may be frequency dependent, what would explain a more marked response when an eventual outcross occurs in a predominantly selfed population rather than from customary outcrossers. In an interesting study on factors controlling outcrossing frequencies in *Datura stramonium*, Motten and Antonovics (1992) pointed out that allogamy can be favored in a predominantly selfing species, through the production of variable progeny in generations **after** outcrossing, especially when the populations consist of many different inbred and highly homozygous lines. The relative frequency of outcrossing in this highly selfed species varied between populations and among individuals within populations, due to multiple factors acting simultaneously, such as variation in floral biology, pollinator movements, and spatial distribution of plants.

A surprising result obtained in the present work was the detection of another source of variation within strains of this autogamous species, i.e., mutations in isoenzyme modifier genes, genes showing epigenetic activity changing the relative mobility of isoenzymes. Unfortunately, the minute size of *R. hypocarpium* flowers does not permit controlled crosses to test this hypothesis. Nevertheless, the variation observed in the progeny of three strains, and possibly four if we include JEAM 546, cannot be explained by segregation after crosses. On the other hand, it is highly improbable that such variation could be originated by mutation in structural genes controlling GOT isoenzymes, since no heterozygote was detected in the progeny of homozygous plants.

The occurrence in plants of isoenzyme modifier genes is known for a long time. These genes have a postsynthesis action, including aggregation of molecules, addition of radicals, adenylation, deamination, phosphorylation, changes in tertiary structure

of enzymes, thus altering their electrophoretic mobility (MacDonald and Brewbaker, 1974; Harris and Hopkinson, 1976; Tyson et al., 1978; Rich et al., 1979; Scandalios, 1983; Hickenbick et al., 1992). This type of variation was observed in 14% to 18% of the strains analyzed for their GOT patterns, or in 12% to 15% if we consider all 26 strains analyzed.

We cannot evaluate the importance of these mechanisms for the species, since it is not clear what effect these mechanisms of postsynthesis modification of isoenzymes could possibly have on plant fitness. However, it should be emphasized that this phenomenon was not observed in a specific population, but was observed in at least three geographically distant localities. We are yet unable to determine if this phenomenon occurs preferentially in more conservative isoenzyme systems such as GOT, or if we detected it in this system because a larger number of strains and of progenies were analyzed for their GOT patterns.

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RESUMO

Vinte e duas linhagens de *Relbunium hypocarpium*, coletadas em cinco localidades do Cone Sul, foram estudadas quanto ao padrão isoenzimático das glutamato oxaloacetato transaminases (GOT, EC 2.6.1.1) por eletroforese horizontal em gel, com a finalidade de obter dados mais precisos sobre o sistema de reprodução e sobre a origem da variabilidade existente nesta espécie cleistógama. Foram estudadas plantas adultas, F₁ ou F₂ de plantas da natureza, e suas progênies. Quando alguma variação foi observada na progênie, as mesmas plântulas ou plântulas irmãs foram também analisadas quanto às isoesterases (EST, EC 3.1.1). Os resultados obtidos mostram que *R. hypocarpium*, espécie cleistógama, reproduz-se preferencialmente por autofecundação, embora pelo menos um evento de fecundação cruzada, ocorrido na natureza, tenha sido detectado em uma linhagem (cerca de 4% das linhagens). Surpreendentemente, foi verificado que uma fonte adicional de variabilidade no sistema das GOT, observada em cerca de 14% a 18% das linhagens, deve-se à ocorrência de modificadores das isoenzimas, possivelmente modificadores pós-síntese e cujo significado adaptativo não podemos, até o momento, explicar.

REFERENCES

- Barrett, S.C.H. and Eckert, C.G. (1990). Variation and evolution of mating systems in seed plants. In: *Biological Approaches and Evolutionary Trends in Plants* (Kawano, S., ed.). Academic Press, London, pp. 229-254.
- Cavalli-Molina, S. and Winge, H. (1988). Phenetic relationships among populations of the autogamous plant *Relbunium hypocarpium* (Rubiaceae). *Rev. Brasil. Genet.* 11: 401-418.
- Cavalli-Molina, S., Motta, V.E.P., Schiengold, M. and Winge, H. (1989). Identical isoenzyme patterns in sib plants of *Relbunium hypocarpium* (Rubiaceae). *Rev. Brasil. Genet.* 12: 361-368.
- Ehrendorfer, F. (1955). Revision of the genus *Relbunium* (Endl.) Benth. et Hook f. (Rubiaceae-Galieae). *Bot. Jb.* 76: 516-533.
- Harris, H. and Hopkinson, D.A. (1976). *Handbook of Enzyme Electrophoresis in Human Genetics*. Amsterdam, North Holland.
- Hickenbick, M.C.M., Flores, A.I.P., Cavalli-Molina, S., Souza-Chies, T.T. and Albarus, M.H. (1992). Mode of reproduction and seed production in *Paspalum dilatatum* poir virasoro biotype-Dilatata group (Gramineae). *Rev. Brasil. Genet.* 15: 85-102.
- MacDonald, T. and Brewbaker, J.L. (1974). Isozyme polymorphism in flowering plants. IX. The E5-E10 esterase loci of maize. *J. Hered.* 65: 37-42.
- Mariath, J.E.A. (1990). Ontogenia, embriologia e biologia floral de *Relbunium hypocarpium* (Rubiaceae-Rubiaceae). Doctoral Thesis. Universidade de São Paulo, São Paulo, SP.
- Mariath, J.E.A., Cavalli-Molina, S., Schiengold, M., Motta, V.E.P., Freitas, L.B., Winge, H. and Venturelli, M. (1987). Estudos morfológicos e genéticos em *Relbunium hypocarpium* (Rubiaceae): biologia floral, variabilidade isoenzimática e regulatória. XXXVIII Congresso Nacional de Botânica. Resumos (Soc. Botânica do Brasil e Univ. de São Paulo, eds.), pp. 344.
- Motta, V.E.P. (1981). Desenvolvimento ontogenético e modificações dos padrões isoesterásicos de *Relbunium hypocarpium* (Rubiaceae). Master's Thesis. Universidade Federal do Rio Grande do Sul, Porto Alegre, RS.
- Motten, A.F. and Antonovics, J. (1992). Determinants of outcrossing rate in a predominantly self-fertilizing weed, *Datura stramonium* (Solanaceae). *Amer. J. Bot.* 79: 419-427.
- Poulik, M.D. (1957). Starch gel electrophoresis in discontinuous system of buffers. *Nature* 180: 1477-1479.
- Richards, A.J. (1986). *Plant Breeding Systems*. G. Allen & Unwin, London, XIV + 529 pp.
- Rick, C.M., Tanksley, S.D. and Fobes, J.F. (1979). A pseudoduplication in *Lycopersicon pimpinelifolium*. *Proc. Natl. Acad. Sci. USA* 76: 3435-3439.
- Scandalios, J.G. (1969). Genetic control of multiple molecular forms of enzymes in plants: a review. *Biochem. Genet.* 3: 37-79.
- Scandalios, J.G. (1983). Molecular varieties of isozymes and their role in studies of gene regulation and expression during eukaryote development. *Isozymes: Current Topics in Biological and Medical Research, gene expression and development* 9: 1-31.
- Schiengold, M. (1985). Efeito da regulação gênica na variabilidade isoesterásica de *Relbunium hypocarpium* (Rubiaceae). Master's Thesis. Universidade Federal do Rio Grande do Sul, Porto Alegre, RS.
- Schiengold, M. and Winge, H. (1990). Methodology: a similarity coefficient of gene regulation. *Rev. Brasil. Genet.* 13: 855-860.
- Stebbins, G.L. (1957). Self-fertilization and population variability in the higher plants. *The Amer. Natur.* 91: 337-354.
- Tyson, H., Taylor, S.A. and Fields, M.F. (1978). Segregation of the environmentally induced mobility shifts in flax genotroph peroxidase isozymes. *Heredity* 40: 281-290.