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Speciation in *Virgilia* (*Fabaceae*): allopatric divergence followed by introgression?

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Abstract: Levels of variation revealed by starch gel-electrophoresis were compared with morphological and chemical variation within and among the species and subspecies of *Virgilia*: *V. oroboides* subsp. *oroboides, V. oroboides* subsp. *ferruginea* and *V. divaricata*. The data sets exhibited concordance in that all point to a very close relationship between the taxa. Differences are mostly quantitative and an overlapping east-west gradient of character variation is indicated. Analysis of morphological and chemical characters showed that *V. oroboides* subsp. *ferruginea* and *V. divaricata* are relatively distinct, whereas allozyme analysis indicated a high degree of genetic similarity among populations of these two taxa. The observed pattern of variation suggests relatively recent speciation with subsequent introgressive hybridisation resulting in a geographical and ecological gradient.

Virgilia is a taxonomically isolated genus within the *Fabaceae* subfam. *Papilionoideae* (tribe *Podalyrieae*) and is endemic to the south-western and southern coastal regions of South Africa. The genus had been treated for a considerable time as monotypic until a second species was described in 1934 (ADAMSON 1934). At present, three taxa are recognised, namely *V. divaricata* ADAMSON, *V. oroboides* (BERG.) SALTER subsp. *oroboides*, and *V. oroboides* (BERG.) SALTER subsp. *ferruginea* B.-E. VAN WYK. The taxonomic revision by VAN WYK (1986) was based on an analysis of the considerable morphological variation that exists within the genus. Each taxon comprises a number of geographically isolated populations. *Virgilia o.* subsp. *oroboides* is limited to the south-western Cape coastal region, from the Cape Peninsula to Swellendam *V. divaricata* occurs in the southern and eastern Cape, from Knysna to Port Elizabeth and, *V. o.* subsp. *ferruginea* is geographically intermediate between the other two taxa and occurs from Mossel Bay to George.

Limited morphological variation occurs within populations but distinct differences between populations are common. The flowers and bark of *V. o.* subsp. *ferruginea* are similar to those of *V. divaricata*, but the general morphology is within the variation range found in *V. o.* subsp. *oroboides* (VAN WYK 1986). Alkaloid data (GREINWALD & al. 1989, VEEN & al. 1991) show no qualitative differences between

species and subspecies but the three taxa can be differentiated by quantitative differences in some alkaloids. VAN WYK (1986) postulated that *V. o.* subsp. *ferruginea* may have originated as a hybrid between the other two species. However, the alkaloid patterns give no definite evidence for such a postulation, but confirms the affinity of subsp. *ferruginea* with *oroboides* rather than *V. divaricata* (VEEN & al. 1991).

Taxonomic schemes that are exclusively based on morphological data do not always reflect the true evolutionary relationships in taxa where relatively little morphological divergence has taken place, or in cases where taxa correspond as a result of convergent evolution. The motivation for this study was to determine if closely related species, such as *V. o.* subsp. *oroboides* and *V. divaricata*, which would generally be accepted as "good species" on morphological grounds, are indeed genetically isolated from one another. This would give a better idea of the evolution of Cape fynbos legumes within a species-rich area. The aims of this study were to explore the following ideas: 1) that the current taxonomy of *Virgilia* can be verified by an electrophoretic study (or that the taxa should be regarded as distinct species); 2) that speciation in *Virgilia* may reflect recent divergence along a geographical (and ecological) gradient, so that all three forms should best be considered as allopatric subspecies; and 3) that *V. o.* subsp. *ferruginea* is of hybrid origin and that introgression took place over a long period of time. We present an analysis of allozyme variation patterns among representatives of the *Virgilia* complex. These patterns are interpreted with reference to morphology, chemistry and geography.

Material and methods

Leaf material of 250 individual plants were investigated from nine populations of *Virgilia*. The species and subspecies investigated in this study and their sources are denoted in Fig. 1 and Table 1.

Collection, tissue preparation, extraction buffers, electrophoresis, staining of gels, interpretation of results, locus nomenclature and statistical analysis follow VAN DER BANK & al. (1995a). The following buffer systems were used: CT – a continuous buffer (pH = 6.1) system (CLAYTON & TRETIAK 1972); MF – a continuous buffer (pH = 8.6) system (MARKERT & FAULHABER 1965); PO – a discontinuous buffer (electrode pH = 8.2; gel pH = 8.7) system (POULIK 1957); RW – a discontinuous buffer (electrode pH = 8.0; gel pH = 8.7) system (RIDGWAY & al. 1970) and TC – a continuous tris, citric acid buffer (pH = 6.9) system (WHITT 1970).

The methods used to determine relationships between the species and subspecies consisted of a) interpreting electromorphs on gels in terms of Mendelian genetics, b) computing allelic frequencies at various loci, c) converting allelic frequencies into a measure of genetic distance (*D*) and similarity (*I*) coefficients among species and populations. NEI's (1972, 1978) and NEI & al.'s (1983) genetic distances were used to produce phenograms using DISPAN (Copyright 1993 by TATSUYS OTA and the Pennsylvania State University, USA).

NEI (1973) showed that the total gene diversity (heterozygosity of alleles pooled over all samples) could be partitioned into within- and between-populations. These relative contributions of diversity (to the total gene diversity) offer an indication of the extent of genetic differentiation that may be present among samples. The method of CHAKRABORTY & al. (1982) was utilised in this investigation to analyse the gene diversity within and

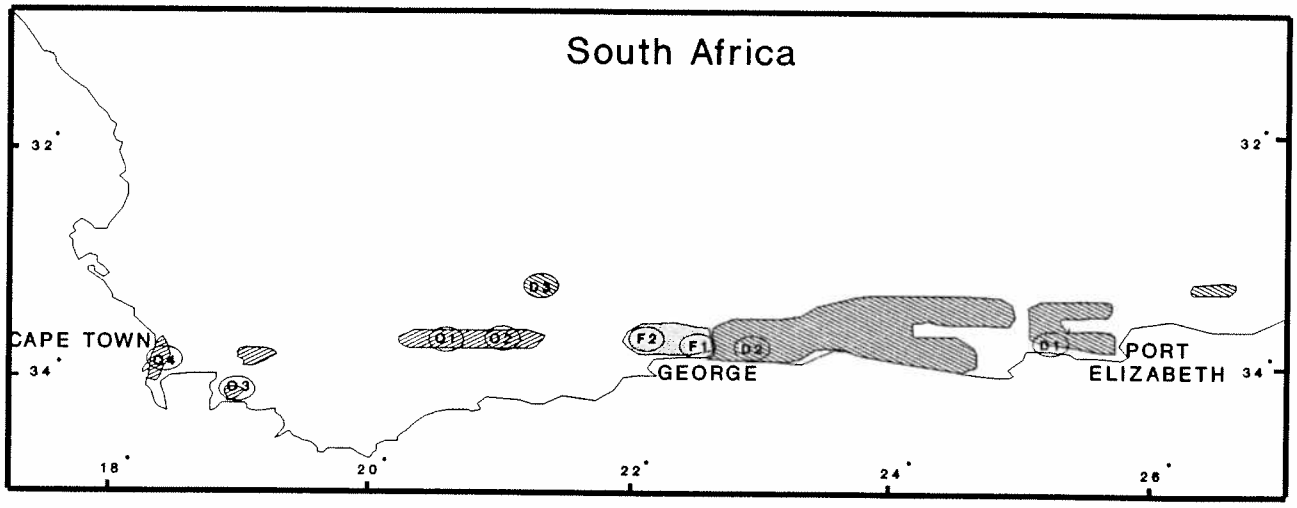


Fig. 1. The geographical distribution of the genus *Virgilia*. Localities sampled are: *V. oroboides* subsp. *oroboides*: (O1) Tradouws Pass; (O2) Glenstream (Swellendam); (O3) Betty's Bay; (O4) Window Stream (Kirstenbosch); *V. oroboides* subsp. *ferruginea*: (F1) Victoria Bay; (F2) Ruitersbos; *V. divaricata*: (D1) Van Stadens River; (D2) The Craggs (Knysna) and (D3) Seweweekspoort

Table 1. Source, location and number of individuals sampled per population of *Virgilia* studied

Species	Abbreviation	Source	No.
Virgilia divaricata	D1	Van Stadens River 33 25 CC	25
V. divaricata	D2	The Craggs, Knysna 34 23 AC	25
V. divaricata	D3	Seweweekspoort 33 21 AD	25
V. oroboides subsp. *ferruginea*	F1	Victoria Bay 34 22 BA	25
V. oroboides subsp. *ferruginea*	F2	Ruitersbos 33 22 CC	25
V. oroboides subsp. *oroboides*	O1	Tradouws Pass 33 20 DC	50
V. oroboides subsp. *oroboides*	O2	Glenstream, Swellendam 33 20 DD	25
V. oroboides subsp. *oroboides*	O3	Betty's Bay 34 18 BD	25
V. oroboides subsp. *oroboides*	O4	Window Stream, Kirstenbosch 33 18 CD	25

between *V. divaricata*, *V. o.* subsp. *oroboides* and *V. o.* subsp. *ferruginea* from allele frequency data. We used the method employed by Nason & al. (1992) to indicate population- and taxon-specific unique alleles.

Results

Nineteen protein coding loci provided interpretable results in all the *Virgilia* populations analysed. Genetic variation within species and subspecies was observed at nine (47%) of the protein coding loci studied (Tables 2, 3). Population- or taxon-specific unique alleles are indicated in Table 3, and the intermediate position of these alleles at some loci in *V. o.* subsp. *ferruginea* is depicted in Fig. 2. *Virgilia divaricata* had the highest frequencies of unique alleles at the *Aat* and *Acp* loci (Fig. 2 a, b), whereas *V. o.* subsp. *oroboides* had high frequencies at *Pep-B* and *Per-1* (Fig. 2 c, d). The taxa differed in numbers and frequencies of alleles (Table 3). The maximum number of alleles for any given taxon was three, and the average heterozygosity (*H*) values ranged between 0.122 and 0.159 (Table 4). The percentage of polymorphic loci (*P*) was the same for all the populations (31.58), except for the F2 population (Table 4) where it was 36.84%. Similarly, the mean number of alleles per locus ($A = 1.47–1.63$) and *H* values (12.2–15.9%) were nearly identical for the nine populations analysed (Table 4).

Genotypic frequencies deviated from expected Hardy-Weinberg proportions at *Aat* (D2; D3; F1; F2; O1; O4), *Cap-1* (D1; D2; O1–4), *Pep-B* (O1; O3), *Per-1* (D1; D2; F2; O4), and *Per-3* (O2) (Table 3). Allele frequencies for all taxa and populations at the *Acp, Gpi,* and *Per-2* loci complied to Hardy-Weinberg proportions and relatively large deficiencies of heterozygotes ($d = -0.007$ to -0.687) occurred at the *Aat, Cap-1, Pep-B, Pep-S, Per-1* and *3* protein coding loci (Table 3). Individual heterozygosity (*h*) values obtained for each polymorphic locus (Table 3) range from 0.039 to 0.663. The B-allele was not found in *V. o.* subsp. *oroboides* samples at the *Per-3* protein coding locus.

Table 2. Enzymes, locus abbreviations, (* polymorphic loci), enzyme commission numbers (E. C. No.) and buffers used in the study of *Virgilia*. See material 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	MF (8.6)
	Cap-2		CT (6.1)
Guanine deaminase	*Gda*	3.5.4.3	CT (6.1)
Glucose-6-phosphate isomerase	* *Gpi*	3.5.1.9	PO (8.7)
General protein	*Prot*		MF (8.6)
Isocitrate dehydrogenase	*Idh*	1.1.1.42	TC (6.9)
Malic enzyme	*Me*	1.1.1.38	RW (8.7)
Menadione reductase	*Mnr*	1.6.99.–	MF (8.6)
Peptidase, substrate:			
L-leucylglycylglycine	* *Pep-B*	3.4.–.–	MF (8.6)
L-phenylalanyl-L-proline	*Pep-D*		
leucyl-tyrosine	* *Pep-S*		
Peroxidase	* *Per-1, -2, -3*	1.11.1.7	PO (8.7), MF (8.6)
Phosphogluconate dehydrogenase	*Pgd*	1.1.1.44	PO (8.7)
Superoxide dismutase	*Sod-1, -2*	1.15.1.1	MF (8.6)

Table 3. Relative allele frequencies, x^2 values, coefficients of heterozygosity deficit or excess (d) and individual heterozygosities (h) at polymorphic loci. * Unique alleles for some populations

Locus	Allele	D1	D2	D3	F1	F2	O1	O2	O3	O4
Aat	A*	0.389	0.355	0.435	0.217	0.224	0.195	0.063		0.175
	B	0.417	0.597	0.435	0.543	0.672	0.659	0.874	0.857	0.625
	C	0.194	0.048	0.130	0.240	0.104	0.146	0.063	0.143	0.200
x^2		0.859	23.139	12.446	19.552	15.901	35.328	0.490	1.037	15.992
d		–0.128	–0.687	–0.425	–0.638	–0.504	–0.615	0.103	–0.222	–0.536
h		0.637	0.516	0.605	0.600	0.487	0.507	0.227	0.245	0.539
Acp	A	1.000	0.827	1.000	0.900	0.980	1.000	1.000	1.000	1.000
	B*		0.173		0.100	0.020				
x^2			0.092		0.123	0.010				
d			–0.059		–0.111	0.020				
h			0.286		0.180	0.039				
Cap-1	A	0.300	0.194	0.360	0.125	0.125	0.448	0.395	0.250	0.217
	B	0.420	0.612	0.480	0.625	0.589	0.188	0.368	0.589	0.543
	C	0.280	0.194	0.160	0.250	0.286	0.364	0.237	0.161	0.239
x^2		20.932	9.894	0.434	3.840	3.510	12.262	6.736	15.515	8.461
d		–0.023	–0.178	–0.023	–0.176	0.093	–0.307	–0.193	–0.114	–0.276
h		0.655	0.549	0.614	0.531	0.555	0.631	0.652	0.564	0.600
Gpi	A	0.933	0.963	0.933	0.700	0.960	1.000	0.980	0.980	1.000
	B*	0.067	0.037	0.067	0.300	0.040		0.020	0.020	
x^2		0.077	0.040	0.077	0.639	0.043		0.010	0.010	
d		0.071	0.038	0.071	0.206	0.042		0.020	0.020	
h		0.124	0.071	0.124	0.420	0.077		0.039	0.039	
Pep-B	A	1.000	1.000	1.000	1.000	0.942	0.490	0.818	0.880	0.826
	B*					0.058	0.510	0.182	0.120	0.174
x^2						0.097	5.111	0.543	9.648	3.584
d						0.061	–0.320	0.222	–0.621	–0.395
h						0.109	0.500	0.298	0.211	0.287
Pep-S	A	0.731	0.879	0.900	0.967	0.860	0.436	0.750	0.643	0.844
	B	0.269	0.121	0.100	0.033	0.140	0.564	0.250	0.357	0.156
x^2		0.007	7.620	0.185	0.018	6.292	0.312	0.032	0.093	1.335
d		–0.023	–0.513	0.111	–0.034	–0.502	0.081	–0.048	–0.067	–0.289
h		0.393	0.212	0.180	0.064	0.241	0.492	0.375	0.459	0.264
Per-1	A	0.538	0.852	0.538	0.675	0.778	0.551	0.761	0.827	0.604
	B*	0.154	0.074		0.200				0.038	0.292
	C	0.308	0.074	0.462	0.125	0.222	0.449	0.239	0.135	0.104
x^2		7.810	27.204	0.066	2.239	8.816	0.420	3.728	1.139	17.362
d		–0.350	–0.438	–0.071	0.182	–0.571	–0.093	–0.403	0.167	–0.614
h		0.592	0.263	0.497	0.489	0.346	0.495	0.364	0.297	0.539
Per-2	A	1.000	0.979	1.000	1.000	0.852	0.980	0.960	0.980	1.000
	B*		0.021			0.148	0.020	0.040	0.020	
x^2			0.011			0.817	0.010	0.043	0.010	
d			0.021			0.174	0.020	0.042	0.020	
h			0.041			0.252	0.039	0.077	0.039	
Per-3	A	0.275	0.333	0.325	0.474	0.308	0.222	0.365	0.380	0.286
	B*	0.225	0.093	0.100	0.052	0.384				
	C	0.500	0.574	0.575	0.474	0.308	0.778	0.635	0.620	0.714
x^2		1.957	1.301	0.675	0.117	0.544	0.551	8.908	1.392	1.890
d		–0.198	–0.126	–0.007	–0.056	–0.013	–0.143	–0.585	–0.236	–0.300
h		0.624	0.551	0.554	0.548	0.663	0.346	0.464	0.471	0.408

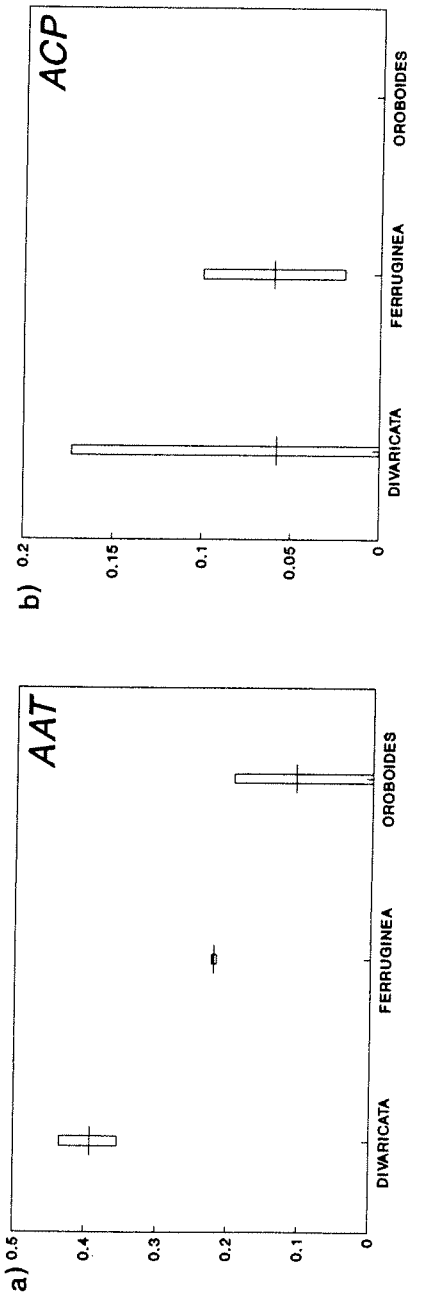


Fig. 2. Frequencies of unique alleles at populations, showing the intermediate position of *Virgilia oroboides* subsp. *ferruginea*

Table 4. Mean number of alleles per locus (*A*), percentage of polymorphic loci (*P*) using the 0.95 criterion, average heterozygosity per locus (*H*), Nei's (1972) genetic distance values below diagonal and genetic identity (*I*) values above diagonal

Population	D1	D2	D3	F1	F2	O1	O2	O3	O4
A	1.53 (± 0.19)	1.63 (± 0.19)	1.47 (± 0.18)	1.58 (± 0.19)	1.63 (± 0.17)	1.47 (± 0.16)	1.53 (± 0.16)	1.53 (± 0.16)	1.47 (± 0.18)
P	31.58	31.58	31.58	31.58	36.84	31.58	31.58	31.58	31.58
H	0.159 (± 0.061)	0.131 (± 0.0.47)	0.136 (± 0.054)	0.149 (± 0.053)	0.146 (± 0.050)	0.158 (± 0.055)	0.135 (± 0.048)	0.122 (± 0.044)	0.139 (± 0.051)
D1	–	0.988	0.995	0.985	0.988	0.967	0.982	0.980	0.989
D2	0.012	–	0.988	0.990	0.990	0.951	0.985	0.987	0.990
D3	0.005	0.012	–	0.985	0.984	0.961	0.982	0.977	0.986
F1	0.015	0.010	0.015	–	0.985	0.940	0.977	0.979	0.987
F2	0.012	0.010	0.016	0.015	–	0.952	0.984	0.984	0.983
O1	0.034	0.050	0.040	0.062	0.050	–	0.980	0.972	0.970
O2	0.018	0.015	0.018	0.023	0.016	0.021	–	0.996	0.990
O3	0.020	0.013	0.023	0.021	0.016	0.029	0.004	–	0.990
O4	0.011	0.010	0.014	0.013	0.017	0.030	0.010	0.010	–

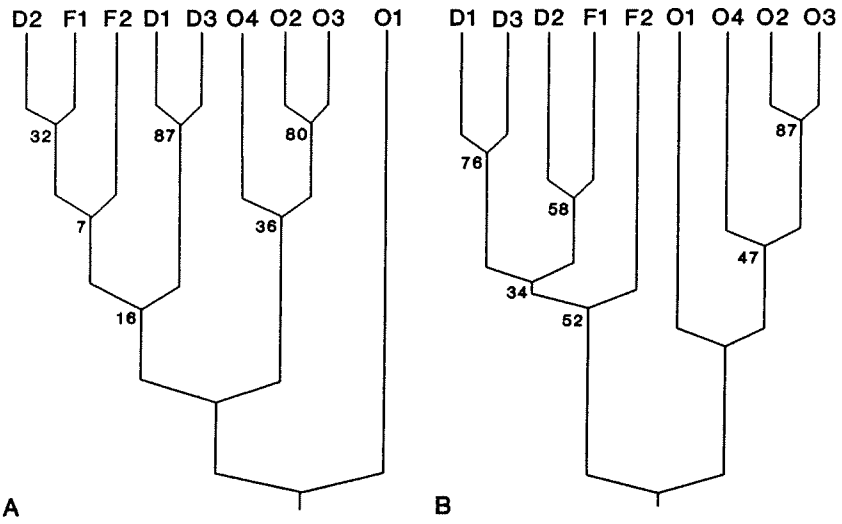


Fig. 3. Dendrograms showing bootstrap numbers and phylogenetic relationships between populations and taxa using: *A* Nei's (1978) and *B* Nei & al's (1983) genetic distance values. See Table 1 for abbreviations of populations

The *I* values (Table 4) ranged from 0.940 to 0.996 (Nei 1972) between populations. The smallest mean *I* value was obtained between *V. o.* subsp. *oroboides* and *V. o.* subsp. *ferruginea* (0.973) and the largest value was calculated between *V. divaricata* and *V. o.* subsp. *ferruginea* (0.987). The smallest *D* values were encountered between *V. o.* subsp. *ferruginea* and *V. divaricata* (0.013). The largest values were obtained between *V. o.* subsp. *oroboides* and *V. o.* subsp. *ferruginea* (0.027). The

relative gene diversities (excluding monomorphic loci) within populations were 90.5% (± 0.015) and 9.5% (± 0.016) among populations. Dendrograms constructed using NEI's (1972, 1978) and NEI & al.'s (1983) genetic distance values (Fig. 3 a and b, respectively) show the main groupings were: a) *V. divaricata* with *V. o.* subsp. *ferruginea*, and these taxa separated from b) *V. o.* subsp. *oroboides*.

Discussion

Genetic variation. Deviations of allele frequencies from expected Hardy-Weinberg proportions occurred at *Aat, Cap-1, Pep-B, Per-1* and *3* (Table 3) due to relatively large deficiencies of heterozygotes at these loci. Since heterozygote deficiencies were not observed at all the loci, nonrandom mating, gene flow and drift were probably not factors, since these processes should affect all loci equally and the observed deficiencies may be due to selection and/or mutations (SOLTIS & SOLTIS 1988). Since *Virgilia* consists of highly localised populations (found in areas of about 1–5 hectare), the WAHLUND (1928) effect can be excluded. The polymorphic loci were not always polymorphic for 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 0.663 (Table 3). These values compare favourably with results for four populations of *Aspalathus linearis* (BURM. f.) DAHLG.(0.039–0.595) reported by VAN DER BANK & al. (1995b).

Previous studies on 669 taxa reported $P = 35\%$, $A = 1.52$, and $H = 0.113$ (see GODT & HAMRICK 1991). The values obtained in the present study ($P = 31.58$–36.84%, $A = 1.58$–1.63, $H = 0.149$–0.146 for *V. o.* subsp. *ferruginea*; $P = 31.58\%$, $A = 1.47$–1.63, $H = 0.131$–0.159 for *V. divaricata* and $P = 31.58\%$, $A = 1.47$–1.53, $H = 0.122$–0.158 for *V. o.* subsp. *oroboides*) differ from these values but fall within the range of means reported by HAMRICK (1979) for vascular plants ($P = 22.0$–75.3%, $A = 1.35$–2.56, $H = 0.079$–0.354). At the *Per-3* locus, the *V. o.* subsp. *oroboides* populations lack the B-allele whereas the *V. divaricata* and F1 populations lack the B-allele at the *Pep-B* locus. This result suggests that *V. o.* subsp. *oroboides* is monophyletic, but that the *V. divaricata* and *V. o.* subsp. *ferruginea* populations are paraphyletic (but see discussion on introgression below).

Genetic differentiation. NEI's (1972) *I* value is widely used to express genetic similarity between populations or species of plants. The mean *I* values ranged from 0.940 to 0.996 (Table 4) and it was slightly higher ($I = 0.986$) within each taxon than between taxa ($I = 0.979$). CRAWFORD (1983) reported high genetic similarities between conspecific populations with *I* values of more than 0.900 in the majority of comparisons. He suggested that the very high genetic identity values for subspecies or varieties of angiosperms were probably the result of either or both of the following: (1) recent divergence with insufficient time for divergence at isozyme loci, and (2) possible hybridisation between the taxa to prevent divergence.

(1) Recent divergence. GOTTLIEB (1981) calculated the mean *I* value for 21 pairs of congeneric plant species at 0.67 (± 0.04), 0.975 among populations of 13 self-fertilising species, and 0.956 for 14 outcrossers. This supports the view that populations of different species (congeneric populations) are considerably more genetically differentiated than conspecific populations (GOTTLIEB 1981). In addition, LINHART & PREMOLI (1993) obtained a relationship between *I* values and

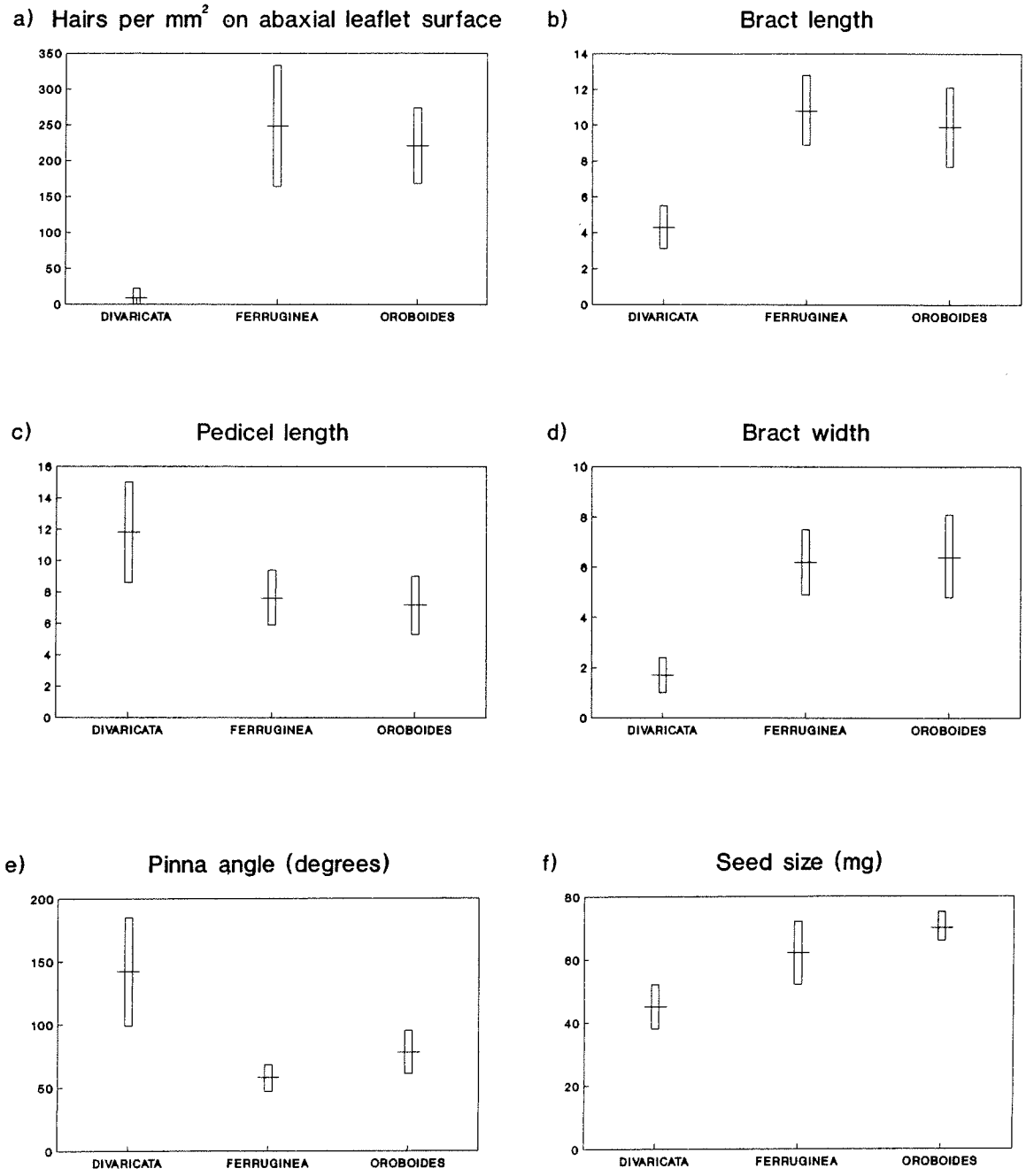


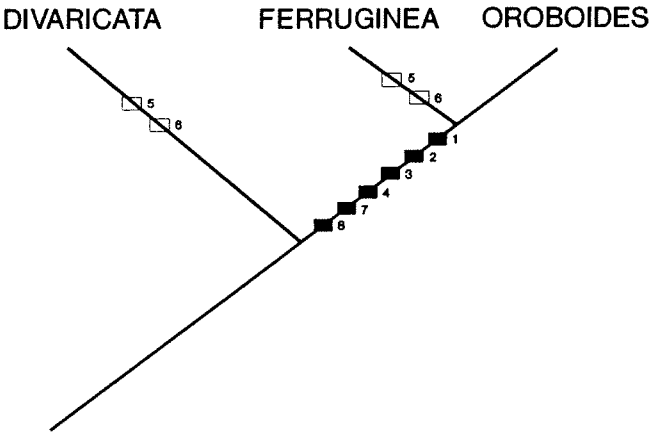
Fig. 4. Graphical summary of salient morphological characters of the three taxa of *Virgilia*, showing the mean and standard deviation of at least 36 measurements per taxon (at least six populations of each taxon was sampled): *a* trichome density of the abaxial leaflet surface (hairs per mm^2, measured in juvenile plants from a provenance trial); *b* bract length (mm); *c* pedicel length (mm); *d* bract width (mm); *e* angle of pinna apex (degrees), acute in *V. oroboides*, obtuse in *V. divaricata*; *f* seed size (mg per seed)

geographic distance. These authors found that populations which are geographically adjacent are genetically more closely related to each other than to more distant populations. For example, RICHTER & al. (1994) reported that the *I* value between two populations of *Delphinium viridescens* LEIBERG was 1.00. These two populations are only separated by 0.5 km. However, we did not find an association between geographical distance and *I* values for the *Virgilia* populations studied. Good correlation of *I* values were found between the current study and that of CRAWFORD (1983).

High genetic identities, as found between populations from different *Virgilia* species in the present study, are hitherto reported only in special situations. For example, high values were observed for species pairs from a number of genera believed to be related as progenitor-derivatives (CRAWFORD 1983, 1985, 1989; GOTTLIEB 1984a). It was assumed that speciation in these cases has been recent to explain the minimal genetic differences between the species (GOTTLIEB 1984a, STEBBINS 1986, CRAWFORD 1989). Other examples of consistently high genetic identities have also been demonstrated for congeneric endemics (LOWREY & CRAWFORD 1983, HELENURM & GANDERS 1985, SOLTIS 1985, CRAWFORD & al. 1987, WITTER 1987, CRAWFORD 1989, HUBER & LEUCHTMANN 1992). However, allozyme analysis indicated a high degree of genetic similarity among populations of *Chenopodium neomexicanum* STANDLEY and *C. palmeri* STANDLEY ($I = 0.974$), but numerical analysis of morphological characters demonstrated that these two species are relatively distinct (WALTERS 1988). Based on allozyme data from only eight isozymic loci WALTERS (1988) concluded that *C. palmeri* should be reduced to a variety of *C. neomexicatum*. The situation in *Heuchera* (SOLTIS 1985) and *Chenopodium* seems to be of particular interest, because some parallells (e.g., the incongruency of morphological and molecular data) to the situation in *Virgilia* can be seen.

Another approach to determine relationships between taxa is by genetic differentiation values. The greatest amount of genetic variation in a species is derived from the within-population level of organisation (GRANT 1989). In the event of subspecies, where the degree of genetic differentiation is large (compared to that of conspecific populations), the within-population component of genetic variation will be much smaller than the between-population component (NEI 1973). The values obtained by gene diversity analysis, using allele frequency data from the present study, revealed that 95.5% of the total diversity originated from diversity within populations. This is an indication that the differences between the allelic frequencies of the samples are very small, and the values are what one would expect of different populations rather than different taxa. These allozyme similarities were also reflected by the small genetic distance between the taxa studied (Table 4).

Comparative studies using both electrophoretic and morphological data to distinguish between species have often produced conflicting results (where morphological data is more than often incongruent with molecular data). In this regard protein electrophoresis proved to be superior to delineate species boundaries (ANDREWS & BEVERIDGE 1990, CREECH 1991, GAJARDO & BEARDMORE 1993). FERGUSON (1980) believes that morphological and allozymic evidence are often discordant for three reasons: 1) either set of data may be incomplete, 2) convergent evolution of particular morphological characters, and 3) morphological and allozymic char-



Characters	Species			
	Calpurnia	OROBOIDES	FERRUGINEA	DIVARICATA
Morphological Characters:				
1 Leaflet shape (obtuse = 0; acute = 1)	0	1	1	0
2 Pubescence of leaflets (± glabrous = 0; densely hairy = 1)	0	1	1	0
3 Seed size (small = 0; large = 1)	0	1	1	0
4 Bract size (small = 0; large = 1)	0	1	1	0
5 Pollen guide (absent = 0; present = 1)	0	0	1	1
6 Nectar guide (± absent = 0; present = 1)	0	0	1	1
Chemical Characters:				
7 Anthocyanins (peonidine low; peonidin < cyanidine = 0; peonidin high; peonidin > cyanidine = 1)	0	1	1	0
8 Sparteine (relatively high concentrations in twigs = 0; low concentrations in twigs = 1)	0	1	1	0

Fig. 5. Cladogram of relationships in the genus *Virgilia*, based on character states of morphological data (Van Wyk 1986, Greinwald & al. 1989) and chemical data (Greinwald & al. 1989, Veen & al. 1991)

acters which may evolve independently. He further states that allozymic and morphological evidence may only concur in cases where morphological evolution has been time dependent. Selective forces apparently have acted on the morphological phenotype of the three taxa of *Virgilia*, but evidently not enough time has elapsed for the accumulation of different allozyme alleles.

(2) Hybridisation between taxa preventing divergence. *Virgilia o.* subsp. *ferruginea* populations share several salient morphological features with *V. o.* subsp. *oroboides*, but have flower colour characteristics (nectar guide and pollen guide) in common with *V. divaricata*. Morphological and chemical evidence leave little doubt that the major discontinuity lies between *V. divaricata* and *V. o.* subsp. *ferruginea*, and not between the two subspecies of *V. oroboides* (see below). In contrast, geographical distribution (Fig. 1) and allozyme analyses (Table 3) indicate that *V. o.* subsp *oroboides* is distinct (all four populations lack the B-allele at the *Per-3* locus). In addition to this apparent greater genetic discontinuity between the two subspecies, allozyme data (Fig. 2) showed *V. o.* subsp. *ferruginea* as an intermediate taxon. Population F1 of *V. o.* subsp. *ferruginea* is also geographically closer to *V. divericata* (Fig. 1), and lack the B-allele at the *Pep-B* locus (corresponding to the loss of this allele in *V. divaricata*). The F2 population also showed similar frequencies of unique alleles to those obtained in samples of *V. o.* subsp. *oroboides* at this locus, and at *Aat, Acp,* and *Per-2* (Table 3, Fig. 2). However, since no locus was fixed for unique alleles in any of the taxa, we propose that long-term introgression between typical *V. oroboides* and *V. divaricata* has resulted in a group of populations (now formally known as *V. o.* subsp. *ferruginea*), which have become genetically similar, regarding allozyme variability, to *V. divaricata*. If this is true, then populations near the boundary of the two distribution areas (the George-Knysna are) should genetically be more similar than adjacent populations of both species. The phenograms based on genetic distances (Fig. 3 a, b) show that populations F1 and D2 are indeed genetically similar.

Phylogenetic relationships. The most powerful approach in systematics available at present is to analyse both molecular and morphological data. However, there are different opinions as to whether one should pool the data and run one combined analysis or run separate analyses and compare the results, e.g., by consensus. DOYLE (1992) suggests that molecular characters should be included in a combined analysis together with morphological data, and that molecular data should be coded as one multistate character. According to HILLIS (1987) and BREMER & STRUWE (1992) the resulting phylogeny might not be the same as the species phylogeny. The latter authors also suggest that discrepancies between the two data sets can never be identified if only a combined analysis is used. Several methods for dealing with different data sets have been proposed and discussed. HILLIS (1987) and SYTSMA (1990) presented different possibilities for comparing morphological and molecular analyses: 1) combining the two data sets with equal weighting for each character; 2) analysing the two sets independently and constructing a consensus tree; 3) generating a cladogram based on molecular characters and secondarily overlaying the morphological characters; and finally 4) generating a cladogram based on morphological characters and secondarily overlaying the molecular characters. If tree comparisons are chosen, then there are possibilities other than generating consensus trees (MICKEVICH & FARRIS 1981, PAGE 1989). In

this study we found a lack of congruence between tree topologies from genetic and morphological data, but the poor dendrograms (as reflected by the low bootstrap numbers in Fig. 3) should come as no surprise since the three taxa are genetically very similar. It is, however, obvious that the taxa which are not geographically isolated (*V. o.* subsp. *ferruginea* and *V. divaricata*) are genetically closest.

According to Van Wyk (1986) *V. o.* subsp. *ferruginea* has an affinity with *V. o.* subsp. *oroboides* rather than with *V. divaricata*. This assumption was based on detailed variation studies and several numerical analyses, including a discriminant analysis. The most reliable quantitative characters that prove this relationship are graphically summarised in Fig. 4. All morphological and chemical characters which show sufficient discontinuity to allow them to be logically polarised, are shown in Fig. 5 (pedicel length shows some overlap and is excluded). Of these, only two morphological characters separate *V. o.* subsp. *ferruginea* from *V. o.* subsp. *oroboides* (pollen and nectar guides) whereas four morphological characters (leaflet shape, pubescence of leaflets, seed size and bract size) and two chemical characters (average values of peonidin and sparteine concentrations) separate *V. o.* subsp. *ferruginea* from *V. divaricata*. The most parsimonious cladogram for the morphological and chemical data is given in Fig. 5. The association between the two subspecies of *V. oroboides* is also supported by some apomorphic tendencies (e.g., from wood anatomy) and various other characters which we were unable to logically polarise for cladistic analysis. In a stepwise discriminant analysis (Van Wyk 1983), the five characters shown in Fig. 4 a–e were found to have the best discriminatory value. Of these, the pubescence of the abaxial surface of the leaflets (Fig. 4 a) and the dimensions of the bracts (Fig. 4 b, d) were diagnostically different between the two species. It is important to note that these two characters show no clinal variation within taxa, but abrupt discontinuity was found between *V. o.* subsp. *ferruginea* and *V. divaricata*. In other genera of the tribe *Podalyrieae*, the pubescence of the leaflets and the size of the bracts have been traditionally used for their diagnostic value at the species level. Large and sheating bracts are of particular evolutionary significance in the tribe *Podalyrieae* (Van Wyk & Schutte 1995).

Congruence between different data sets is desired (Shaklee & Whitt 1981) in order to resolve "true" phylogeny. However, we were unsuccessful in obtaining congruence among phylogenetic inferences based on different character sets but it is noteworthy that there is congruence between morphological and chemical characters (and between allozyme data and geographical distribution patterns).

Speciation in *Virgilia*. The manner of, and conditions during speciation may influence the extent of the genetic distance after separation. Conditions for the formation of a new species demand the occupation of new or different environments and isolation or semi-isolation, until genetic characters become fixed (Lowe-McConnell 1959). In addition, evolutionary biologists agree that, in order to speciate, gene flow between diverging populations must be reduced. In the present study no evidence for a reduction in gene flow between the three taxa could be found from the allozyme data except for the lack of the B-allele at the *Per-3* locus in the four *V. o.* subsp. *oroboides* populations (Table 3).

Patterns of speciation involving either allopatric, parapatric or sympatric development of reproductive isolation have been invoked to explain the origin of new

species. Allopatric speciation basically occurs in two different ways: 1) speciation by subdivision and 2) speciation by the founder effect (BUSH 1975). The overall pattern of variation in *Virgilia* is typical of most Cape legumes and suggests allopatric speciation (VAN WYK 1986).

By adopting the speciation system of GRANT (1981), modes of plant speciation can be referred to two basic categories: (1) primary speciation (i.e., the process of evolutionary divergence of populations to the species level), and (2) hybrid speciation. Primary speciation may involve either quantum or geographical speciation. Quantum speciation is a rapid and radical process and starts from a semi-isolated peripheral population (local race) of a polymorphic, cross-fertilising ancestral species. Geographical speciation is continuous and runs from polymorphic variation through local and then geographical races to species (GRANT 1981). Hybrid speciation was proposed by VAN WYK (1986) to explain the origin of *V. o.* subsp. *ferruginea*. Although *V. o.* subsp. *ferruginea* combines morphological features of *V. o.* subsp. *oroboides* and *V. divaricata*, comparisons of alkaloid patterns of the taxa showed little intermediacy, and a close relation between *V. o.* subsp. *oroboides* and *V. ferruginea* (VEEN & al. 1991). Evidence of possible hybrid speciation could not be inferred from the present allozyme study because consistent genetic differences (fixed unique alleles) for the *Virgilia* species are lacking.

Three possible explanations for the present patterns of speciation can be given. (1) The loci studied electrophoretically may not be representative of the whole plant genome, and the analysis of such data may result in cladograms that reflect the evolutionary history of genes, but not the organism (DOYLE 1992). The whole plant genome is rarely studied due to constraints in manpower, funding and time. If this is true then a distinction at the species level is appropriate. Since time is the critical factor for allozyme divergences (CRAWFORD 1989), the *Virgilia* species must be the result of recent speciation. As indicated by GOTTLIEB (1984b), relatively large changes in plant morphology are possibly based on a few genetic mutations. For example, morphological differences in shape (of leaves), orientation (of ligulate florets), presence versus absence (of glandular hairs or filiform-florets) as well as ecological preferences are often controlled by one or a few genes. (2) Allopatric speciation of a clinal nature may be involved. The morphological and chemical characters that distinguished the taxa can be seen as geographical gradients, thus a graded pattern associated with geographical gradients. Clinal variation has been described in a large number of species (e.g., MCVEAN 1953, COOK 1962, WOODSON 1964, BENSON & BORRILL 1969, ANTONOVICS & BRADSHAW 1970, THOMAS & BARBER 1974, PRENTICE 1979). In *Virgilia*, morphological characters are geographically correlated along an east-west gradient (VAN WYK 1986, GREINWALD & al. 1989) with abrupt increases in seed size, bract size, hairiness of leaves and duration of flowering occurring from east to west. From the above-mentioned information and the intermediacy of the population-specific unique alleles of *V. o.* subsp. *ferruginea* (Fig. 2), it appears that the concept of geographical gradients may apply in this situation. It is also important to note than no geographical barrier exists between *V. divaricata* and *V. o.* subsp. *ferruginea*. If the enzyme data from the present study reflect the true speciation, then the sparse allozymic variation found among *Virgilia* taxa would be consistent with the assumption that the taxa studied should be regarded as recently diversified subspecies or varieties of a sin-

gle species. (3) Divergence followed by introgression could also account for the little distinction between the taxa studied. The morphology reflects an initial complete divergence into two species (*V. oroboides* becoming morphologically and geographically separated from a more widely distributed *V. divaricata*) with subsequent introgression, resulting in the morphologically "intermediate" *V. o.* subsp. *ferruginea*. This would explain the lack of correlation between the major morphological discontinuity (between *V. o.* subsp. *ferruginea* and *V. divaricata*) and the major genetic discontinuity (between *V. o.* subsp. *oroboides* and the other two taxa).

Conclusions. The current accepted taxonomy of *Virgilia* could not be directly verified by enzyme electrophoresis, and no distinct allozyme evidence was found to indicate that *V. o.* subsp. *ferruginea* is a product of hybridisation between *V.* o. subsp. *oroboides* and *V. divaricata*. The genetic evidence supports three possibilities. Firstly, that the morphological differences can be attributed to geographical gradients and that the taxa should be regarded as actively diversifying conspecific populations or infraspecific taxa. Secondly, that hybridisation and introgression between *V. o.* subsp. *oroboides* and *V. divaricata* has occurred and that natural selection has acted on the hybrid populations to select for the morphological and chemical traits but perhaps not on the allozyme loci. Thirdly, and perhaps more likely, that the close genetic relationships reflect allopatric speciation followed by introgression. The apparent lack of agreement between formal taxonomic rank and genetic distance (as reflected in allozyme variation), should be explored in a wider survey of other Cape legumes. It is also possible that DNA studies may provide stronger phylogenetic inferences.

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