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Allozyme variation in a natural population of *Stryphnodendron adstringens* in the Rio Preto State Park, southeastern Brazil

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ABSTRACT – (Allozyme variation in a natural population of *Stryphnodendron adstringens* in the Rio Preto State Park, southeastern Brazil). Leaves and fruits from 63 *Stryphnodendron adstringens* trees were sampled in the Rio Preto State Park to analyze allozyme segregation, tissue specific expression of allozyme loci, and their genetic parameters. The enzyme systems ADH, EST, ACP, PGM, PGI, GDH, G6PDH, GOT, IDH, LAP, MDH, PER and SKDH were assessed by means of starch-gel electrophoresis. The polymorphic systems PGI, IDH, MDH and GOT demonstrated a dimeric quaternary structure, while EST and PER were monomeric. The total expected genetic diversity (H_E) for leaves and seeds were 0.325 and 0.244 respectively. The effective number of alleles per locus (A_E) was 1.58 in leaves and 1.42 in seeds. The values of H_E and A_E observed in *S. adstringens* were comparatively higher than the average values seen in allozyme studies of other woody plants. The values of the fixation indices for the population, considering leaves ($f = 0.070$) and seeds ($f = 0.107$), were not significant. The high values of genetic diversity and of effective number of alleles per locus, as well as the non-significant fixation index and the adjustments of the Hardy-Weinberg proportions between generations for the *pgi-1*, *mdh-2* and *idh-1* loci, indicated random mating in this population. The enzyme systems EST and PER demonstrated their best resolution in leaf tissues, while the MDH, IDH, PGI and GOT systems demonstrated their best resolution in seed tissues.

Key words - allozyme segregation, genetic diversity, heterozygosity, medicinal plants

INTRODUCTION

Stryphnodendron adstringens (Mart.) Coville (Mimosoideae, Leguminosae) is a small evergreen tree (locally called *barbatimão*) that is widely distributed in the *Cerrado* (Brazilian savanna) biome (Ortiz et al. 2003). It is used in traditional medicinal practices to treat infirmities such as ulcers, sores, hemorrhoids (Barros 1982), gastritis, sore throats (Hirschmann & Arias 1990), leukorrhea, hernias, diarrhea, bleeding, ringworm and ophthalmia (Pio Correa 1926, Almeida et al. 1998). Its racemose andromonoecious inflorescences have small and densely arranged flowers. Cross-pollination is vital for fruit production in this species and the main floral visitors appear to be Hymenoptera; other floral visitors include Diptera, Lepidoptera and Coleoptera. Fruit production is constrained by resource availability (Ortiz et al. 2003).

Stryphnodendron adstringens populations that used to cover extensive *Cerrado* areas are now isolated in small fragments due to deforestation, indiscriminate land use, and urban development. One of the few regions where *Cerrado* vegetation is still well-conserved is in the Rio Preto State Park (RPSP), located in the municipality of São Gonçalo do Rio Preto in the Serra do Espinhaço Reserve Biosphere, 70 km from the city of Diamantina in Minas Gerais State, in southeastern Brazil. The park covers a total area of 12,185 ha. that is mostly covered with *cerrado sensu stricto* and *campos de altitude* vegetation. The fauna and flora in the RPSP are very rich and include many endangered species such as *S. adstringens* (IEF 2011). The Espinhaço Range serves as a watershed for the basins of many central-eastern rivers as well as the large São Francisco River (Saadi 1995), and separates two important biomes in its central and southern portions: the Atlantic Rain forest on the eastern slopes, and the *Cerrado* biome on its western slopes (Melo Júnior et al. 2001).

The genetic resources of medicinal plants (especially arboreal species) are finite, and many *Cerrado* plants have been extensively used without concern for their conservation or renewal. It is essential that our remaining forest resources be prudently used – and genetic studies are fundamental to gaining a full understanding of their evolutive processes and for developing conservation strategies that can guarantee the future of managed species. Isozyme techniques provide a way to assay genetic variation

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levels in natural populations of tropical forest trees and to measure population processes important to ecologists, conservationists, and forest management personnel (Loveless 1992). Very few studies of the levels of genetic variations in woody *Cerrado* plants are available, however. Two studies of genetic diversity in *S. adstringens* have been published, the first was based on microsatellite data (Branco et al. 2010) and the second on RAPD markers (Camillo et al. 2001), but no isozyme studies have been reported.

Due to the fact that the genes that control isozyme expression are manifest in certain development stages and in specific organs and tissues, or in response to certain stimuli (Ramírez et al. 1991), zymograms from extracts of seeds, seedlings and the leaves of adult plants may be quite distinct (Alfenas & Brune 1998). Many enzymes, such as esterases, peroxidases, phosphatases, and peptidases also demonstrated developmental and environmental variations that mimic Mendelian segregation (Conckle 1971b, Kelley & Adamns 1977) and either genetic (Law 1967) or environmental post-translational modifications (Cullis 1977), so that Mendelian analyses are essential in studies of nonspecific enzyme assays (Hart & Langston 1977). The best way to address this problem is to verify through Mendelian analyses that a given set of isozyme variants are true *allozymes*, that is, they are coded for by different alleles at the same locus (Broun & Moran 1979).

The objectives of the present study were to evaluate the tissue-specific expression and segregation of allozyme loci, to measure genetic parameters, and to describe useful allozyme variants for future evaluations of the genetic structure of *S. adstringens* populations.

MATERIAL AND METHODS

The vegetation in the RPSP reserve is well-conserved and *S. adstringens* is very common both inside and outside the park, occurring almost continuously in the municipalities of Olhos D'Água and Diamantina, Minas Gerais State, Brazil. Samples were taken of leaves and fruits (in their final maturation stages) from 63 individual *S. adstringens* trees. The average spacing between the sampled trees was 60 m. The fruits were transported in paper bags and the leaves were immersed in liquid nitrogen until assayed at the Plant Reproduction Laboratory of the Federal University of Viçosa.

The present study focused on the isozyme variants (allozymes) present in this *S. adstringens* population. The analyses were performed using starch-gel electrophoresis technique, extracting the allozymes from the leaves and three seeds from each sampled tree. The proportions of 1 g of leaf tissue or one seed to each 3 mL of extracting solution #1 were used, as recommended by Alfenas et al. (2006). The gels were prepared with 12 g of sucrose and 60 g of starch per 500 mL of gel buffer solution. The buffer systems used followed Soltis et al. (1983) (buffer A) and Shaw & Prasad (1970) (buffer B). A pre-run was performed at 15 mA for 30 min. with buffer A, and for 1 h with buffer B. The extract runs were carried out at 35 mA and lasted about 5 h. The allozyme systems analyzed and the composition of the electrode/gel buffer systems are described in table 1.

The zymograms were analyzed and the allozyme loci identified using the same abbreviations used to designate

Table 1. Enzyme systems and buffer compositions used in the electrophoresis of leaf and seed extracts of *Stryphnodendron adstringens.* (EC = Enzyme commission).

Enzyme	Abbreviation	EC	Electrode/gel buffers*
Alcohol dehydrogenase	ADH	1.1.1.1	B
Esterase	EST	3.1.1.1	A
Acid phosphatase	ACP	3.1.3.2	A
Phosphoglucomutase	PGM	5.4.2.2	A
Phosphoglucose isomerase	PGI	5.3.1.9	A
Glucose dehydrogenase	GDH	1.1.1.47	A
Glucose-6-phosphate dehydrogenase	G6PDH	1.1.1.49	A
Glutamate oxaloacetate transaminase	GOT	2.6.1.1	A
Isocitrate dehydrogenase	IDH	1.1.1.42	B
Leucine aminopeptidase	LAP	3.4.11.1	A
Malate dehydrogenase	MDH	1.1.1.37	B
Peroxidase	PER	1.11.1.7	A
Shikimate dehydrogenase	SKDH	1.1.1.25	B

* Electrode buffer/gel: A = electrode (4.0 g L^{-1} NaOH, 18.55 g L^{-1} boric acid), pH 8.6/gel (dilute 40 mL L^{-1} electrode solution, 1.84 g L^{-1} Tris, 0.69 g L^{-1} citric acid), pH 7.8 (Soltis et al. 1983); B = electrode (Tris 16.35 g L^{-1}, 9.04 g L^{-1} citric acid), pH 7.0/gel (dilute 66.7 mL L^{-1} electrode solution), pH 7.0 (Shaw & Prasad 1970).

each enzyme system (for example, PGI for phosphoglucose isomerase) in lowercase letters in italics, followed by its ascending numerical order beginning with the slower migrating locus (for example, *pgi-1*). The fastest migrating allele at each locus was identified by the letter *a*, while those migrating more slowly followed in alphabetical order. Statistical tests involving allele frequencies were carried out only for loci that presented simple banding patterns that could be easily identified.

Controlled crossing experiments or allozyme studies using haploid tissues (such as pollen) are required to test Mendelian inheritance and to correctly interpret zymograms (Hart & Langston 1977, Broun & Moran 1979, Alfenas & Brune 1998). These studies are difficult to perform in *S. adstringens* as it is an undomesticated, allogamous, and slow growing plant, and most of its racemes failed to produce fruits by self-pollination (Rocha & Moraes 1997, Ortiz et al. 2003).

Brown & Moran (1979) observe that the best way to avoid misinterpreting isozyme data is to use Mendelian analyses to verify that sets of isozyme variants are truly allozymes and that they are coded for by alleles at a single locus. Therefore, in order to correctly identify allozyme inheritance modes without carrying out genetic crosses or studies of haploid tissues, we analyzed the genotypic proportions of the allozymes between generations by measuring the allele frequencies of leaves and seeds from the same plant. Using the observed allele frequencies in the leaves from 63 trees, we calculated the expected genotype frequencies under a Hardy-Weinberg Equilibrium (HWE) for the same size sample of progeny (189 seeds). Individual seeds or seedlings can be assayed as members of progeny arrays (Brown & Moran 1979). The expected allele frequencies of the progeny were then compared with the same sample size of tested progeny using the chi-square test (X^2). Since this species is allogamous and the male parents are unknown, the individuals of the population themselves were regarded as forming the male gamete set (Cruz 2005), and the male allele frequencies were therefore considered equal to those of the females, assuming a HWE.

The genetic parameters of the effective number of alleles, genetic diversity (H_E and H_O) (Nei 1973), and fixation index (Wright 1951) were estimated. The expected proportions of heterozygous loci per individual (H_E) is a composite measure that summarizes genetic variation at the allele level. This parameter, which is often referred to as genetic diversity, was calculated for each locus and averaged over all loci (Berg & Hamrick 1997). The Li & Horvitz (1953) test of the fixation index (*f*) was conducted. Each locus was tested separately and the X^2 value and df's were summed over all loci to give an overall test of the mean multilocus *f*. The statistic analyses were performed using the Genes software system for genetics and statistics. Fisher's exact and chi-squared (X^2) tests were used to calculate the probabilities of the genotypic arrangements observed in the leaves and seeds.

RESULTS AND DISCUSSION

Differences were observed in terms of the active regions and the numbers of loci and alleles between the leaves and seeds for the polymorphic systems of malate dehydrogenase (MDH), phosphoglucose isomerase (PGI), isocitrate dehydrogenase (IDH), glutamate oxaloacetate transaminase (GOT), peroxidase (PER), and esterase (EST); the PGI, IDH, EST and SKDH systems, on the other hand, demonstrated activity at the same loci. The active regions, number of loci, number of alleles, the quaternary structure observed in polymorphic systems, and the quaternary structures recorded in the literature are listed in table 2. The diagrammatic representations of the variation in the polymorphic systems used in the statistic analyses (PGI, IDH, GOT and PER) are presented in figure 1.

Two regions of MDH activity were apparent in the gels of leaves (female parents), while only one region was apparent in the gels of seeds (progeny). Catodal region activity was principally observed in leaves, and seemed to be controlled by a single locus (*mdh-1*); it exhibited a monomeric isozyme pattern in contrast to the dimeric or tetrameric patterns usually observed

Table 2. Active regions, numbers of loci, numbers of alleles per locus, and the quaternary structures observed in *Stryphnodendron adstringens* and the quaternary structures of enzymes reported in the literature.

Enzyme	Active region	Number of loci	Number of alleles per locus	Quaternary structure	
				Observed	*Reported
MDH	2	3	1-2	dimeric/ monomeric	dimeric/tetrameric
PGI	2	2	2	dimeric/monomeric	dimeric
IDH	1	1	3	dimeric	dimeric/oligomeric
GOT	2	3	1-2	dimeric	dimeric
EST	3	4	1-2	monomeric	monomeric/dimeric
PER	3	3	1-2	monomeric	monomeric

* Data from Brune at al. (2006).

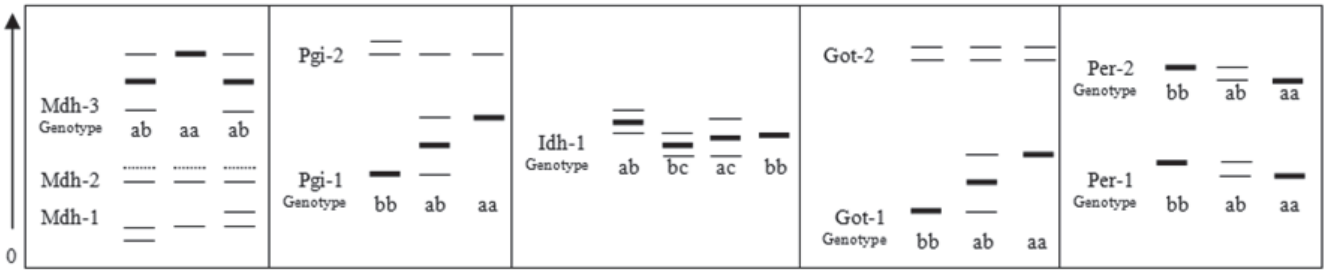

Figure 1. Diagrammatic representation of the variation of the malate dehydrogenase loci (*mdh-1* observed only in leaves, *mdh-2* observed only in seeds, *mdh-3* observed in both seeds and leaves), phosphoglucose isomerase (*pgi-1* and *pgi-2* observed in leaves and seeds), isocitrate dehydrogenase (*idh-1* observed in both leaves and seeds), glutamate oxaloacetate transaminase (*got-1* observed only in seeds, *got-2* and *got-3* observed in both leaves and seeds) and peroxidase (*per*-1 and *per-2* observed in both leaves and seeds); Genotypes of allozyme systems used in the statistic analyses: a = the fastest migrating allele; b = the intermediate migrating allele; *c* = the slowest migrating allele.

(Brune et al. 2006). The monomorphic locus *mdh*-2 in the anodal region was apparent only in seeds, and the dimeric locus *mdh-3* was coincident in leaves and seeds, exhibiting a dimeric pattern with two alleles that were clearly and consistently observed in both organs. MDH has been isolated from different sources, including archaea, eubacteria, fungi, plants, and mammals, and has been described as consisting of two or four subunits (Musrati et al. 1998, Brune et al. 2006).

Two coincident active regions were observed in the PGI gels in both leaves and seeds that seemed to be controlled by a locus and by two alleles each. The *pgi-1* locus exhibited a dimeric pattern with hybrid bands typical of heterozygous individuals and two alleles. The anodal (*pgi-2*) locus showed a banding pattern typical of monomeric enzymes. Although a monomeric pattern of PGI has been observed in microorganisms such as *Archaeoglobus fulgidus* and *Methanosarcina mazei* (Hansen et al. 2005), this enzyme is generally dimeric (Cini et al. 1988, Tekamp-Olson et al. 1988, Sun et al. 1990, Brune et al. 2006). IDH gels of leaves and seeds demonstrated an activity zone controlled by a locus (*idh-1*) with three alleles. The enzyme IDH has been well-studied in fungi and animals and is usually dimeric or oligomeric (Brune et al. 2006); a dimeric structure has been confirmed in plants such as *Cucumis sativus* (Watanabe et al. 2007) and the cherry-tree (Granger et al. 1992). GOT gels showed two active regions in the seeds and one active region in the leaves. The anodal region showed a pattern of two fixed bands that were evident both in leaves and seeds (*got-2* and *got-3*). An active catodal region from seed extracts demonstrated only a single locus (*got-1*) with two alleles and a dimeric band pattern. GOT allozymes are known to have dimeric patterns in plants (Kephart 1990).

Three active regions were observed in the PER gels of the leaves, and two active regions were seen in the seed gels. Although the banding patterns of the progenies (seeds) agreed with those of the trees (leaves), comparisons between leaves and seeds were not possible due to the poor resolution of most of the progeny individuals. A monomeric pattern with one locus (*per-1* and *per-2*) and two alleles was evident in each anodal and intermediate region of leaf extracts, in agreement with the results of Brune et al. (2006). Cherry trees demonstrated a dimeric quaternary structure (Granger et al. 1992), but monomers were observed in *Cucumis sativus*, *Roystonea regia* (Watanabe et al. 2007), and *Allium sativum* (Marzouki et al. 2005).

Three active regions were apparent in the EST gels, and although the numbers of loci and alleles were the same for leaves and seeds, the banding patterns were not clear enough in all individuals to permit statistical analyses. A monomeric pattern was observed with one locus and two alleles in each anodal and intermediate region, while two fixed loci were observed in the catodal region. Esterase is one of the most polymorphic enzyme systems in plants (Weeden & Wendel 1990) and the most investigated system in rice (Endo & Morishma 1983), and monomeric or dimeric variants of these enzymes are usually found in plants (Brune et al. 2006). In the present study, only the *got-1*, *idh-1*, *pgi-1*, *mdh-2*, *per-1* and *per-2* loci banding patterns were clearly and consistently observed in the gels and could be used for statistical purposes. The sample sizes and the allele frequencies of the polymorphic loci analyzed are presented in table 3.

Achromatic bands with no detectable patterns of distribution were observed with glucose dehydrogenase (GDH), alcohol dehydrogenase (ADH), and glucose-6-

Table 3. Allele frequencies of polymorphic loci measured in leaf and seed extracts of *Stryphnodendron adstringens,* and sample sizes (n).

Locus	Alleles	Plant tissue	
		leaves	seeds
got-1	1	–	0.933
	2	–	0.067
	n	–	171
idh-1	1	0.359	0.437
	2	0.576	0.524
	3	0.065	0.056
	n	46	156
mdh-3	1	0.944	0.930
	2	0.056	0.070
	n	62	184
pgi-1	1	0.920	0.894
	2	0.080	0.106
	n	62	184
per-1	1	0.395	–
	2	0.605	–
	n	62	–
per-2	1	0.233	–
	2	0.767	–
	n	60	–

phosphate dehydrogenase (G6PDH). One active region was observed with a fixed pattern of triplex bands in the ADH progeny gels; the bands varied in their color intensities, and the more slowly migrating bands were generally more intensely colored. One active region with a fixed pattern of five consecutive bands was observed in GDH progeny gels, with the third and fifth bands showing much deeper color intensities. The enzyme systems leucine aminopeptidase (LAP), phosphoglucomutase (PGM), shikimate dehydrogenase (SKDH), and acid phosphatase (ACP) were monomorphic.

Surprisingly, the tests of adjustment to the HWE proportions, and the Fisher's and the χ^2 tests were non-significant for all of the allozyme systems analyzed in the different types of tissues and among generations (tables 4, 5). It must be noted, however, that in the HWE tests (as in any other statistical test) the inability to reject the null-hypothesis does not necessarily indicate its validity. It is possible that genotype frequencies in populations in which there are no random matings are distributed in such a way that they mimic multinomial distributions (Li 1988). It is also possible that factors that cause deviations from HWE expectations lead genotype frequencies in opposite directions, with non-significant final results between the numbers of observed and expected genotypes (Workman 1969, Cavalli-Sforza & Bodmer 1971).

The ripe fruits of *S. adstringens* are intensively attacked by several types of insects, and few or even no seeds are left in each pod (Branco et al. 2009), and it is not known if there is a selective environmental factor in this predation or if it is directional. However, as the sampled fruits were harvested in their final maturation stages and the seeds used to estimate progeny allele frequencies had not been submitted to any putative selective pressure, this may have led to non-significant results. It could be postulated that these results would be different if only ripe seeds (that had been submitted to natural selective pressure) had been used. Another point that must be considered is that equality between allele frequencies of males and females was presumed in this study. Thus, the HWE observed between generations might not be real if the allele frequencies between male and female gametes did, in fact, differ. Outcrossing in populations with low effective sizes may serve as a mechanism for accumulating excess heterozygotes, with the allelic frequencies among males and females in a small population differing by chance alone (Balloux 2004, Souza et al. 2004) due to drift processes that result in more frequent crosses between individuals bearing different alleles.

The genetic diversity (H_E), effective number of alleles per locus (A_E), observed heterozygosity (H_O), and the fixation index (f) of the polymorphic loci measured are outlined in table 6. The locus *idh-1* showed the highest H_E values in leaves (0.535) and seeds (0.532). This result was expected due to higher number of alleles in *idh-1* than in the other polymorphic loci. The smallest values were observed with *mdh-2* ($H_E = 0.106$) and *got-1* ($H_E = 0.125$) in leaves and seeds respectively. The total expected genetic diversity considering all the loci was 0.325 in the leaves and 0.244 in seeds. The effective number of alleles per locus (A_E) for the population was 1.58 in the leaves and 1.42 in seeds. The A_E values observed in *S. adstringens* were higher, and the H_E values smaller, than the average values reported in allozyme studies as compiled by Hamrick et al. (1992) that evaluated woody plant species. Long-lived woody species have, on the average, higher effective number of alleles per locus ($A_E = 1.24$) and more genetic diversity ($H_E = 0.177$) than other life forms. The f values measured in *S. adstringens* were non-significant for all loci considering leaves ($f = 0.070$) and seeds ($f = 0.107$). Nevertheless, the probability

Table 4. Absolute genotype frequencies measured in leaves and seeds, Fisher's exact and χ^2 tests for adjustments of the genotype frequencies observed in *Stryphnodendron adstringens* to the proportions expected under a Hardy-Weinberg equilibrium (HWE). $P(N_{Aa}/n_A)$ = condicional probability; P(acum) = accumulated probability; * = simplified model that considers only two alleles (*1* most frequent allele, *2* allele representing the others).

Locus	Plant tissue	Genotype			$P(N_{Aa}/n_A)$	P(acum)	χ^2	$P(\chi^2)$
		11	*12*	*22*				
got-1	seeds	150	19	2	0.134	0.163ns	2.236	0.135ns
**idh-1*	leaves	16	21	9	0.208	0.762ns	0.196	0.658ns
**idh-1*	seeds	59	72	25	0.121	0.737ns	0.149	0.699ns
mdh-3	leaves	55	7	0	0.836	1.0 ns	0.222	0.638ns
mdh-3	seeds	160	22	2	0.170	0.218ns	1.474	0.225ns
pgi-1	leaves	53	8	1	0.286	0.325ns	1.045	0.307ns
pgi-1	seeds	147	35	2	0.301	1.0 ns	0.003	0.959ns
per-1	leaves	12	25	25	0.091	0.286ns	1.518	0.218ns
per-2	leaves	3	22	35	0.284	1.0 ns	0.037	0.847ns

Levels of significance: ns = not significant; α = 5%.

Table 5. χ^2 test for adjusting the genotype frequencies observed in *Stryphnodendron adstringens* to the Hardy-Weinberg equilibrium (HWE) proportions among the generations.

Locus	χ^2	P
pgi-1	3.2011a	0.2018ns
mdh-3	3.6908a	0.1580ns
idh-1	3.2565b	0.6605ns

Degrees of freedom: a = 2; b = 5. Levels of significance: ns = not significant; α = 5%.

of the non-significance of the fixation index of seeds ($P = 0.0918$) was comparatively smaller than that of leaves ($P = 0.8653$). The total fixation index observed for leaves and seeds was considerably smaller than that observed using microsatellite data ($f = 0.3529$) (Branco et al. 2009). These differences were due the higher numbers of alleles per locus of microsatellite markers, which increased the numbers of expected heterozygous loci and consequentially increased the positive values of the genetic parameter *f*.

Table 6. Genetic diversity (H_E), effective number of alleles (A_E), observed heterozygosity (H_O), and the fixation index (f) measured in the leaves and seeds of *Stryphnodendron adstringens*. ( ) χ^2 test probability.

Locus	H_E	A_E	H_E	A_E	H_O		f	
	leaves		seeds		leaves	seeds	leaves	seeds
mdh-3	0.106	1.12	0.131	1.15	0.112	0.119	-0.060ns (0.6339)	0.089ns (0.2274)
idh-1	0.535	2.15	0.532	2.14	0.5	0.451	0.066ns (0.9402)	0.152ns (0.0655)
pgi-1	0.148	1.17	0.189	1.23	0.129	0.190	0.129ns (0.3097)	-0.004ns (0.8563)
per-1	0.478	1.91	–	–	0.403	–	0.156ns (0.2191)	–
per-2	0.357	1.55	–	–	0.366	–	-0.025ns (0.8464)	–
got-1	–	–	0.125	1.14	–	0.111	–	0.114ns (0.1360)
Average	0.325	1.58	0.244	1.42	0.302	0.218	0.070ns (0.8653)	0.107ns (0.0918)

χ^2 = test; ns = not significant; α = 5%.

The higher values of genetic diversity and effective number of alleles per locus, the non-significant fixation index, and the adjustments of the HWE proportions between generations observed for the loci *pgi-1*, *mdh-2* and *idh-1* all indicate random mating in this population of *S. adstringens*. The allozyme systems EST and PER were more clearly determined in leaf tissue, while the MDH, IDH, PGI, and GOT systems were more clearly determined in seed tissue.

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