SPECIATION AND POPULATION GENETICS

Palaeoclimate-induced range shifts may explain current patterns of spatial genetic variation in renosterbos (*Elytropappus rhinocerotis*, Asteraceae)

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The impact of Pleistocene climatic fluctuations on the distributions of plant species in the Greater Cape Floristic Region is largely unknown. We used a molecular fingerprinting tool, inter-simple sequence repeat (ISSR) PCR to examine the spatial distribution of genetic variation in the common and widespread shrub *Elytropappus rhinocerotis*. We wished to test the hypothesis that refugia for the species were located in areas which were buffered from marked variations in precipitation between glacial and interglacial periods. Populations from less protected areas, in contrast, should have suffered size reductions or extinctions during the dry Holocene optimum. We detected a large amount of genetic variation in the species, which was apportioned largely amongst individuals within populations rather than amongst populations or regions, as expected for an outcrossing and well-dispersed plant species. However, there was significant spatial structure and an uneven distribution of diversity across the range. Geographic distance is a very poor predictor of genetic distance between localities, especially towards the east of the range. This may be due to range alteration over the time-scale reflected by ISSR polymorphism. Inter-SSR variation declined from south to north in the western arm of the range, consistent with the prediction of Holocene aridification starting first and being most extreme in the north. Areas shown by the marker to harbour populations with high levels of variability include most parts of the eastern arm of the range, and the Kamiesberg highlands. Possible explanations for the observed patterns of ISSR variation are discussed.

KEYWORDS: Asteraceae, Cape flora, climatic history, *Elytropappus rhinocerotis*, ISSR, late Quaternary, phylogeography, spatial structure

INTRODUCTION

The Cape Floristic Region (CFR) occupies the south-western tip of the African continent (Fig. 1) and is remarkable both for its species richness and its island-like levels of endemism (Goldblatt, 1978; Linder, 1985, 2003). It shares the defining ecological factor of a winter-rainfall regime with the succulent-karoo vegetation of Namaqualand (Fig. 1; Marloth, 1929) which also has unusually high plant species diversity and endemism (Hilton-Taylor, 1996). Together these regions could be said to form a "greater Cape flora", which is distinct from the adjacent summer-rainfall vegetation (Fig. 1; Bayer, 1984, Born & al., 2007). The winter-rainfall region is topographically, edaphically and climatologically diverse, and comprises many vegetation types. Succulent karoo grows in the hottest and most arid parts, fynbos and renosterveld occur in fire-prone areas of high to intermediate rainfall, and afromontane forest is confined to fire-protected sites with high precipitation (Cowling & Holmes, 1992). In the CFR, vegetation boundaries are correlated with precipitation (Cowling & Holmes, 1992), and so species ranges are likely to have been affected by past alteration in rainfall regimes. Although southern Africa has not been glaciated during the Quaternary, temperatures during the Last Glacial Maximum (LGM, 24–18 thousand years ago) are thought to have been reduced by ca. 5°C–6°C (Heaton & al., 1983; Talma & Vogel, 1992), coupled with alteration in both amount and seasonality of precipitation (van Zinderen-Bakker, 1976; Barrable & al., 2002).

Palaeoclimatic data indicate that changes in precipitation regime may not have been uniform across the area, especially between the current winter and all-year rainfall zones (Fig. 1; Meadows & Baxter, 1999; Barrable & al., 2002). However, our knowledge of late Quaternary environmental change in southern Africa is limited because continuous and precisely-dated palaeoenvironmental records are scarce (Meadows & Sugden, 1993) compared with, for example, temperate areas of the northern hemisphere (Bennett, 1997). Molecular data potentially provide an independent means of inferring population history, because historical patterns of gene flow as well as population events such as bottlenecks and long-distance colonisation leave their signatures in the genetic structure of modern populations (Avise & al., 1987).

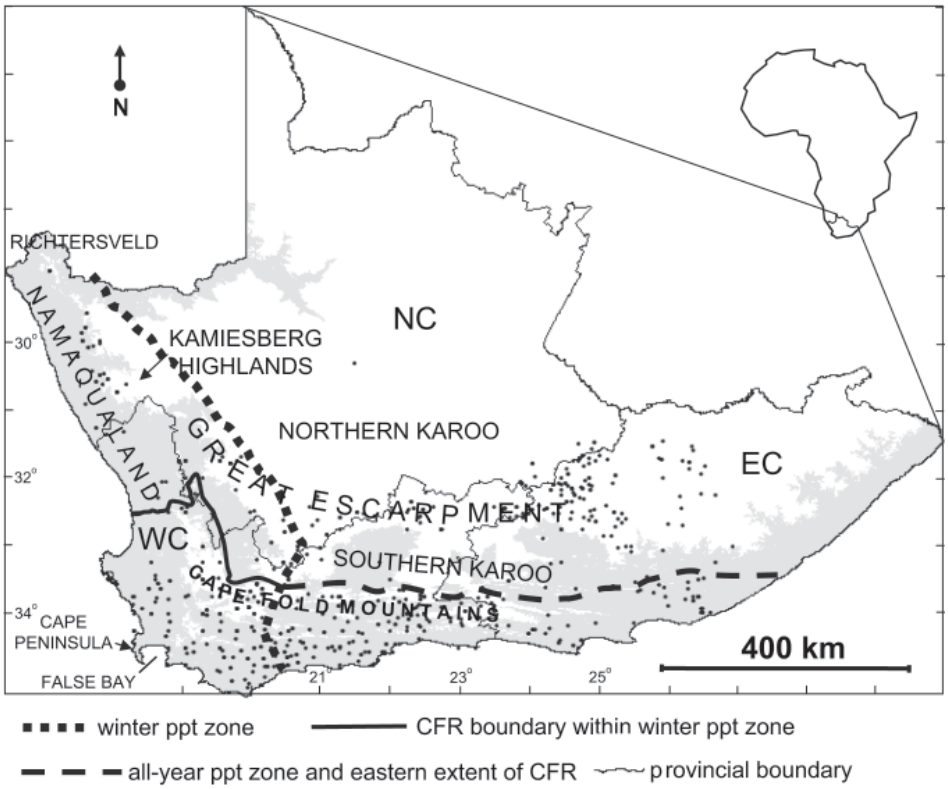


Fig. 1. The location of the Cape Provinces of South Africa. Grey shading indicates areas with elevation less than 800 m above mean sea level. Dots indicate records of *E. rhinocerotis* occurrence as mapped by the PRECIS (National Herbarium, Pretoria [PRE] Computerised Information Service) and ACKDAT (Rutherford & al., 2003) databases. Inland and to the east of the winter precipitation zones (shown with thick solid and broken lines), rainfall occurs predominantly in the summer months. CFR, Cape Floristic Region; EC, Eastern Cape; NC, Northern Cape; ppt, precipitation; WC, Western Cape.

We used the PCR-based hypervariable ISSR technique (Zietkiewicz & al., 1994), which produces dominant markers from multiple anonymous sites located between microsatellite repeats in the plant nuclear genome (Wang & al., 1994), to examine spatial genetic structure in *Elytropappus rhinocerotis* (L. f.) Less. This species, commonly known as "renosterbos", is a member of an endemic Cape lineage in the daisy tribe Gnaphalieae (the everlastings). It is a long-lived woody shrub which is the dominant member of the vegetation type renosterveld (Cowling & Holmes, 1992). The adult plant is drought-resistant due to a deep taproot (Scott & van Breda, 1937), but the seedlings are killed by even slight moisture stress (Levyns, 1956). During its juvenile phase, it is also intolerant of shading and so does not survive competition from other plants (Levyns, 1956).

Renosterbos is widespread across the CFR lowlands, but also occurs outside of the CFR, in more arid parts of the Cape provinces (Fig. 1). Here it is confined to sites where aridity is probably ameliorated by high elevation, raised water tables or orographic precipitation. For example, at the extreme north of its range in the mountainous Richtersveld desert, less than 10 plants were observed on the summit of the Vandersterrberg (1,380 m).

The distribution of renosterbos suggests that it requires more moisture than succulent karoo plant species but less than most fynbos taxa (Levyns, 1938; Cowling & Holmes, 1992) and it is often found in an ecotone between the two vegetation types. Since vegetation records of past climate change are most sensitive near ecotones (Peteet, 2000), the ecotonal ecology of renosterbos may make it a good model species for examining distributional history.

Renosterbos may also be one of the few Cape plants that is represented in the fragmentary palaeoenvironmental record. Being putatively wind-pollinated (M. Koekemoer, pers. comm.) it is thought to be one of the sources of the *Stoebe*-type pollen found in Quaternary palynological sediments from around southern Africa (e.g., Deacon & al., 1983; Bousman & al., 1988; Meadows & Sugden, 1988; Scott & Bousman, 1990; Scott & al., 2004).

There is at present in the western arm of renosterbos' distribution, an increase in both temperature and aridity moving from the Cape Peninsula in the extreme south, towards the Richtersveld in the north (Fig. 1). However, this aridity gradient may not always have been present, and Namaqualand appears to have been substantially wetter during the phases of the last glacial period (van Zinderen-

Bakker, 1976; Cockroft & al., 1987; Meadows & Baxter, 2001). Palynological evidence from the last glacial period, including records of *Stoebe*-type pollen, indicates that between approximately 70–17 thousand years (ky) ago, CFR elements existed far to the north of their current extent (Shi & al., 1998, 2000; Dupont & al., 2005). Midgley & Roberts (2001) used bioclimatic envelope modelling to estimate the extent of the main CFR vegetation types over the last 21 ky. Their model predicts that renosterveld may have been present throughout the Richtersveld at the LGM. If this is correct then increasing Holocene aridity over the last ca. 12 ky must have subsequently caused large-scale extinction of renosterbos populations in this area, leading to the current highly fragmented distribution there. Such a demographic scenario should result in northern Namaqualand populations showing signals of a genetic bottleneck (reduced genetic variation and greater isolation among populations) relative to southwestern populations. The vegetation of the southern part of the western arm would not have been affected to the same degree by Holocene aridity (Meadows & Baxter, 1999).

The climatic history of the eastern arm of the distribution range (from Cape Agulhas eastwards) is more difficult to reconstruct. Despite indications of some alteration to both temperature and precipitation regimes (Martin, 1968; Deacon & al., 1983; Partridge & al., 1999), conditions may never have been harsh enough to cause extinction of renosterbos populations. Complex climatic regimes as well as the nature of palaeoclimatic data make it difficult to predict past distribution changes of any species in the eastern arm, which will also be influenced by ecological interactions such as competition. Such interactions may have been more important during times (and in regions) where climatic constraints were less severe.

Several authors have used dominant, multilocus, anonymous markers to examine historical demography and detect refugial or recently founded populations (e.g., Amsellem & al., 2000; Clausing & al., 2000; Hess & al., 2000; Stehlik & al., 2001; Holderegger & al., 2002; Stehlik & al., 2002; Knowles & Richards, 2005). Since genetic diversity takes a long time to accumulate and is sensitive to reductions in population size (Widmer & Lexer, 2001), areas which have housed large, stable populations for long periods of time should be characterised by greater variability than areas which have experienced drastic or persistent reduction in numbers (Nei, 1975). In addition to having higher diversity, refugial areas are also likely to be genetically distinct (Ferris & al., 1999) due to isolation from other populations. In contrast, localities which have been recently colonised should possess a reduced subset of the loci present in the source populations (Hewitt, 1996). We surveyed localities from across the entire distribution range of *E. rhinocerotis* in order to answer the following questions:

(1) What are the spatial patterns of genetic diversity and relatedness within the species?

(2) Does the western arm of the distribution exhibit a gradient of genetic variation indicating greater recent bottlenecks in the north than in the south?

(3) Which areas harbour higher-than-average levels of ISSR variability, indicating that they may have housed large, stable populations of *E. rhinocerotis* throughout the period of time reflected by the genetic marker?

MATERIALS AND METHODS

Sampling, DNA extraction and ISSR amplification. — To comprehensively survey spatial genetic structuring in *E. rhinocerotis*, we sampled multiple localities across the entire known distribution range, including isolated "edge" regions, and ensuring that there were no large intervening areas left unsampled (Fig. 2). This entire-range sampling allows the inference of broad-scale patterns of spatial genetic variation (Arafeh & Kadereit, 2006), albeit at the cost of denser within-population sampling. In total, 107 samples and 26 localities were included in the analysis (Table 1).

Young shoots were collected into silica gel for drying prior to DNA extraction by the CTAB method of Doyle & Doyle (1987) and a single voucher specimen per locality was deposited in the Compton Herbarium (NBG), Cape Town (Table 1). Three ISSR primers were selected from the University of British Columbia Biotechnology Laboratory Primer set 9. The primer sequences are $(GA)_8$-A (primer 812), $(AG)_8$-YC (primer 835), and $(CA)_8$-RT (primer 846). Amplifications were performed in either a Hybaid PCR Sprint (Fisher Scientific International) or an ABI GeneAmp® (Applied Biosystems) thermal cycler. Reaction volumes were made up to 25 µl with PCR-grade autoclaved water. Reaction mixtures consisted of 1× reaction buffer, $MgCl_2$ at 3 mM (primers 812 and 835) or 2.5 mM (primer 846); 0.15, 0.1 and 0.2 mM of each dNTP (primers 812, 835 and 846, respectively); 0.4 µM of primer and 0.03, 0.04 and 0.024 units of Biotaq™ (Bioline) per µl (primers 812, 835 and 846, respectively). The thermal cycler profiles consisted of an initial denaturation for 1.5 min. at 94°C followed by 35 cycles of: 1 min. at the primer-specific annealing temperature, 1 min. at 72°C and 30 sec. at 94°C. This was followed by 2 min. at 52°C and 3 min. at 75°C. The annealing temperatures were 54°C, 50°C and 53°C for primer 812, 835 and 846, respectively.

Band visualisation and assessment of locus identity. — Banding profiles were visualised by electrophoresis in 5% TBE buffer using 1.5% (weight by volume) 12-lane agarose gels. Gels were run at 65–80 mV until the bromophenol blue loading-buffer dye band had advanced 8 cm from the wells (approximately 3 hours), stained with

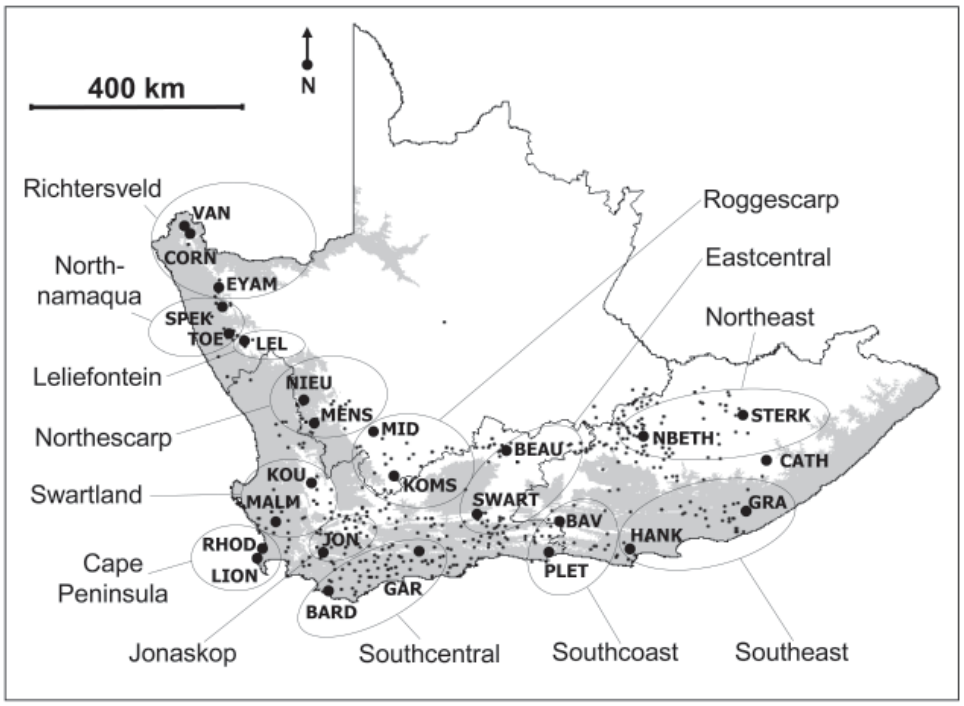


Fig. 2. The Cape Provinces of South Africa showing sampled localities, indicated by black dots with names in upper case. Grey shading indicates areas with elevation less than 800 m above mean sea level. The regions into which samples were grouped for regional analyses are indicated by the ellipses. Names of regions are given in lower case with initial capitalised letter.

Ethidium Bromide and photographed under UV light. Multiple gel images were printed at different levels of contrast and brightness to facilitate detection of both bright and faint bands (Zietkiewicz & al., 1994). For each primer, one or two individual plants that exhibited a range of fragment sizes were chosen as reference samples and their banding profiles were used in lieu of size standards. The sizes of bands in reference sample profiles were measured against Eco-RV-digested λ DNA size standards in a separate gel. Extracted *E. rhinocerotis* DNA samples were labelled with a random letter code. This resulted in sample identity being unknown to ensure objective band scoring. In each scoring gel, reference samples occupied three lanes while samples to be scored were run in two non-adjacent replicate lanes per gel, similar to van der Kloet & Paterson (2000). Each fragment size was assumed to represent a separate ISSR locus and individuals were coded for the phenotype band present (1) or absent (0) at each locus.

Reproducibility of fragments was tested by rigorous replication of all steps in the generation and visualisation of ISSR bands. Each primer-sample combination was repeated in at least two and up to 20, separate PCRs, and visualised in at least two separate scoring gels.

Data analysis. — Multivariate analyses for measuring broad patterns of spatial genetic structure included all 107 samples. Pairwise distances were calculated using the index of Jaccard (1908) which takes into account only shared band presences, and the simple matching coefficient (SM) which uses both shared presences and absences (Sneath & Sokal, 1973), both calculated in NTSYS-2.1 (Rohlf, 2000). Uncorrected P distances (the observed proportion of bands not shared by two accessions) were calculated in Splitstree 4 v. 4.6 (Huson & Bryant, 2006). The dis-/similarity matrices were clustered using UPGMA (NTSYS-2.1) and Neighbour Joining (NJ; Splitstree 4 v. 4.6). The goodness-of-fit of the UPGMA dendrograms was measured by their cophenetic correlation with the original dissimilarity matrix in NTSYS-2.1. The robustness of the NJ groupings was quantified with 1,000 bootstrap replicates in Splitstree 4 v. 4.6 (Huson & Bryant, 2006). Principal co-ordinates analysis (PCoA) scatterplots based on Jaccard's index and the SM coefficient were produced in NTSYS-2.1 (Rohlf, 2000).

Genetic structure was measured as the partitioning of genetic variation within and among groups (AMOVA) in ARLEQUIN V.2.0 (Excoffier & al., 1992; Schneider & al., 2000). The three localities represented by a single sample each were excluded from AMOVA analysis. Levels of significance of the variance components were assessed via 10,000 nonparametric randomisation permutations as implemented in ARLEQUIN. Four different grouping structures were assessed (Table 2). Firstly, samples were grouped by locality to assess the amount of ISSR variance within the average locality. Secondly, the distribution

range was divided into eastern and western arms, corresponding to the winter precipitation zone (western arm; Fig. 1) and the all-year precipitation zone (eastern arm; Fig. 1). The third and fourth AMOVA structures estimated the amount of variation partitioned across groupings that were suggested by the multivariate analyses (see Results).

Analysis of regional patterns of ISSR variation. — The entire-range sampling strategy precludes inference of population-level parameters due to low numbers of samples for each locality. However, the AMOVA indicated that nearly 80% of the variation measured in the entire species sample is represented within localities (see Table 2 and Results). In renosterbos, any given locality is thus largely representative of the total variation (Gregorius, 1988). Although statistically significant, differentiation among localities is small and so we considered it biologically reasonable to group individuals from neighbouring localities into "regions" for further analysis. This serves to increase sample sizes so that diversity estimates are based on larger (and equal) numbers of individuals. Grouping samples into regions also allows estimation of genetic relationships among different regions. Regional groupings were made up of samples from nearest-neighbour localities less than 250 km apart. To maintain equal sample sizes, some individuals were excluded from the regional analyses. Samples to be excluded were chosen randomly.

Any specific arrangement of individuals into a regional grouping structure may not be congruent with the overall pattern of genetic structure. It is also possible that different arrangements of localities into regions might produce different results. To test this, we examined multiple different nearest-neighbour allocations of individuals to regions and repeated diversity calculations for every arrangement. For example, in the eastern arm, samples can be grouped into regions which include only localities aligned along two east-west axes, one for the coastal and one for the inland mountain ranges (Fig. 2). Alternatively, there could be several groupings, each running north-south from the escarpment to the coastline. We tested multiple types of grouping arrangements, as well as multiple different arrangements within either east-west or north-south types of groupings. We also tested a similar range of types of arrangements in the western arm of the range. All regional grouping structures resulted in the same patterns of range-wide diversity, which are all consistent with the conclusions we draw (see Discussion), so we present the results only from one grouping arrangement, that depicted in Fig. 2. This arrangement includes 103 *E. rhinocerotis* individuals grouped into 13 regions.

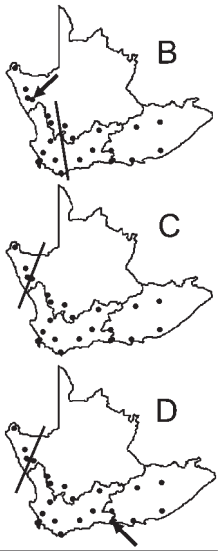
Table 1. Sampling localities for *E. rhinocerotis*.

Locality	n	Locality, province	Region[a]	Voucher no.[b]	Co-ordinates (long./lat.)
BARD	3	Baardskeerdersbos, WC	Southcentral	*Bergh 301*	19.58°/–34.59°
BAV	5	Baviaanskloof, western end, EC	Southcoast	*Bergh 238*	23.58°/–33.49°
BEAU	5	Beaufort West, Karoo, WC	Eastcentral	*Bergh 795*	22.56°/–32.20°
CATH	1	Cathcart, north of Stutterheim, EC	—	*Bergh 746*	27.11°/–32.32°
CORN	1	Cornellsberg, Richtersveld, NC	Richtersveld	*Desmet 3160*	17.18°/–28.57°
EYAM	1	Eyams, Richtersveld, NC	Richtersveld	*Desmet 3273*	17.62°/–29.35°
GAR	5	Garcia's Pass, Muiskraal, WC	Southcentral	*Bergh 270*	21.22°/–33.92°
GRA	5	Coombs, near Grahamstown, EC	Southeast	*Bergh 728*	26.78°/–33.29°
HANK	3	Hankey, near East London, EC	Southeast	*Bergh 692*	24.82°/–33.91°
JON	10	Jonaskop, near Villiersdorp, WC	Jonaskop	*Bergh 871*	19.52°/–33.92°
KOMS	3	Komsberg Pass, Roggeveld, NC	Roggescarp	*Bergh 374*	20.76°/–32.67°
KOU	5	Koue Bokkeveld, WC	Swartland	*Bergh 201*	19.26°/–32.82°
LEL	8	Leliefontein, Kamiesberg, NC	Leliefontein	*Bergh 76*	18.08°/–30.31°
LION	3	Little Lion's Head, Cape Town, WC	Cape Peninsula	*Bergh 992*	18.35°/–34.01°
MALM	5	Malmesbury, WC	Swartland	*Bergh 880*	18.72°/–33.47°
MENS	5	Farm Mensieskraal, NC	Northescarp	*Bergh 62*	19.31°/–31.73°
MID	5	Middelpos, Roggeveld scarp, NC	Roggescarp	*Bergh 990*	20.25°/–31.91°
NBETH	3	Nieuw-Bethesda, Sneeuberge, EC	Northeast	*Bergh 817*	25.06°/–31.97°
NIEU	3	Nieuwoudtville Waterfall, NC	Northescarp	*Bergh 980*	19.12°/–31.32°
PLET	3	Plettenberg Bay, South coast, EC	Southcoast	*Bergh 478*	23.57°/–33.96°
RHOD	4	Rhode's Memorial, Cape Town,WC	Cape Peninsula	*Bergh 950*	18.46°/–33.96°
SPEK	5	Spektakel Pass, Namaqualand, NC	Northnamaqua	*Bergh 319*	17.71°/–29.70°
STERK	5	Sterkstroom, Stormberg mts, EC	Northeast	*Bergh 751*	26.66°/–31.63°
SWART	3	Swartberg Pass, WC	Eastcentral	*Bergh 614*	22.11°/–33.38°
TOE	3	Farm Toeneus, Kamieskroon, NC	Northnamaqua	*Bergh 964*	17.82°/–30.22°
VAN	5	Vandersterrberg, Richtersveld, NC	Richtersveld	*Bergh 966*	17.07°/–28.43°
Total	107	26 populations			

Abbreviations: EC, Eastern Cape; n, no. of sampled individuals; NC, Northern Cape; WC, Western Cape.
[a]Region to which a locality was assigned for genetic diversity calculations; see Fig. 2.
[b]All vouchers deposited at the Compton Herbarium (NBG).

Table 2. AMOVA design and results.

	Source of variation	d.f.	Sum of squares	Variance components	% total variation	Φ-statistics	*p*
A.	**Total (23 localities)**						
	Among localities	22	359.56	1.28	20.98	0.209	<0.0001
	Among individuals	185	892.96	4.83	79.02	—	—
B.	**Western (12 localities)[a] vs. Eastern (11 localities)[b]**						
	Among West vs. East	1	18.60	0.01	0.18	0.002	0.1770
	Among localities within groups	21	340.95	1.28	20.87	0.210	<0.0001
	Within localities	185	892.96	4.83	78.95	0.210	<0.0001
	Western vs. Eastern localities but with LEL (arrow, Fig. B) included with Eastern localities						
	Among West vs. (East + LEL)	1	23.76	0.06	0.94	0.009	0.0090
	Among localities within groups	21	335.79	1.25	20.39	0.206	<0.0001
	Within localities	185	892.96	4.83	78.67	0.213	<0.0001
C.	**Northwestern grouping (localities VAN, SPEK & TOE) vs. all other localities**						
	Among Northwest vs. Rest	1	33.47	0.39	6.04	0.060	0.0010
	Among localities within groups	21	326.08	1.19	18.61	0.200	<0.0001
	Within localities	185	892.96	4.83	75.34	0.250	<0.0001
D.	**Northwestern grouping vs. locality PLET (arrow, Fig. D) vs. the rest of the sample**						
	Among groupings	2	55.23	0.50	7.78	0.078	<0.0001
	Among localities within groups	20	304.32	1.14	17.62	0.191	<0.0001
	Within localities	186	892.96	4.83	74.60	0.254	<0.0001



Note: All statistical significance values were determined by comparison against 10,000 random permutations of samples and localities, while keeping sample numbers at each locality constant.
[a]Western grouping: VAN, SPEK, TOE, LEL, NIEU, MENS, KOU, MALM, RHOD, LION, JON, BARD.
[b]Eastern grouping: MID, KOMS, GAR, SWART, BEAU, BAV, PLET, HANK, NBETH, GRA, STERK.

The frequency of band presence at each locus was calculated among samples from each region. This produced interval (as opposed to binary) data for each locus which was used to calculate a pairwise Euclidean distance matrix and clustered using UPGMA in NTSYS-2.1 (Rohlf, 2000). A Principal Components Analysis (PCA; Hotelling, 1933) as implemented by STATISTICA version 6.1 was performed on the interval data and the results mapped for visual inspection using synthesis mapping, as in Piazza & al. (1981) and Cavalli-Sforza & al. (1994). This is a technique for graphical display of complex multivariate data. Each map represents one Principal Components (PC) axis with the position of a sample on that axis indicated by a greyscale shade. The shading scale was chosen by dividing each PC axis into nine segments and assigning each segment a shade from white at one extreme of the axis to black at the other. We mapped the applicable greyscale shade onto the geographic centre of each region, which is the point midway between all localities included in that region, weighted according to number of samples from each locality (as in Templeton & al., 1995). Values were interpolated onto the map space between geographical centres using inverse weighting of the distance to the three nearest neighbour geographical centres in ArcView GIS 3.3 with a Spatial Analyst extension (Environmental Research Institute, Inc.).

Measures of phenotypic rather than genetic diversity were used in order to reduce the number of genetic assumptions required (e.g., no analysis assumed Hardy-Weinberg genotype proportions). The following diversity measures were calculated: the percentage of loci that are polymorphic (P) for each region; Shannon's diversity index (H′; Shannon & Weaver, 1949) calculated as in King & Schaal (1989); and the average pairwise SM distance between all individuals in a region (Amsellem & al., 2000).

Isolation-by-distance analyses. — Isolation-by-distance (IBD) between individual samples and between localities was tested via Mantel's (1967) test using NTSYS-2.1 PC (Rohlf, 2000). Significance was determined by comparing the observed Mantel test statistic M_R with the distribution of values obtained from 10,000 random Monte Carlo permutations of one of the matrices (Rohlf 2000) in the same software. Great-circle geographic distances between localities were calculated in Arcview 3.3. Geographic distances between individuals from the same locality were set at zero.

Directional autocorrelation between genetic and geographic distance was examined over eight distance classes of 250 km each, as in Oden & Sokal (1986). The Mantel statistic M_R was calculated for each geographic distance-class matrix versus the ISSR distance matrix, tested for significance as described above, and plotted against geographic distance. Significance values were corrected using the sequential Bonferroni technique of Holm (1979).

RESULTS

Band replicability across PCRs and gels. — The number of bands we obtained per ISSR primer is unusually high (Wolfe & Liston, 1998) and more comparable to that found in typical AFLP studies. However, all bands were present in all replicates; these results may be attributable to species-specific SSR abundance and genomic distribution and/or our particular band-detection methods. Only bands that are always very faint when they are detectable vary in their observable presence, and this may be related to the limitations of agarose gel visualisation. These faint bands were not included in the analyses and all scored ISSR loci were reproducible between template concentrations, gels and replicate PCR's. For some samples, such as the five reference samples, this involved successful reproduction of identical banding patterns in over 20 PCR reactions and 50 scoring gels. We produced 220 reliable ISSR loci overall, ranging from ca. 300 to ca. 2,000 base pairs in length. Of these, 79 come from primer 812, 73 from primer 835 and 68 from primer 846. We included all 220 bands in our analyses as our statistical measures make minimal assumptions about the genetic nature of the ISSR bands (Lynch & Milligan, 1994). No two individuals share the same ISSR banding pattern. Only one band is present in every individual, making the directly observed proportion of polymorphic loci 99.5%. The proportion of bands present in > 5% and < 95% of the sample is 0.73. Several loci are unique to a single locality (data not shown) but none of these is present in more than two individuals from that locality, and possessing unique loci is correlated with sample size so is probably not informative in this dataset.

Analysis of all individuals. — Figure 3 shows the unrooted NJ tree based on uncorrected P distances. Geographically coherent clusters are labelled on Fig. 3. The only locality for which all sampled individuals group together is PLET from the south coast. This locality is extremely distinct in all multivariate analyses, and although it is represented by only three samples, many other localities have similarly few samples which do not group together or form a distinctive cluster. Several MENS samples from the northern CFR also group together, as do five of the ten samples from JON near the southwest of the Cape Fold mountain belt. There is also a grouping which includes five of the seven samples from the Cape Peninsula (localities RHOD and LION) as well as two samples from nearby KOU. However, the cluster that has greatest geographical coherence contains 93% of the individuals from the extreme northwest of the range (localities VAN, SPEK, EYAM, CORN, and TOE). These localities all occur to the north of the Kamiesberg massif. Samples from LEL, collected on the Kamiesberg itself, are distributed throughout the tree, frequently grouping with samples from the far eastern or southern extreme of the range.

Although none of these clusters has bootstrap support above 50%, these same groupings were recovered in all multivariate analyses: NJ (Fig. 3), UPGMA and PCoA (data not shown). The UPGMA topologies from both the Jaccard and SM coefficient are essentially the same as that in Fig. 3. The cophenetic correlations of the UPGMA dendrograms are r = 0.526 (Jaccard's index) and r = 0.634 (SM coefficient). The first three axes of the principal co-ordinate scatterplots summarised 7.4% (Jaccard's index) and 10.6% (SM) of the variation in the data. The main feature of all the individual-level multivariate analyses, clearly visible in Fig. 3, is that there is very little geographical structuring of genetic variation in *E. rhinocerotis*. No localities or regions form well-supported groupings, and most clusters consist of individuals from geographically distant localities across the distribution range.

Analysis of Molecular Variance (AMOVA). — The AMOVA indicates that nearly 80% of ISSR variance is represented among individuals within localities (Table 2A). However, there is no significant apportionment of variance between the summer-arid and the all-year rainfall zones (Fig. B within Table 2). There is statistical support for the stronger association of LEL with eastern localities: if LEL is included with the eastern rather than western samples, the west versus east component, though very small, acquires statistical significance (Table 2B). The strongest genetic disjunctions suggested by the multivariate analyses occur between northwestern localities (those north of LEL), and all others, and between PLET and surrounding localities. In order to quantify these breaks, we performed AMOVAs with localities divided accordingly (Table 2C and D). These arrangements showed the greatest structure, with nearly 6% of the variance attributable to the division between northwestern localities and the rest, and 7.8% attributable to a division of localities into northwestern versus PLET versus the rest.

We also used AMOVA to examine ISSR structure within the eastern and western arms of the range separately, divided according to Fig. B in Table 2. The amount of ISSR variation apportioned among western localities only (excluding LEL) is 23.32%, while that apportioned among eastern localities only is 17.87% (including LEL) and 19.99% (excluding LEL; each separate AMOVA with $p < 0.0001$).

Isolation-by-distance analyses. — The Mantel test indicates a statistically significant but negligible trend of increasing genetic distance with untransformed geographic distance between individual *E. rhinocerotis* plants ($M_R = 0.13$, $p = 0.001$. The spatial autocorrelation analysis (Fig. 4) shows that the slight effect of decreasing relatedness with distance operates most strongly over distances of about 250 km, but once samples are more than 750 km

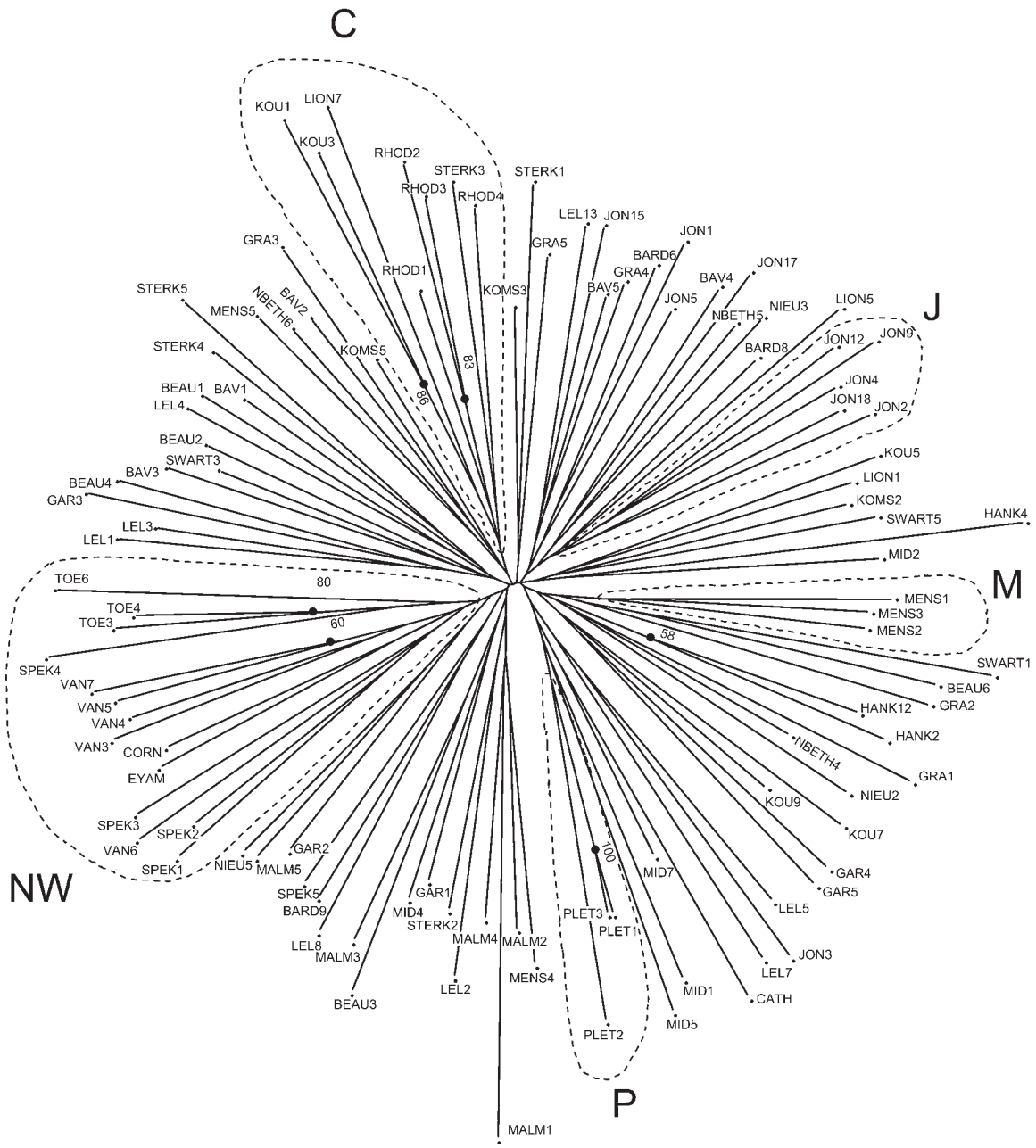


Fig. 3. Unrooted neighbour-joining tree of all 107 renosterbos accessions based on uncorrected (observed) P distances as calculated in Splitstree 4 v.4.6. Clusters enclosed in dotted lines indicate localities or regions whose samples group together (C, five of the seven samples from the Cape Peninsula and two samples from the nearby locality KOU; J, five of the ten samples from locality JON; M, three of the five samples from locality MENS; NW, 14 of the 15 samples from the northwesternmost localites, i.e., those north of LEL; P, the three samples from locality PLET). Bootstrap values are shown only for those nodes (represented by dots) which received > 50% support (1,000 replicates).

apart this relationship is no longer significant (filled symbols in Fig. 4). This indicates that samples which are just over 750 km apart are as distinct as, for example, samples 1,500 km apart. The pattern of IBD holds true for the western and eastern arms separately (broken lines, Fig. 4), but the trend is stronger and operates over greater distances in the western arm.

Regional patterns: synthesis mapping. — Figure 5 shows synthesis maps of band frequencies for each region. The first three Principal Component axes account for 40.3% of the total variation. Each axis needs to be interpreted independently and indicates the degree to which a region is different from other regions on that axis only (Cavalli-Sforza & al., 1994). By far the strongest signal in

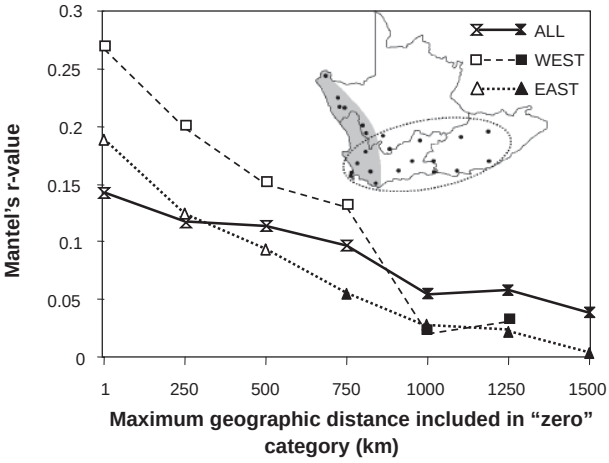


Fig. 4. Spatial correlogram of Mantel's r-statistic with increasing geographic distance. Open symbols indicate statistical significance ($p < 0.01$) after sequential Bonferroni correction. Closed symbols indicate correlations which are not significant even at $p = 0.05$. "ALL" indicates all localities included in analysis; "WEST" indicates localities from the western arm of the distribution only (indicated in the figure by the shaded region) and "EAST" indicates only localities from the eastern arm of the distribution (those included in the ellipse in the inset figure).

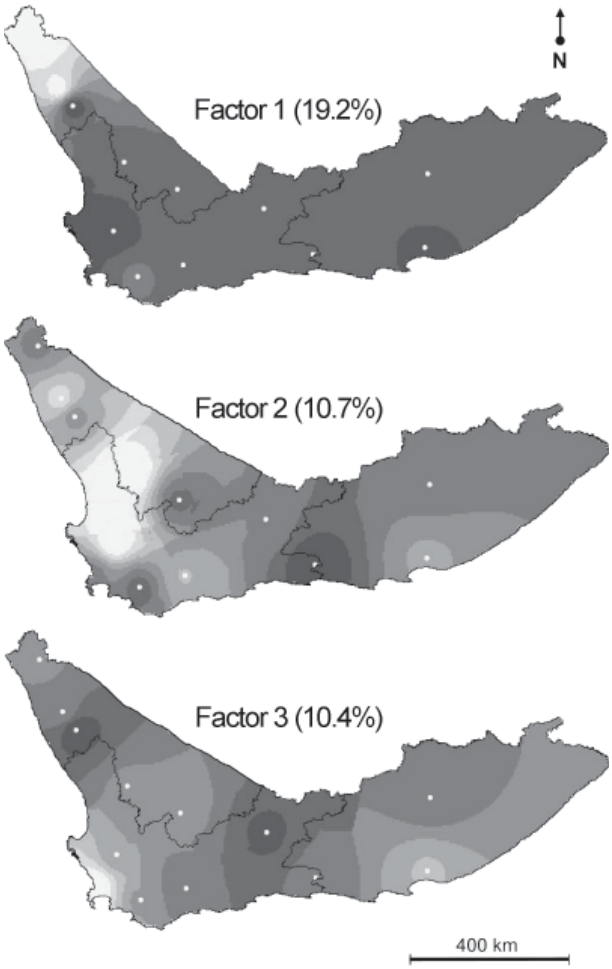


Fig. 5. Synthesis maps of the first three principal components factor scores of ISSR band frequencies for each region. The geographic centre of each region is indicated by a white dot. Shading in each map represents the range of factor co-ordinate values, arbitrarily set at black for highest and white for lowest values, interpolated via inverse distance weighting to the three nearest neighbour regions. The shades are independent in each map and indicate only similarity amongst regions for the particular PC axis being represented. Actual values of the factor co-ordinates are not relevant to interpreting the figures and only indicate whether regions have similar or different scores for each factor. The distinctness of the northwestern localities is the strongest signal in PC1, and may account for most of the variance explained by this axis, and therefore most of the variance in the data. Leliefontein is distinct from its neighbours on all three axes. Factor 2 indicates distinctness of regions Swartland and Northescarp, which occur towards the same end of this axis as Northnamaqua. An ISSR discontinuity between the central part of the eastern arm and the regions to the west and east of this is also evident on Factor 2 around the Southcoast region. Principal component 3 echoes this ISSR discontinuity, but places it more strongly on the Eastcentral region and also shows a grade in affinity between a highly distinctive Cape Peninsula region and other regions in the southwest.

the regional PCA is the distinctness of the northwestern localities, which accounts for most of the variation in the most influential axis, Factor 1.

Regional patterns: spatial structure and ISSR diversity. — The grouping of localities into regions increases the apportionment of ISSR variance within groups, as measured by AMOVA, to 87.38% ($p < 0.0001$). The proportion of ISSR variability among individuals within regions as estimated by H′ (King & Schaal, 1989) is 71.3%.

Figure 6 shows the UPGMA cluster diagram of regions. The greatest distance in the phenogram separates the northwestern grouping (Richtersveld and Northnamaqua) from the rest. The association of the Kamiesberg locality with southern localities, which was found with individual-level analyses, is true at the regional level also, as Leliefontein clusters together with the geographically distant Northeast region.

The values of H′, P and average pairwise SM for each region are shown in Fig. 7. The different measures agree well in the ranking of regions by ISSR diversity. There is a very uneven distribution of variation across the range of the species, but all of the regions with below-average diversity occur in the western arm of the range (regions Richtersveld, Northnamaqua, Northescarp, Jonaskop and the Cape Peninsula). However, Leliefontein and Swartland, also from the western arm, have the two highest diversity measures. Roggescarp and Southcoast are the

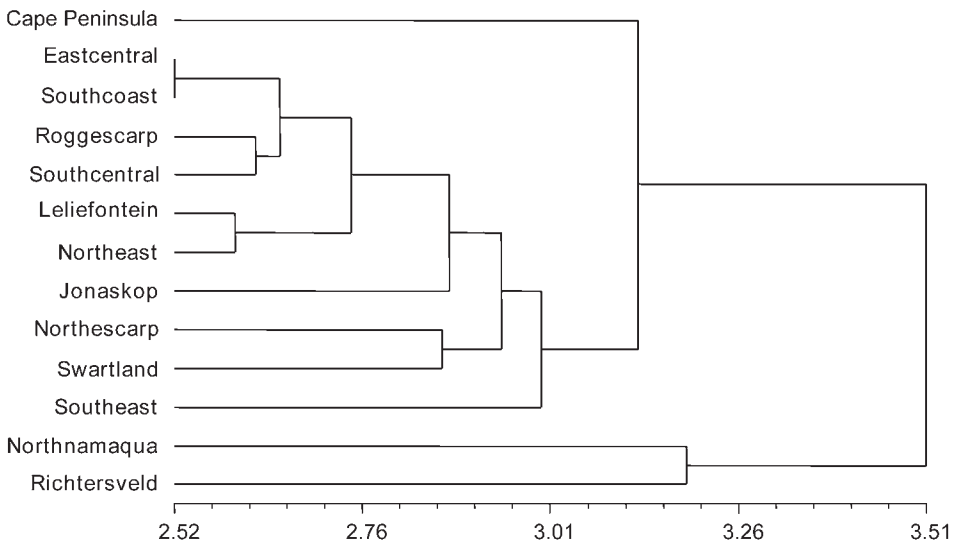


Fig. 6. UPGMA clustering of regions (see Fig. 2) based on Euclidean distances calculated from band frequencies at each locus. The cophenetic correlation is r = 0.918.

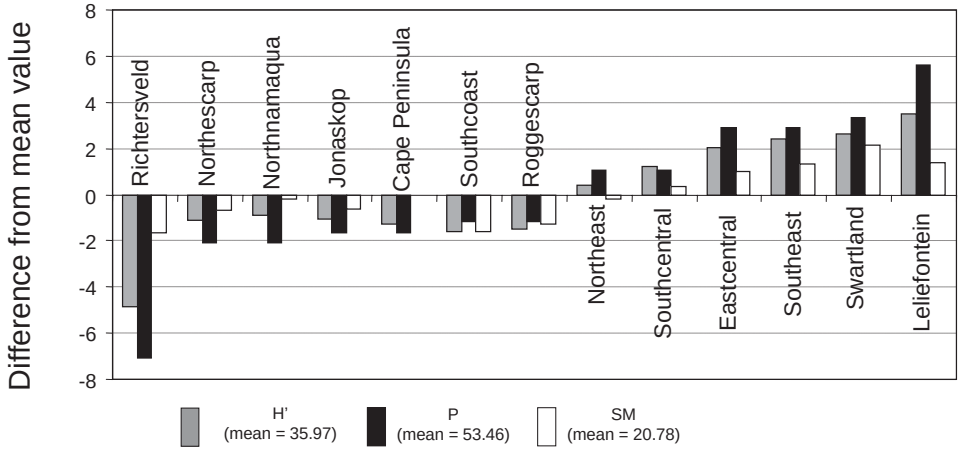


Fig. 7. Diversity measures for regions (each region consists of eight individuals from one or more localities, as shown in Fig. 2). Each bar represents the difference between the region's value and the mean value for all regions. P, proportion of loci which are polymorphic within a region; H′, Shannon's index based on the natural logarithm; and SM, the mean pairwise dissimilarity between individuals from a region, based on the simple matching coefficient and expressed as a percentage.

two regions from the eastern arm with below-average diversity; all other regions from the eastern arm have values that fall above the mean.

Is there a north-south gradient of genetic variation in the western arm? — Within the western arm, ISSR diversity does appear to decrease as one moves northwards, except for the high-diversity region Leliefontein (Fig. 7). To put a statistical value on this observation, we regressed Shannon's index and average SM dissimilarity per region against geographic distance from False Bay (Fig. 1) for all the regions from the western arm (Richtersveld, Northnamaqua, Leliefontein, Northescarp, Swartland, Cape Peninsula and Jonaskop). There is no significant decrease in diversity moving northwards. However, excluding Leliefontein from the analysis gives a significant negative correlation ($R^2 = 0.81$, $p < 0.01$ for Shannon's index and $R^2 = 0.65$, $p < 0.02$ for SM) despite the reduction in degrees of freedom resulting from removal of a data point.

DISCUSSION

Despite low numbers of samples from each locality, our study achieved range-wide sampling of the species' distribution which allowed us to examine broad-scale patterns of spatial genetic structure. Renosterbos harbours a

large amount of ISSR variation, and this variation is not strongly structured according to the current geographical distribution of its populations. The lack of strong geographic structure is probably compounded by high levels of recombination among ISSR loci. Such recombination could be a product of high levels of gene-flow and high outbreeding rates, and is to be expected for hypervariable, recombining, multilocus and dominant markers in plant species which are outcrossing and well-dispersed (Nybom, 2004). Both pollen and seeds will contribute to dispersal of nuclear loci such as ISSRs (Wang & al., 1994) and several characteristics of *E. rhinocerotis* are likely to increase average dispersal distances. Firstly, wind-dispersal is likely to be an efficient method for translocating both pollen and seeds; secondly, adult renosterbos plants have a very large seed output (potentially thousands per plant per year); and thirdly, there is an initial period of physiological seed dormancy which spreads germination over several years after seed is shed (Levyns, 1929). As a result, only geographic structure that is either recent or persistent (or both) is likely to be reflected in the ISSR data.

Patterns of genetic diversity and relatedness across the range. — To a large degree, localities are representative of the total variation in the species, and both AMOVA and H′ gave similar proportions for the amount of within-locality variation. This type of spatial structure (high within-population variability) was also found in the individual-level multivariate analyses, as indicated by the low cophenetic correlations for the UPGMA dendrograms (Rohlf, 2000), the small proportion of the variation explained by PCoA, and the low bootstrap values (NJ). Shared band absences may be informative, as indicated by the marginally better fit of the SM phenogram to the data, and as proposed by Rieseberg (1996) for within-species comparisons.

Despite the overall lack of population genetic structure, a significant amount of ISSR variation is still allocated among groups, whether these are localities or regions. This is clear from the AMOVA and from the fact that all analyses recovered the same geographic pattern. This indicates a degree of spatial ISSR structure in *E. rhinocerotis* essentially due to the distinctness of the samples from the extreme northwest of the range, the uniqueness of PLET samples, and the far stronger association of LEL samples (or the region Leliefontein as a whole) with samples from the far eastern part of the distribution range. However, this pattern is not congruent with the current geographical arrangement of populations, and this is lent statistical support by the fact that there is only a very weak signal of IBD. What little signal of IBD is present in the data is due only to differences between individuals, not between localities, so grouping localities into geographic regions is not likely to violate the spatial structure that is present. Regional groupings allowed more robust sample sizes for diversity estimates, and more rigorous examination of how ISSR diversity varies from region to region. The ranking of regions by amount of ISSR diversity was in agreement across different diversity estimators (Fig. 7). We will discuss the possible causes for the ISSR patterns in the western and eastern arms of the range separately.

The western arm of the distribution. — This part of the range exhibits a very strong signal of genetic disjunction between northernmost localities and all the rest.

Except for Leliefontein, ISSR diversity decreases moving northwards, as expected if diversity is linked to the increasing aridity gradient in the western arm. Our data are thus consistent with the scenario that Holocene aridity began in the north and spread southwards. The palaeoclimatic models predict a greater degree and earlier onset of aridification in the north (van Zinderen-Bakker, 1976), which would have caused vicariance and population-size bottlenecks in populations whose ancestors had previously enjoyed more mesic conditions in Namaqualand during the last glacial period. The distinctness of the northwestern populations is thus consistent with long-term and persistent isolation, which may well date back to the start of the Holocene.

An alternative scenario, which does not require a change in precipitation in the northwest, is that northwestern populations are the result of long-distance colonisation events, perhaps from the Kamiesberg, with subsequent differentiation by genetic drift in isolation. If this were the case, we would expect northwestern localities to carry a reduced subset of the ISSR bands present in the source localities, and to show greatest genetic affinity with these source populations. Such clear indications of sources for the northwestern localities are not evident in our data; but if colonisation events happened sufficiently far back in time, genetic association between source and sink localities might not be detectable, especially with high recombination rates amongst phylogenetically unordered markers. We may be unable to distinguish vicariance from ancient dispersal, but would expect that if Richtersveld samples were derived from multiple independent samples, they would collectively harbour greater ISSR diversity, and show lower genetic similarity to each other, than we observed. A survey of cpDNA sequence variation (N.G. Bergh, unpubl. data) confirms that Namaqualand localities have far lower haplotype diversity than LEL, and indicates that this may be due to historical fragmentation.

Alternatively, ISSR variation may simply reflect current ecological conditions, with the correlation of diversity and aridity in the western arm arising from populations being smallest and most isolated in the most arid parts of the range (i.e., those from the most extreme environment in the Richtersveld). Unfortunately, it is not possible to test

whether unordered markers such as ISSRs reflect current conditions or historical demographic processes. Correlation of genetic variation with population size is well documented in the conservation genetic literature (e.g., Frankham, 1996) so diversity differences amongst localities may simply reflect ongoing population size effects. Some of the northern localities, especially VAN, are very small, while LEL, the genetic exception, has potentially a very large effective size. However, we did not attempt to quantify population sizes of our localities.

The greater IBD in the western arm than the eastern arm, as indicated both by the Mantel test and by the greater apportioning of AMOVA variation amongst western localities, may indicate greater historical site-fidelity here. Populations in the western arm thus may have existed in their current localities for longer periods than those in the east, perhaps due to a lack of suitable sites for colonisation.

The fact that the strongest genetic disjunction occurs between LEL and TOE, and that the region Leliefontein has the highest levels of ISSR diversity as estimated by all three diversity measures, indicates that LEL is unaffected by whatever factors influenced the northwestern populations. The Kamiesberg highlands, due to their altitude, are substantial cooler and moister than the surrounding plains (Schulze, 1995). Despite being only 48 km from TOE, LEL is ca. 700 m higher, and amelioration of aridity with increasing altitude is a well-known environmental phenomenon. For this reason, Midgley & Roberts' (2001) model predicts that fynbos would have survived on the top of the Kamiesberg even during the hottest and driest period of the current interglacial (the Holocene Altithermal ca. 8–4 ky; Partridge, 1993). This serves as additional support for the hypothesis that aridity is a strong reason for the genetic distinctiveness of the northwestern localities.

The high ISSR diversity evident in LEL may also be in part a result of Kamiesberg populations acting as a sink for propagules from multiple surrounding areas, due to the better habitat offered by the moister highland areas. LEL samples may thus have genetic affinities with a wide range of source localities. If this is the case, the ISSR data indicates source localities to the south and east of LEL, and this is consistent with the current prevailing wind direction in the region (Desmet & Cowling, 1999), which is likely to move seeds in a predominantly northerly direction.

The uniqueness and high ISSR diversity of LEL samples are also consistent with the inference that this area provided stable habitat for renosterbos throughout the Holocene. We can speculate that the greater genetic affinity of LEL with southeastern samples may be due to greater opportunity for gene exchange to the south than with shrinking and isolated northern populations. However, it is interesting that LEL shows genetic affinity with populations from the far eastern end of the range rather than with closer localities such as NIEU and MENS (Fig. 6). The latter are the most obvious means of connecting LEL to the eastern arm via gene flow, but such a Great-Escarpment gene-flow corridor is not indicated by the data. It is thus tempting to hypothesise a historical expansion of renosterbos into the Northern Karoo, connecting the Kamiesberg highlands directly with northeastern localities such as STERK. A palaeoclimatic incursion of the winter-rainfall region into what is currently the summer-rainfall interior of South Africa (Northern Karoo; Fig. 1) has previously been hypothesised by van Zinderen-Bakker (1976), and Dowson (1988) suggested that the reduced temperatures of the last glacial may have allowed an eastward and northward shift in the winter-rainfall vegetation types. The possibility of such a shift, however, remains speculative without further palaeoclimatic data.

The summit of the Kamiesberg houses outliers of the Cape flora (Marloth, 1908; Pearson, 1912; Adamson, 1938; Weimarck, 1941; van-Wyk & Smith, 2001) which have been hypothesised to be the remnants of a once more widespread CFR (e.g., Rourke, 1990). The alternative explanation would be that they are the result of independent long-distance colonisation events from the south. The high diversity and affinity of Leliefontein samples with eastern localities data may be more consistent with longstanding isolation of a large, genetically diverse population, perhaps even with an historical extension of the range into the Northern Karoo (Fig. 1) as discussed above, and our data may thus represent the first genetic indication that the Kamiesberg is a historical refugium for Cape floral elements in arid Namaqualand. In this regard our ISSR data also lend support to the idea that the CFR may in the past have extended further to the north than it does at present.

The eastern arm of the range.— As discussed above, our data show no evidence of a gene flow route between the Kamiesberg and the southeast via the Great Escarpment. Figure 5 shows that there is no detectable ISSR connectivity between the regions that make up this putative "corridor". Instead of a pattern of east-west connectivity along the Great Escarpment, the dendrogram in Fig. 6 indicates that Great Escarpment regions such as Roggescarp, Eastcentral and Northeast are placed closest to various other regions (respectively, Southcentral, Southcoast, and Leliefontein). Similarly, there is no detectable east-west corridor linking regions along the south coast, and this is true for all schemes for grouping localities into regions. This lack of correlation between geographic proximity and genetic relatedness is clear from the low level of IBD. Either patterns of gene flow are not easily predictable from geography, or other factors, such as historical range shifts or genetic recombination of ISSR markers, have obscured or prevented the development of such geographically predictable patterns.

In the eastern arm, regions are generally characterised by high levels of ISSR variation and there are no areas which are very distinct in this regard, unlike the Kamiesberg in Namaqualand. This may indicate either high historical population sizes or good gene flow amongst localities, or both, for most localities in the southwestern Cape and at the eastern extreme of the species' range. However, the central area (region Southcoast comprising localities PLET and BAV) and also region Jonaskop have lower ISSR variability, while the southwest and the far eastern part of the range have very high diversity estimates in many grouping arrangements tested.

The southwestern-most Cape is predicted to have experienced relatively mesic climatic conditions throughout the late Quaternary, due to the area remaining within the influence of moisture-bearing westerlies at both glacial and inter-glacial extremes (Meadows & Baxter, 1999). Jonaskop may be an exception to this as it occurs in the rainshadow on the north-facing slopes of part of the Cape Fold Mountain belt and houses an ecotonal vegetation ranging from fynbos on the summit to succulent karoo vegetation in the arid Little Karoo. The effects of aridity may explain why it has much lower levels of variation than other southwestern regions. Parts of the Great Escarpment may also represent plausible long-term habitat for renosterbos due to their higher altitudes attenuating any arid periods. However, the far eastern arm of the range has not been explicitly considered as a potential long-term refuge for Cape floral elements, although there is palaeoecological evidence of *Stoebe*-type pollen presence throughout the late Quaternary in places (Scholtz, 1986; Bousman & al., 1988; Meadows & Sugden, 1988; Scott & al., 2005). These deposits might have been produced by the summer-rainfall relative, *Stoebe vulgaris* Levyns, but there are additional indications that *E. rhinocerotis* has had a long history in the area that today forms its eastern extent. Levyns (1935) speculated that renosterbos originated in the eastern part of the range, since its phenology appears to conform more closely to the current climate in the east. She also described renosterbos as being ecologically more "at equilibrium" with other components of the eastern vegetation, while in the west it often dominates renosterveld communities and has a more "invasive" character. More tellingly, *E. rhinocerotis* has a close association only in the eastern part of its range with a flightless insect, the locust *Lentula obtusifrons* Stål., which has no other food source (Smit, 1935). Although we have no information on the palaeodistribution of *L. obtusifrons*, it has never been collected west of 21° E longitude (S. Antunes and M. Picker, unpublished data). Such an obligate association argues for a long sympatric history, possibly only in the east of the renosterbos' range.

The presence of *Stoebe*-type pollen and renosterbos charcoal fossils imply that *E. rhinocerotis* may have been widespread across large parts of the eastern arm during the late Quaternary (Martin, 1968; Deacon & al., 1983; Scholtz, 1986). However, climatic fluctuations may have prevented continuous presence of renosterbos in the central part of the region. This central part not only has lower ISSR diversity, but also is genetically distinct. Locality PLET is an extreme genetic outlier amongst all renosterbos localities. The reasons for this are unclear, and it would be difficult to support speculation based only on the three PLET samples. However, regional diversity statistics for the centre of the eastern arm calculated without PLET but using, for example, samples from BAV and HANK, still find a reduction in ISSR variability here. Thus there may be a real historical reason for lower diversity in the southern coastal region. Palynological and other evidence indicates that temperate afromontane forests were greatly restricted during the last glacial period when *Stoebe*-type pollen was abundant (Butzer & Helgren, 1972; Martin, 1968; Scholtz, 1986). However, increases in moisture during phases of the Holocene and the resulting expansion of forests (Martin, 1968; Partridge & al., 1999) may have displaced the shade-intolerant renosterbos (Levyns, 1956) from the southern coastal mountains. Subsequent contraction of these forests due to late-Holocene aridification, together with anthropological deforestation, could have resulted in later recolonisation and the more recent populations could have lower genetic variation.

Greater isolation among western than among eastern localities (separate AMOVAs; Fig. 6) is consistent with a scenario in which some eastern populations only recently colonised their current positions, since this would not allow time for isolation by distance to become established.

CONCLUSIONS

Our interpretation of historical patterns reflected in the ISSR dataset is that in the western arm, climatic changes were either of greater amplitude or of consistent direction while in the east, climatic conditions may have oscillated between dry/wet conditions with no consistent directionality to the change. This may be the reason for what appears to be a clearer picture of demographic history in the genetic structure of western localities.

In the western arm, populations appear to be restricted both geographically and in terms of size. The most obvious explanation for this is reductions in population sizes due to aridity, both today and historically. The Kamiesberg samples are the exception that seems to prove the rule. In the eastern arm, populations appear less restricted in terms of size but distributional shifts may have played a greater role, and we hypothesise that biotic interactions may have been more important here.

Phylogenetically unordered, dominant markers such as ISSRs have limited utility for phylogeographic inference. Nevertheless, this research constitutes a first examination of the distribution of genetic variation in a member of the unique Cape flora. Our results are largely consistent with predictions of how palaeoclimatic changes might have structured genetic variation in renosterbos. We present hypotheses about putative changes in distribution of this typical Cape plant species in response to late-Quaternary climate changes in the Cape provinces of South Africa. These hypotheses may be tested by future phylogeographic, population genetic or palaeoecological research in the region.

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