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Biochemical Genetic Variation in Four Wild Populations of *Aspalathus linearis* (Rooibos Tea)

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Key Word Index—*Aspalathus linearis*; Fabaceae; Rooibos tea; genetic diversity; electrophoresis; genetic distance.

Abstract—Four morphologically different populations of *Aspalathus linearis* were examined by horizontal starch gel electrophoresis to assess levels and patterns of genetic variation and to estimate the amount of genetic differentiation within and between populations. Leaf extracts were surveyed for 13 enzymes, and gene products revealed genetic variation at six (40%) of 15 protein coding loci. The percentages of polymorphic loci range from 6.67 to 26.67% (0.95 criterion), values of 1.07–1.40 were obtained for the mean number of alleles per locus, and average heterozygosities per locus were calculated at 0.016–0.096. The mean genotypic distance index (Nei, 1978) between populations was 0.034. Enzyme electrophoresis appears to be a suitable method for studying genetic variation in *A. linearis*.

Introduction

Aspalathus linearis (Burm. f.) Dahlg. is a leguminous shrub indigenous to the western Cape, South Africa, and is one of the relatively few economically important plants that has made the transition from a local wild resource to an important cultivated crop in the 20th Century. The extraordinary variation in this species was described by Dahlgren (1968), who gave a detailed account of the morphology and geographical distribution of various wild forms and different tea types (e.g. red, black, grey and red-brown types). The uncertainty about the natural affinities within the species is clearly reflected in the taxonomic changes that were made in a later revision (Dahlgren, 1988) and also in attempts by Van der Westhuizen and Taylor (1971, unpublished notes) to describe various wild forms of the red type in the Clanwilliam area. Despite the detailed work of Dahlgren (1968, 1988), the identity and affinities of the various forms are poorly understood and the biodiversity within this polymorphic species remains largely unexplored.

Of all the wild forms, it is only one of the red tea types (the so-called "Rocklands" type) that has been selected for commercial cultivation, but further selection may be possible and indeed desirable to improve production characteristics, such as uniformity, yield and disease tolerance. The genetic integrity of various wild types may be at risk of introgression with the cultivated type, which is now planted in areas where it did not occur naturally. This paper is a first attempt at documenting genetic diversity in *A. linearis*, and is aimed at evaluating the use of horizontal starch gel electrophoresis in biosystematic studies of this economically important species.

The study of enzyme polymorphism proved to be a practical way for examining genetic variation in natural and artificial populations (Cheliak and Pitel, 1984; Bergmann *et al.*, 1990) and for the identification of cultivars in several crops (Torres, 1983; Weeden, 1989). The main advantage is that isozymes are unlinked characters, often hardly or not environmentally influenced. Furthermore, since many isozymes show co-dominance, identification of heterozygotes is possible (Loos, 1993). We

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examined four geographically isolated populations of *A. linearis*, chosen to represent the extreme forms within the range of variation. Because isozymes were previously used to characterise population genetics of many plant species (e.g. Hamrick *et al.*, 1979; Loveless and Hamrick, 1984), ample contrasts were available to interpret and explain population genetic parameters obtained for *A. linearis*.

Materials and Methods

Leaf material of 106 individual plants were investigated from four morphological and geographically distinct populations (Table 1). All four of these populations comprised relatively few individuals, and the total populations were sampled at Pakhuis and Franschhoek Pass.

Collection, tissue preparation, extraction buffers, electrophoresis, staining of gels, interpretation of results, locus nomenclature and statistical analysis follow Van der Bank *et al.* (1995). The following buffer systems were used: **CB**—a discontinuous buffer (electrode pH = 8.1; gel pH = 8.4) system (Cooke and Buckley, 1987); **CT**—a continuous buffer (pH = 6.1) system (Clayton and Tretiak, 1972); **HC**—a continuous buffer (pH = 5.7; 6.5) system (Stuber *et al.*, 1977); **MF**—a continuous buffer (pH = 8.6) system (Markert and Faulhaber, 1965); **PO**—a discontinuous buffer (electrode pH = 8.2; gel pH = 8.7) system (Poulik, 1957); and **TC**—a continuous Tris–citric acid buffer (pH = 6.9) system (Whitt, 1970).

The method that was used to determine relationships between the populations consisted of (a) interpreting electromorphs on gels in terms of Mendelian genetics, (b) computing allelic frequencies at various loci, and (c) converting allelic frequencies into a measure of genetic distance (*D*) among populations, using Rogers' (1972) and Nei's (1978) methods.

TABLE 1. LOCALITIES (from north to south) AND DESCRIPTIONS OF *ASPALATHUS LINEARIS* POPULATIONS STUDIED

Population	N	Locality	Description
Gifberg Pass	49	(31°49′S; 18°40′E)	Prostrate; up to 0.6 m wide and 0.2 m high; bright green leaves; resprouter.
Pakhuis Pass	18	(32°09′S; 18°59′E)	Erect; up to 1.5 m tall; bright green leaves; reseeder.
Elandskloof Pass	25	(32°40′S; 19°09′E)	Erect; up to 1.5 m tall; leaves somewhat glaucous; resprouter.
Franschhoek Pass	17	(33°54′S; 19°09′E)	Procumbent; 0.14 m wide and 0.14 m high; bright green leaves; resprouter.

Results

Genetic interpretation of isozyme phenotypes were based on the quaternary structure of each enzyme and expression in other plant species. Fifteen enzyme coding loci provided interpretable results in all of the *A. linearis* populations analysed, and these data could be used for comparative studies and to calculate the extent of differentiation between populations. Additional loci were observed for *ACP*, *CK*, *DDH*, *EST* and MNR; they were either not resolved or could not be scored consistently in all individuals for all populations. No activity was found when gels were stained for the following proteins: alcohol dehydrogenase (E.C. 1.1.1.1), adenylate kinase (E.C. 2.7.4.3), L-lactate-dehydrogenase (E.C. 1.1.1.27), general protein, peptidase (E.C. 3.4.—.—) using glycyl-L-leucine, leucylglycylglycine, leucine–alanyl and L-phenylalanyl-L-proline as substrates, 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44) and shikimate dehydrogenase (E.C. 1.1.1.25).

Nine of the 15 loci (60%) displayed monomorphic gel banding patterns (Table 2) and genotypic frequencies deviated from Hardy–Weinberg proportions at the following loci (Table 3): *CAP-2* (Franschhoek Pass, Elandskloof Pass); *LAP* and *PEP-S* (Gifberg Pass, Franschhoek Pass, Elandskloof Pass); *PER-1* (Gifberg Pass) and *PER-2* (Pakhuis Pass, Elandskloof Pass). Allele frequencies for all four populations at the *MDH* locus complied with Hardy–Weinberg proportions whereas relatively large deficiencies of heterozygotes resulted in deviations of allele frequencies from expected Hardy–Weinberg proportions at the *CAP-2*, *LAP*, *PEP-S*, *PER-1* and *-2* enzyme coding loci (Table 3). The Pakhuis Pass population was monomorphic at all of the loci studied, except for *PER-2*, where there was a deficiency of heterozygotes. The

TABLE 2. ENZYMES, LOCUS ABBREVIATIONS, ENZYME COMMISSION NUMBERS (E.C. No.) AND BUFFERS USED IN THE STUDY OF *ASPALATHUS LINEARIS*. See Materials and Methods for abbreviations of buffers used

Enzyme	Locus	E.C. No.	Buffer (pH)
Aspartate aminotransferase	(AAT)	2.6.1.1	MF (8.6)
Acid phosphatase	(ACP)	3.1.3.2	CT (6.1)
Cytosol aminopeptidase	(CAP-1)	3.4.11.1	CB (8.4)
	*(CAP-2)		CB (8.4)
Glucose-6-phosphate isomerase	(GPI)	3.5.1.9	PO (8.7)
Guanine deaminase	(GDA)	3.5.4.3	HC (5.7)
Isocitrate dehydrogenase	(IDH)	1.1.1.42	TC (6.9)
Leucine aminopeptidase	*(LAP)	3.4.11.1	MF (8.6)
Malate dehydrogenase	*(MDH)	1.1.1.37	HC (6.5)
Malic enzyme	(ME)	1.1.1.38	HC (5.7)
Peptidase (substrate: leucyl–tyrosine)	*(PEP-S)	3.4.—.—	MF (8.6)
Peroxidase	*(PER-1, -2)	1.11.1.7	CB (8.4), PO (8.7)
Phosphoglucomutase	(PGM)	5.4.2.2	HC (6.5)
Superoxide dismutase	(SOD)	1.15.1.1	TC (6.9)

* = Polymorphic loci.

TABLE 3. RELATIVE ALLELE FREQUENCIES, χ^2 VALUES, COEFFICIENTS OF HETEROZYGOSITY DEFICIENT OR EXCESS (*d*) AND INDIVIDUAL HETEROZYGOSITIES (*h*) FOR POLYMORPHIC LOCI

		Population			
Locus	Allele	Gifberg Pass	Pakhuis Pass	Elandskloof Pass	Franschhoek Pass
CAP-2	f*A*	1.000	1.000	0.905	0.941
	f*B*			0.095	0.059
χ^2				4.203	17.000
d				−0.447	−1.000
h				0.172	0.111
LAP	f*A*	0.541		0.470	0.470
	f*B*	0.275	1.000	0.412	0.412
	f*C*	0.184		0.118	0.118
χ^2		21.644		20.665	20.665
d		−0.351		−0.605	−0.605
h		0.598		0.595	0.595
PEP-S	f*A*	0.949	1.000	0.780	0.882
	f*B*	0.051		0.220	0.118
χ^2		6.626		10.574	17.000
d		−0.368		−0.650	−1.000
h		0.097		0.343	0.208
MDH	f*A*	0.980	1.000	0.980	0.971
	f*B*	0.020		0.020	0.029
χ^2		0.021		0.010	0.016
d		0.021		0.020	0.030
h		0.040		0.039	0.057
PER-1	f*A*	0.643	1.000	1.000	1.000
	f*B*	0.357			
χ^2		5.994			
d		−0.378			
h		0.459			
PER-2	f*A*	0.125	0.861	0.840	0.618
	f*B*	0.875	0.139	0.160	0.382
χ^2		2.939	10.610	12.333	2.420
d		−0.429	−0.768	−0.702	−0.377
h		0.219	0.239	0.269	0.472

individual heterozygosity (*h*) values obtained for each polymorphic locus (Table 3) ranged from 0.039 to 0.595. The Gifberg Pass population possesses unique alleles at the *PER-1* locus whereas the Franschhoek and Elandskloof Pass populations had unique alleles at the *CAP-2* locus. At the *PER-2* locus, the Gifberg Pass population had the greatest frequency of *B*-alleles whereas the alternative allele (*A*-allele) was predominant in the other three populations. Despite these differences, the mean number of alleles per locus (*A*) was the same for the Gifberg, Franschhoek and Elandskloof Pass populations (1.4%) but less (1.1%) for the Pakhuis Pass population (Table 4). This phenomenon also prevailed in that the percentage of polymorphic loci (*P*) and average heterozygosity per locus (*H*) values were nearly identical for the former three populations.

The genetic distance values ranged from 0.002 to 0.079 (Nei, 1978) and from 0.028 to 0.121 (Rogers, 1972) between populations with a mean genotypic distance index of 0.034 (Nei, 1978). Similar *D* values between populations were obtained with both methods (e.g. with the largest *D* value between the Pakhuis and Gifberg Pass populations, smallest *D* value between the Franschhoek and Elandskloof Pass populations, etc.).

TABLE 4. MEAN NUMBER OF ALLELES PER LOCUS (*A*), PERCENTAGE OF POLYMORPHIC LOCI (*P*) USING THE 0.95 CRITERION, AVERAGE HETEROZYGOSITY PER LOCUS (*H*), ROGERS' (1972) GENETIC DISTANCE BELOW DIAGONAL AND NEI'S (1978) UNBIASED GENETIC DISTANCE VALUES ABOVE DIAGONAL

Population	Gifberg Pass	Pakhuis Pass	Elandskloof Pass	Franschhoek Pass
A	1.40	1.07	1.40	1.40
	(±0.16)	(±0.07)	(±0.16)	(±0.16)
P	26.67	6.67	26.67	26.67
H	0.094	0.016	0.095	0.096
	(±0.049)	(±0.016)	(±0.046)	(±0.049)
Gifberg Pass	—	0.079	0.050	0.027
Pakhuis Pass	0.121	—	0.023	0.024
Elandskloof Pass	0.097	0.060	—	0.002
Franschhoek Pass	0.073	0.066	0.025	—

Discussion

Deviations of allele frequencies from expected Hardy–Weinberg proportions occurred at five of the six polymorphic loci (Table 3) due to deficiencies of heterozygotes at these loci. Heterozygote deficiencies may be due to non-random mating, selection, gene flow, mutations and genetic drift (Soltis and Soltis, 1988). However, since heterozygote deficiencies were not observed at all the loci, non-random mating, gene flow and drift were probably not factors, since these processes should affect all loci equally. Thus the heterozygote deficiency at the Gifberg, Franschhoek and Elandskloof Pass populations may be the result of natural selection and mutations or a combination thereof. Because only one polymorphic locus (*PER-2*) was observed in the Pakhuis Pass population, it is unknown whether the observed deficiency of heterozygotes in this population is due to any one of the factors listed above. Little genetic variation exists in the Pakhuis Pass population, where individuals had an *A* value of 1.07, whereas it was 1.40 for the other three populations (Table 4). The alleles for individuals from the former population were fixed except at the *PER-2* locus. Hamrick and Godt (1990) report a value for *A* of 1.31 and a *P* value of 20% for inbreeding species. The value of *P* obtained for the Pakhuis Pass population was only 6.67% and for the other three populations 26.67%. The *H* values for the Pakhuis Pass

population was 1.6% and it varies between 9.4 and 9.6% for the other populations. Previous studies of other flowering plants (dicotyledons) reported *P* values of 31.1%, and an *H* value of 0.059 (Nevo *et al.*, 1984). Respective values obtained in the present study for Gifberg, Elandskloof and Franschhoek Pass, therefore, compare favourably with these values, but it is less ($P=6.67$; $H=0.016$) for the Pakhuis Pass population. Several factors may account for the lower allozyme variation obtained at this population (e.g. genetic bottlenecks associated with dispersal and subsequent establishment, or the gradual isolation of larger populations into smaller ones as a result of climatic changes or increasing fire frequencies). These ideas will be explored in a wider survey (Van der Bank *et al.*, in prep.).

The distribution of total gene diversity within and among populations of *A. linearis* differs for the four populations studied. All six polymorphic loci were not present in every population, thus the value of *h* at several loci in some populations is zero, and where polymorphism occurred, *h* values ranged from 0.039 to 0.595 (Table 3). These values compare favourably with results for four populations of *Virgilia oroboides* (0.039–0.652) reported by Van der Bank *et al.* (1995). Short-lived herbaceous species with a predominantly sexual, animal-outcrossing mode of reproduction and gravity-dispersed seeds have a mean *H* value of 0.127, mean *P* value of 33% and an *A* value of 1.16 at the population level (Godt and Hamrick, 1991). Thus, the values obtained for these parameters in this study are less, with the Pakhuis Pass population having the lowest values. Long-lived species tend to maintain higher levels of genetic variation within their populations (Hamrick, 1979) presumably because of the large number of generations present in any given population (Levin, 1978) and endemic species also tend to have less variation than widely distributed species. This could explain the relatively high *H* values (9.4–9.6%) for the Gifberg, Elandskloof and Franschhoek Pass populations (which are resprouters, and comprises various generations), compared to that of the Pakhuis Pass population (1.6%) which is a reseeder.

The Gifberg Pass population possesses unique alleles at the *PER-1* locus whereas the Franschhoek and Elandskloof Pass populations (which are both from the southern part of the distribution area) had unique alleles at the *CAP-2* locus. There are also population differences in the distribution within and among populations for polymorphic loci. At the *PER-2* locus, the Gifberg Pass population had the greatest frequency of *B*-alleles whereas the alternative allele was predominant in the other three populations. The pattern of diversity seems to suggest abrupt genetic changes, perhaps as a result of strong selection or genetic bottlenecks. The largest *D* value was obtained between the Pakhuis and Gifberg Pass populations and the smallest *D* value between the Franschhoek and Elandskloof Pass populations (Table 4). No obvious agreement was found between this result and various other population parameters (Table 1), which suggest that the populations diverged independently. This is not a surprising result since the populations were specifically chosen to represent the extremes in the range of morphological and geographical variation within the species.

In conclusion, this is the first account of electrophoretic variants in *A. linearis*, and we have established the feasibility of the method for studying genetic variation in the species. A wider survey is now needed to test various hypotheses regarding the effects of selection and fire-survival strategy on genetic diversity. Answers to these questions are of theoretical interest in understanding more about the evolution of Cape legumes in fire-prone environments, and could also be useful in gaining insight into natural affinities within the *A. linearis* complex. At the same time, these wider studies may be of considerable practical value in terms of the selecting and breeding of desirable biotypes for commercial cultivation.

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