A traitor in the ranks:

hybridisation between two formerly allopatric *Protea* species

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Abstract

Hybridisation can lead to the extinction of species through outbreeding depression, hybrid vigour and, in some cases, the formation of hybrid swarms. Species in the Cape Floral Kingdom are at particular risk of hybridising with one another as the floral diversity of the region is recent, with the result that isolating mechanisms are less likely to have had the time to evolve. We investigated a case of putative hybridisation between locally indigenous *P. lepidocarpodendron* and the introduced *P. nerifolia* in the Silvermine Nature Reserve on the Cape Peninsula, South Africa. A continuous cline in morphological and genetic character variation, identified using ordinations and Bayesian provenance estimation procedure, provides strong evidence of hybridisation between these two species. Certain morphological characters, including capitulum length and various qualitative morphological characters are useful in discriminating between the parent *Protea* species and may provide a means for identifying hybrids in the field. Based on these characters, as well as the genetic data, the scale of hybridisation is inferred to be locally extensive. Evidence for extensive hybridization in this study highlights the potential genetic permeability of species boundaries in *Protea* and draws attention to the inadequacies of using a biological species concept. We conclude that the planting of indigenous species close to wild populations of congeners may pose a significant threat to the conservation of the Proteaceae.

Introduction

Humans have been translocating plant species since at least as far back as the development of agriculture, spreading crop and weed species across the globe as more and more people abandoned a nomadic lifestyle for an agricultural one (Colledge *et al.* 2004). With the advent of global exploration, and later the establishment of international trade routes, so began a massive redistribution of plant species around the world (Mooney & Cleland 2001, Abbott *et al.* 2003). The threat of exotic and invasive species has been highlighted as a major threat to "biodiversity, ecosystem integrity, agriculture, fisheries, and public health," but the genetic risks of translocations have historically received far less attention (Lee 2002).

Introduction of individuals from different species, or of different populations, can lead to the genetic swamping of local genotypes through hybridisation (Levin *et al.* 1996, Hufford & Mazer 2003). The genetic introgression of introduced genes is exponential because as the number of hybrids increase, so the potential for cross-pollination increases (Levin *et al.* 1996). Hybrids between the introduced and local genotypes may either show decreased (outbreeding depression) or increased (heterosis) fitness relative to the parent genotypes. Outbreeding depression may result because of the dilution of genes for local adaptations or as a result of hybrid breakdown, in which locally adapted genes are reshuffled during sexual recombination (Levin 2000, Hufford & Mazer 2003). Alternatively, hybrids can show increased fitness relative to their parent species because they may be more genetically variable, they may possess novel interactions between genes, they may have lost the deleterious effects of recessive alleles or they have received beneficial genes from both parent species (Lee 2002). When hybrids are fitter than parent species, hybrid swarms can arise. These are completely hybrid populations and they may be highly invasive, such as has been shown in a number of studies (Levin *et al.* 1996, Vila & D'Antonio 1998, Allendorf *et al.* 2001, Bleeker 2003). From a conservation point of view, both outbreeding depression and hybrid vigour are undesirable, as

they can result in the extinction of the local species (Levin *et al.* 1996, Rhymer & Simberloff 1996). As human-induced introductions, and habitat fragmentation and modification continue to increase, so hybridisations between formerly genetically isolated species are likely to become ever more common (Rhymer & Simberloff 1996, Allendorf *et al.* 2001, Lamont *et al.* 2003).

From a theoretical viewpoint translocations present an excellent opportunity to test the nature of plant species concepts. The most widely accepted species concept by evolutionary biologists today, is the biological species concept (Mayr 1942, Mallett 2005). Recently, Mallett (2005) contended that hybridisation and introgression challenge "the 'reality' of biological species". Hybridisation has been shown to be more common amongst recently diverged taxa (Mallett 2005) and is more likely to occur between species that evolved in allopatry as it is less likely that barriers to reproduction have developed in the limited time since divergence (Levin 2000, Moyle *et al.* 2004). The high species diversity of the Cape Floristic Region has been suggested to have evolved fairly recently (Richardson *et al.* 2001, Linder 2003), and it is, thus, highly likely that hybridisation is possible between many of the species occurring here. Little is known about reproductive barriers of the species of the Cape Floristic Region (Linder 2003), and the translocation of species in the region could provide more insight in this regard.

Between 1960 and 1970 a pine plantation was cleared at the Silvermine Nature Reserve on the Cape Peninsula and the area re-vegetated with plants from the government nursery (T. Rebelo *pers. comm.*). One of the species introduced to this area as part of a restoration project was *Protea nerifolia*, a species that naturally occurs on the mountains from Hottentots Holland to Port Elizabeth, but which is historically absent from the Cape Peninsula (Rebelo 1995) (Fig. 1.). A congener, *Protea lepidocarpodendron,* is indigenous to the Peninsula, and has a disjunct distribution, occurring on the Peninsula and from the Kogelberg to the Kleinrivier mountains (Rebelo 1995) (Fig. 2.). The two species differ morphologically in

a number of respects, the most obvious of which are their differences in involucral bract colour and length of flowerhead. *Protea nerifolia* has cream to carmine involucral bracts and flowerheads 100-180 mm long, while *P. lepidocarpodendron* has cream to pink upper involucral bracts, contrasting brown lower involucral bracts, and flowerheads 90-110 mm long (Rebelo 1995). Since the introduction of *P. nerifolia* to Silvermine, two fires have passed through the area, and putative offspring of *P. nerifolia* are still present as evidenced by their pink to red involucral bracts (*pers. obs.*). However, it appears that hybridisation may have taken place between these two species of *Protea,* as individuals outside the site to which *P.nerifolia* was introduced are showing traits characteristic of this species such as pink involucral bracts, and lack of traits characteristic of *P. lepidocarpodendron* such as a defined band of brown lower involucral bracts (T. Rebelo *pers. comm.*). Using a joint approach using morphological and genetic characters, we therefore investigated whether hybridisation is in fact occurring between these two species, and if so, what morphological characters could be used to identify hybrids. We also attempted to determine the spatial extent of hybridisation, as this has direct implications for *Protea* farming and the planting of non-local indigenous species near to wild populations of *Protea* in the Cape Floristic Region.

Methods

Sampling

Sampling was conducted at a site within the Silvermine sector of the Table Mountain National Park, Cape Town, South Africa (S 34°05'37", E 18°24'48") (Fig. 3). Six grid plots (A – F) were sampled along a transect beginning at the site of original introduction of *P. nerifolia* (plot A) and ending approximately 500 m away in a stand of *P. lepidocarpodendron* plants in which no putative "morphological hybrids" were visible (plot F). Although plot A is located on the site of the original *P. nerifolia* plantation, not all individuals making up the present population of the plot are necessarily pure *P. nerifolia* since they represent the second generation since the time of introduction. Each plot, except for the first plot at the *P. nerifolia* source population, measured 15x15 m and was divided up into a grid of nine squares of 5x5 m. Sampling was conducted by choosing plants nearest to all of the corners of each 5x5 m square giving a total of 16 plants per plot. If a number of plants were of equal distance to a square corner, then preference was given, firstly, to "morphological hybrid" individuals, or if none were present, to individuals with the most open flowers. As there were only a few, scattered individuals in the *P. nerifolia* source population, 16 individual plants were sampled at random in this plot. Three large, healthy-looking, older leaves were selected on each plant and placed in bags containing silica gel for preservation. One healthy, mature flowerhead was removed from each plant so that various morphological measurements could be made.

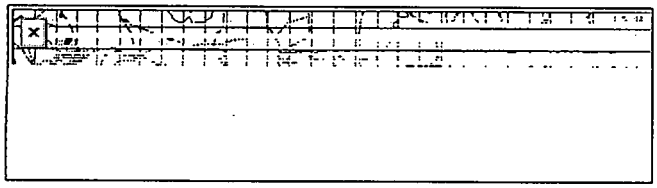

Fig. 1. *Protea nerifolia* distribution in the Cape Floristic Region. .

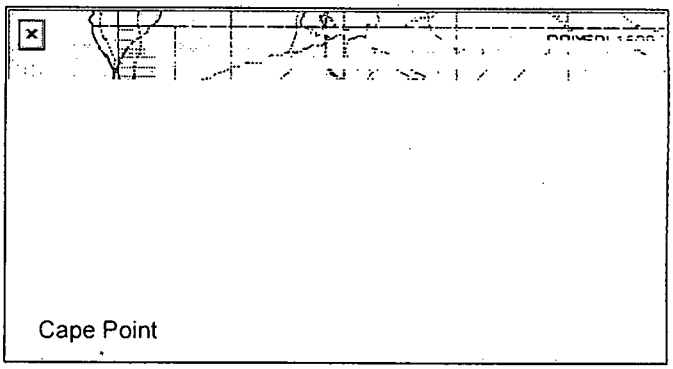

Fig. 2. *Protea lepidocarpodendron* distribution in the Cape Floristic Region.

Source: Protea Atlas Project – Interim distribution maps (http://protea.worldonline.co.za/idm_ca.htm).

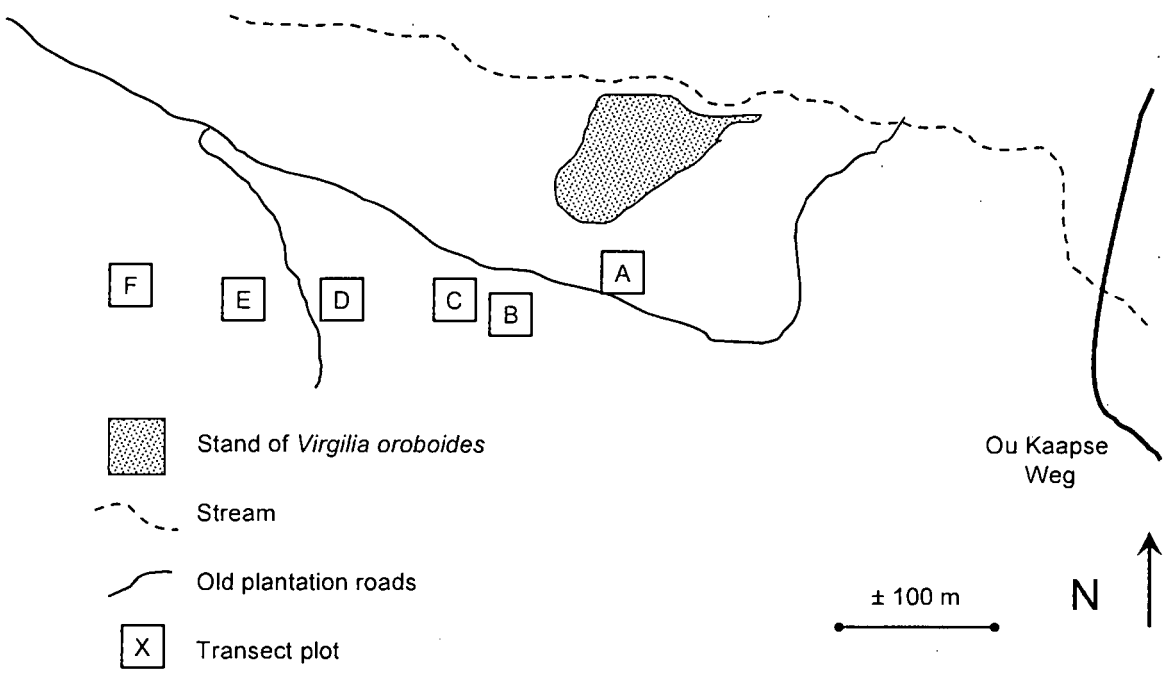

Fig. 3. Sampling site within the Silvermine Nature Reserve, South Africa.

Morphological and phenological measurements

Table 1. Morphological traits and their respective states measured for each individual sampled in the six transect plots.

Characters	State / units measured in
Colour of lower involucral bract (lower involucral bract defined as being 2.5 cm from the base of the flowerhead).	0 = mostly pale with brown along the margins. 1 = entirely brown.
Reflexion of tips of lower involucral bracts.	0 = not reflexed. 1 = reflexed.
Hairiness of lower involucral bract margin.	0 = glabrous. 1 = hairy.
Angle of the tip of a lower involucral bract (the largest angle measurable from margin to margin by placing the tip of the bract at the centre of a protractor).	degrees
Colour of upper involucral bracts (upper involucral bracts defined as those reaching to the apex of a mature flowerhead).	0 = carmine / pink 1 = cream
Hairs on upper involucral bracts.	0 = black hairs only along margin. 1 = black/white hairs extending from the margin a few cm down the upper bracts.
Angle of the tip of an upper involucral bract	degrees
Width of one upper involucral bract halfway down its length divided by the width 1 cm from the tip of the bract.	cm
Curling of the upper involucral bracts of flowers from the previous season.	0 = erect, not curling. 1 = curling.
Length of the capitulum.	cm
Length of a perianth tube from its base to the apex of the awn.	cm
Length of the style from the base of the ovary to the apex of the stigma.	cm
Presence of a purple band (± 4 mm long) a few mm below the stigma.	0 = absent (white). 1 = present (purple band present).
Colour of the awn.	0 = brown-yellow. 1 = white.

Eight qualitative, as well as six quantitative characters were measured on each flowerhead as described in Table 1.

Molecular data collection

Total DNA was extracted from ± 30 mg of silica gel-dried leaf material using a modified version (a spatula tip of polyvinylpyrrolidone (PVP) added to leaf sample before addition of liquid nitrogen) of the protocol described by Gawel & Jarret (1991). DNA extractions were performed on a minimum of 12 and maximum of 14 of the 16 samples in each transect plot. Concentrations of total DNA for each sample were quantified using an ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware USA). Samples were diluted to a concentration of 10 ng/µl.

Thirteen primers from the University of British Columbia ISSR primer set # 9 (http://www.biotech.ubc.ca/naps) were screened for successful PCR amplification using a set of eight samples representing both putative parent species (Table 2). Primers were optimised changing annealing temperature, amount of DNA template used and concentration of $MgCl_2$. Of the primers screened, three produced scorable bands with sufficient variation to be useful at this level. Ultimately, the two primers (813 and 841) that produced the most distinct and variable banding pattern were scored for all samples. Amplification reactions were carried out using an Applied Biosystems Geneamp® PCR System 2700 (Applied Biosystems, Foster City, CA, USA). PCR amplification consisted of an initial denaturation of 1.5 min at 94 °C, followed by 35 cycles each comprising 1.5 min denaturation at 94°C, 1 min at the annealing temperature (52°C for primer 813 and 54°C for primer 841), 1 min at 72°C, 30 s at 94°C, and ending with 2 min at 44°C and 2 min at 72°C. Thereafter reactions were held at 4° C. A negative control containing sterile water instead of DNA extract was included in each PCR set to control for possible contamination. The reaction mixture of 25 µl contained approximately 10 ng genomic DNA, 1 mM $MgCl_2$, 1x PCR buffer (Eisenberg Bro. Co., Givat Schmuel, Israel), 1 µl dNTP and 0.15 µl SuperTherm *Taq* DNA polymerase (Eisenberg Bro. Co., Givat Schmuel,

Israel). Amplification products (2µl) were electrophoresed on 50 ml 2 % agarose gels, stained with 1.7 µl ethidium bromide (10 mg/µl), and run at 80 V in a 0.5x TBE buffer solution for roughly two hours. A single reference sample was run in three different wells on each gel, two on the margins and one in the centre. Five minutes prior to removing the gel, 10 µl of ethidium bromide was added to the gel tank. Gels were visualised under a UV light.

Bands were manually detected using the programme UVIDoc 12.6 for Windows (Cambridge, U.K.). Homology of bands was ensured through inspection of bands produced by the reference samples. Band presence or absence was scored in a binary manner for all individuals to yield a matrix with 36 0/1 characters.

Table 2. List of 13 primers from the University of British Columbia primer set # 9 that were screened for distinct, variable bands for detecting hybridisation
(* indicates primer produced distinct, polymorphic bands, ** indicates primer used for scoring).

Primer	Sequence	Primer	Sequence
812	$(GA)_8A$	866*	$(CTC)_8$
813**	$(CT)_8T$	868	$(GAA)_6$
835	$(AG)_8YC$	880	$GGA(GAG)_2AG(GA)_2$
841**	$(GA)_8YC$	881	$(G)_3(TGGG)_2GTG$
853	$(TC)_8RT$	888	$BDB(CA)_7$
856	$(AC)_8YA$	891	$HVH(TG)_7$
857	$(AC)_8YG$		

Data analysis

We investigated the pattern of hybridisation between *P. nerifolia* and *P. lepidocarpodendron* using a number of approaches. Ordination methods were used to investigate the pattern of morphological and genetic character variation in individuals across the transect. To explore the morphological variation of sampled individuals across the transect, a principal component analysis (PCA) of the quantitative morphological data was performed in Statistica v7.0 (StatSoft, Inc, Tulsa, USA). To the same end, we used a principal

coordinates analysis based on Simple Matching Coefficient in NTSYSpc v2.10 (Rholf 1997). Spatial variability in genetic composition of sampled individuals was investigated using a pairwise similarity matrix obtained using Jaccard's Similarity Coefficient in NTSYSpc v2.10 (Rholf 1997). The similarity matrices were then plotted by principal coordinate analysis (PCO) implemented in NTSYSpc v2.10 (Rholf 1997).

To explore the spatial structure of genetic variation, the likelihood calculator DOH (http://www.biology.ualberta.ca/jbrzusto/Doh.php) was used. The likelihood calculator uses the spatial variation of genotypes of individuals to determine the plot from which sampled individuals were most likely to have come, through the use of an assignment index, A, as described on the website. The assignment calculator was set at ploidy = 1 because ISSR markers are dominant (Wolfe at al. 1998a). DOH also calculates a genetic distance measure, d_{xy}, between plots, ($d_{x,y} = (A_{x,y} + A_{y,x}) / 2$, where x and y represent different plots, and A is an index of how much more likely genotypes of individuals sampled in plot x are actually in plot x, than in plot y). To test for a correlation between geographical distance and genetic distance, a Mantel test was performed in NTSYSpc v2.10 (Rholf 1997).

Because two generations had passed since the introduction of *P.* nerifolia, it was no longer possible to assign individuals parent or hybrid status. For this reason a model-based approach implemented in NewHybrids v1.1 beta (http://ib.berkeley.edu/labs/slatkin/eriq/index.htm) was used to investigate the genetic history of all individuals. The model considers a group of individuals as comprising two species, and hybrids of various classes (e.g. F1 and F2), after a specified number of generations within the framework of Bayesian model-based clustering, the programme calculates the posterior probability of an individual belonging to each of the various classes (in this case parent *P. nerifolia*, parent *P. lepidocarpodendron*, F1 hybrid, F2 hybrid). This provides an estimate of the genetic history of each individual rather than simply assigning individuals as hybrid or pure. As we knew for how many generations hybridisation is likely to

have occurred, we assumed only two hybrid classes (F1 and F2). Markov chain Monte Carlo (MCMC) simulation used for investigating the hybrid status of all sampled individuals included a burn-in of 17 000 sweeps, followed by 30 000 sweeps, and used the default Jeffrey's priors for calculating π (*pi*; the posterior probability of the mixing proportions) and θ (*theta*; the posterior probability of the allele frequencies) (Anderson & Thompson 2002).

We wished to identify characters that best differentiated between the two parent species, and thus have utility for detecting hybrids in practice. Thus, a discriminant function analysis was performed in Statistica v7.0 (StatSoft, Inc., Tulsa, USA) on the quantitative morphological data for individuals identified as 'pure' *P. nerifolia* and 'pure' *P. lepidocarpodendron* by the Bayesian analysis. A minimum posterior probability of 0.8 was used to define individuals as 'pure' species. Capitulum length and perianth length were inverse transformed (1/x) as these characters were found to have non-normal distributions. Chi-square tests were used to determine which qualitative morphological character traits best discriminated between the parent species. Once again, only 'pure' individuals as defined above were used in this analysis.

Using morphological traits identified above, the population was surveyed in order to identify the full extent of introgression. Eight linear transects , starting at plot A, and heading out in different bearings (20°, 60°, 100°, 140°, 180°, 220°, 260° and 340° from N) were conducted. Every individual (*P.nerifolia, P. lepidocarpodendron* or putative hybrid) encountered along and within 2 m to each side of the transect was scored for the four quantitative morphological characters found to be best at discriminating between the two parent species. The distance of each individual from plot A was also recorded. 'Morphological' hybrids were then mapped using Arcview 3.3 (ESRI, Redlands CA, USA).

Results

Ordinations

The ordinations of morphological data showed most of the separation of plots along the first principal axis (Fig's 4&5). Individuals from plots A and F were generally located at the extremes of the first principal axis and did not overlap with one another. Individuals from the remaining plots formed a continuum between the extremes of plots A and F, but most individuals showed more overlap with those in plot F. This was not readily apparent from the PCO of the qualitative morphological data, but this was because most individuals from plots B to F possess exactly the same qualitative morphological characters and are thus projected on exactly the same points (Fig. 5). The genetic data showed a very similar pattern to the morphological data (Fig. 6). This was supported by the significantly positive, although weak, correlations between the first principal axis values of the morphological data and those of the first principal axis of the genetic data ($r^2 = 0.376$; $p < .000$ for factor 1 of the ISSR markers versus PC1 of the quantitative morphological data; $r^2 = 0.256$; $p < .000$ for factor 1 of the ISSR markers versus factor 1 of the qualitative morphological data). This implies the morphological grade was genetically, and not environmentally based.

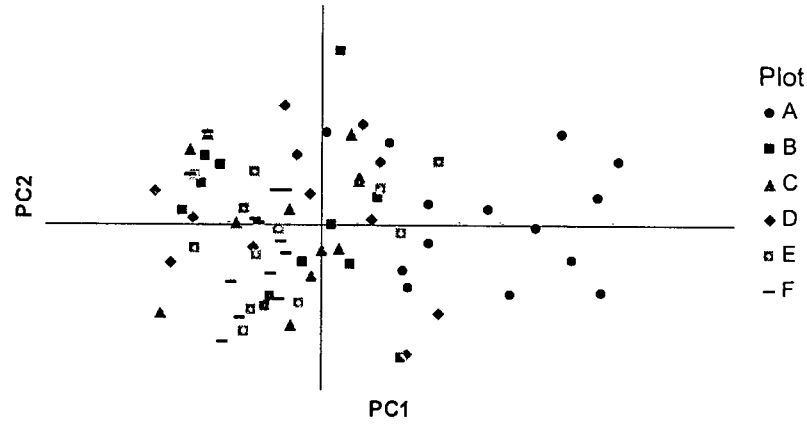

Fig. 4. Plot of the first and second principal components of a principal component analysis of the quantitative morphological characters for each individual sampled in the six transect plots. The plot from which each individual was sampled is indicated by a unique symbol. PC 1 and PC 2 explain 37.70 % and 20.08 % of the variance in the data respectively.

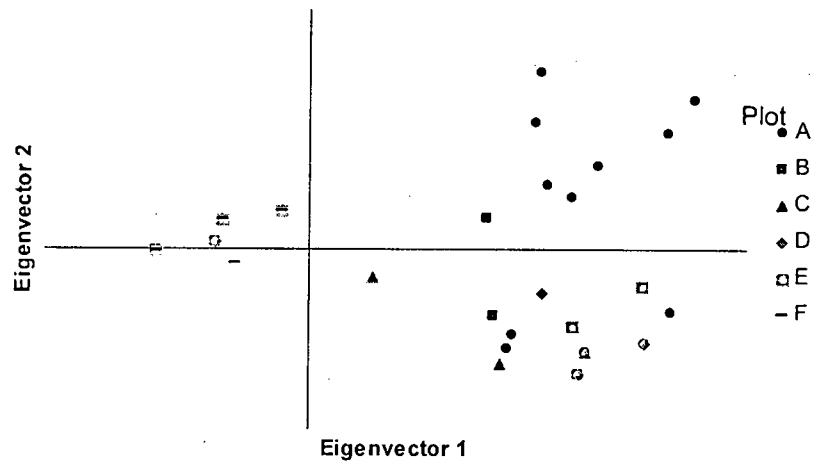

Fig. 5. Plot of the first and second eigenvectors of a principal coordinate analysis of the qualitative morphological characters for each individual sampled in the six transect plots. The plot from which each individual was sampled is indicated by a unique symbol.

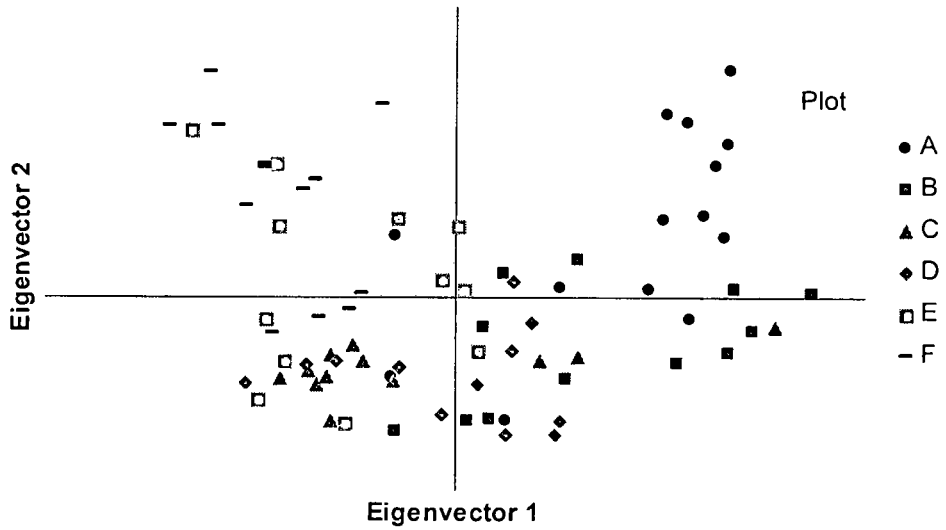

Fig. 6. Plot of the first and second eigenvectors of a principal coordinate analysis of the ISSR marker binary matrix for each individual sampled in the six transect plots. The plot from which each individual was sampled is indicated by a unique symbol.

Spatial distribution of ISSR variation

The spatial structuring of the ISSR data was well illustrated by the DOH analysis, which correctly assigned a high proportion of genotypes to the plots from which they were sampled (Table 3). Gene introgression was shown to be spatially related by a strong correlation between the inter-plot genetic distances, which were calculated on the basis of assignment probabilities, and the geographic distance between plots (Mantel test: $r^2 = 0.256$, n = 75, $p < 0.0001$) (Fig. 7).

Table 3. Plot assignment of sampled individuals using the Doh assignment calculator. The top row represents the plot to which individuals were assigned and the first column represents the plot from which they were sampled.

	A	B	C	D	E	F
A	11	0	0	2	1	0
B	1	9	1	0	1	0
C	1	0	8	0	3	0
D	0	1	3	6	2	0
E	1	2	2	2	1	4
F	0	0	1	0	3	9
	14	12	15	9	11	13

44

12

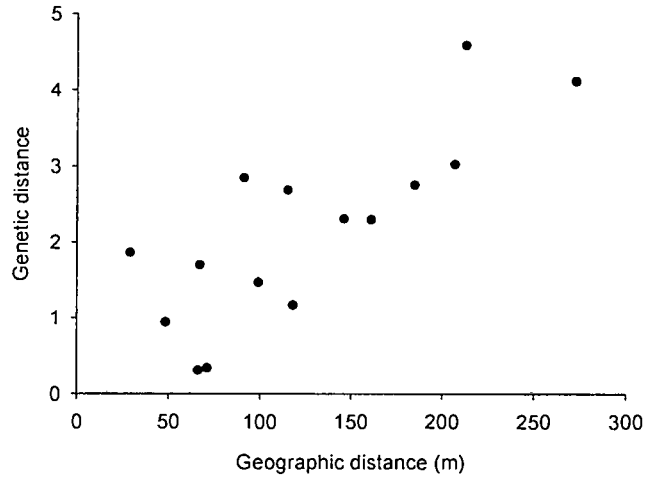

Fig. 7. The geographic distance between plots versus the associated genetic distance between plots (as calculated in DOH: see methods for description).

Identification of hybrids

NewHybrid (Anderson & Thompson 2002) identified a high proportion of the individuals in plots A and F as having a high posterior probability of being *P. nerifolia* or *P. lepidocarpodendron* respectively (Fig. 8). Most individuals from the plots between A and F were assigned fairly high posterior probabilities of being F1 or F2 hybrids. The proportion of individuals in a plot with at least some posterior probability of being *P. nerifolia* steadily decreased to zero moving from plot A to F. By contrast, individuals generally had increasingly higher posterior probabilities of being *P. lepidocarpodendron* with increasing distance from plot A. These spatial patterns of ISSR variation, however, are dependent on very few loci, as shown by the Kullback-Leibler divergence value of each locus (Fig. 9). These divergence values are a relative distance measure between the prior and posterior distributions and give an idea of the relative usefulness of a locus in discriminating between two species (Kullback & Leibler 1951). Two loci (n and o) were the most informative in separating the two *Protea* species, and another three, or possibly four, loci were of intermediate use (a, c, m and ad). Of these informative loci, all except 'ad' came from primer 813.

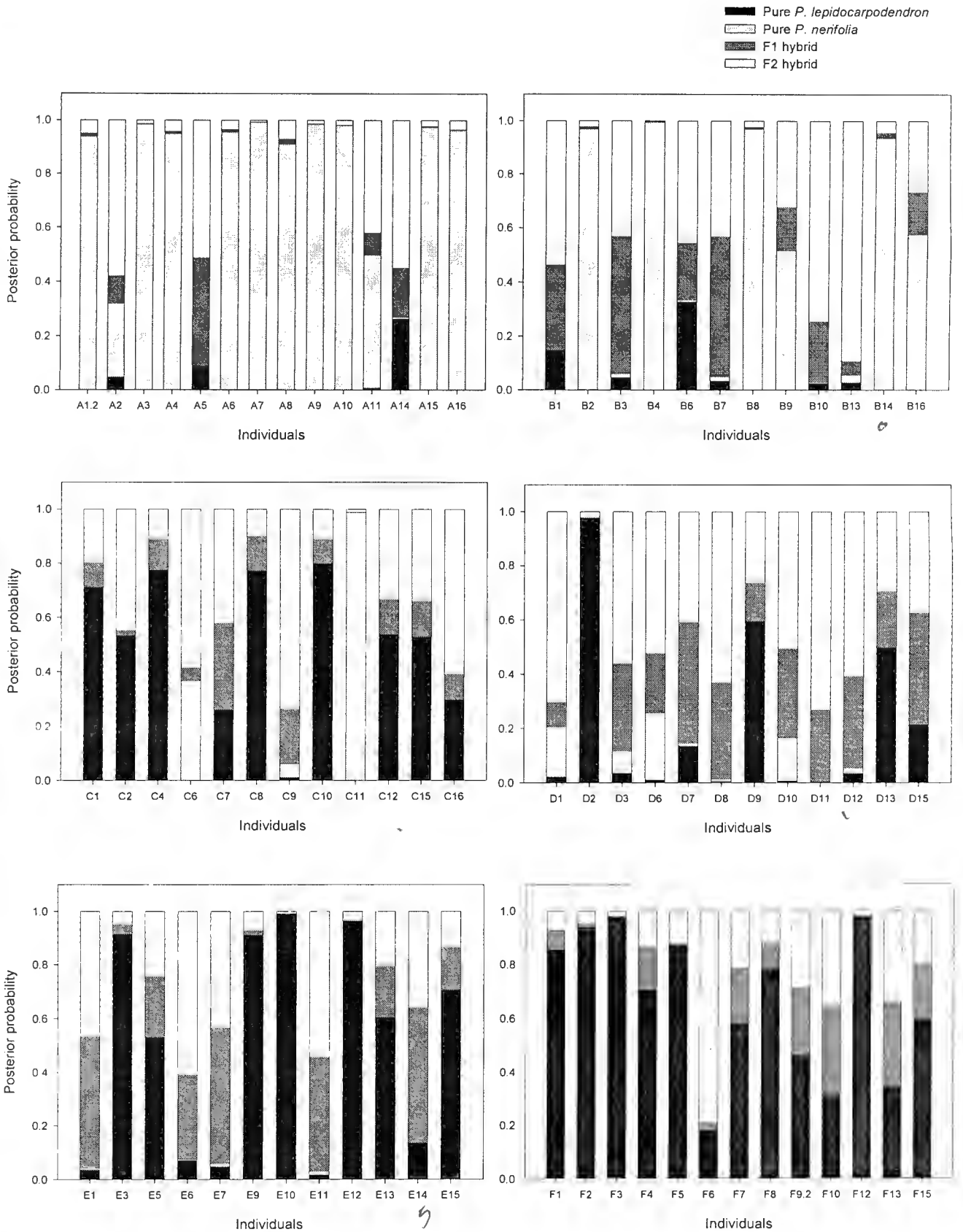

Fig. 8. Posterior probabilities of specific genotype frequency categories for all individuals sampled in the transect plots. Neri = *P. nerifolia*, Lepi = *P. lepidocarpodendron*, F1 = first generation hybrid, F2 = second generation hybrid.

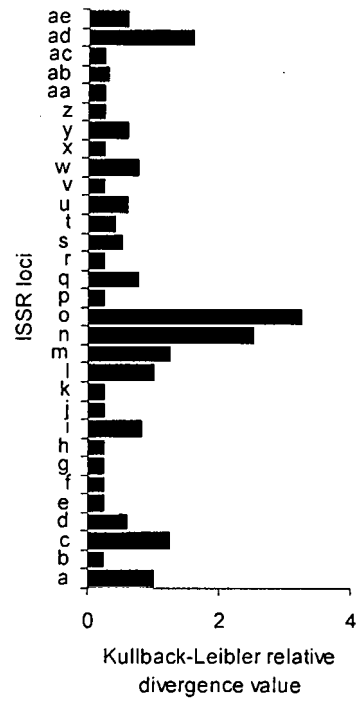

Fig. 9. Relative usefulness of each locus in separating out the two species of *Protea* as calculated in NewHybs (Anderson & Thompson 2002) – measured using the Kullback-Leibler divergence values of each locus.

Identifying hybrids in the field

A discriminant function analysis (DFA) and chi-square tests identified five characters as being especially useful in discriminating between parent species, and these had great utility in identifying hybrids in the field. The DFA identified capitulum length as the single morphological character most effective at identifying individuals as either *P. nerifolia* or *P.lepidocarpodendron* (Wilk's λ: 0.401, Wilk's λ Capitulum length: 0.549, $F(6,19) = 4.728$, $p < .01$). A one-way ANOVA showed capitulum length of *P. nerifolia* to be significantly longer than that of both hybrid and *P. lepidocarpodendron* individuals, and that of hybrids significantly longer than in *P. lepidocarpodendron* ($F = 23.88$, d.f. = 72, $p < .0001$) (Fig. 10). Chi-squared tests identified the following traits as being most effective at discriminating between the two parent species: lower involucral bract colour, reflexion of lower involucral bract tips, upper involucral bract colour and curling of upper bracts of previous season's flowers (Table 4).

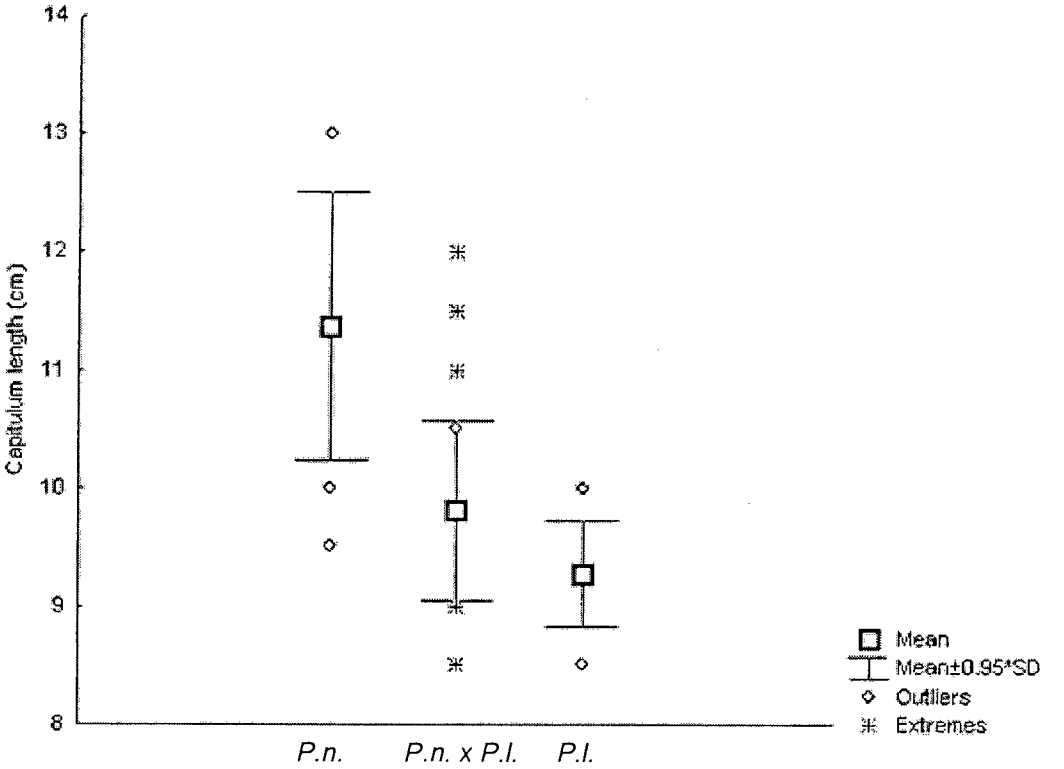

Fig. 10. Capitulum length of individuals identified as being *P. nerifolia*, *P. lepidocarpodendron*, or a hybrid (either F1 or F2 generation) by the programme NeyHybs (Anderson & Thompson 2001). (*P.n.*: Mean = 11.37 cm, sd = 1.2 cm, n = 15; *P.n.* x *P.l.*: Mean = 9.82 cm, sd = 0.80 cm, n = 49; *P.l.* : Mean = 9.27 cm, sd = 0.47 cm, n = 11).

Table 4. Frequencies of qualitative morphological states in the 'pure' parent species as identified by the Bayesian analysis. X^2 tests of association were used to test whether a particular trait was observed more often than expected in *P. nerifolia* and less often than expected in *P. lepidocarpodendron* individuals or vice versa. Associated X^2 and p values given for each trait (d.f. = 3; for all characters). The percentage of each genotype with a particular trait is also given, with the actual number of individuals found with that trait in brackets. Significant traits given in bold type, those used for assessing spatial extent of introgression highlighted in grey.

Trait	State	*P.nerifolia*	*P.lepido-carpodendron.*	X^2	*P*
Colour of lower involucral bracts	Pale with brown margin	80 % (12	0	16.34	p < .001
	Entirely brown	20 % (3)	100 % (11)	0	
Reflexion of tips of lower involucral bracts	Not reflexed	73 % (11)	0	13.98	p < .01
	Reflexed	27 % (4)	100 % (11)	0	
Hairiness of lower bract margin	Glabrous	40 % (6)	0	5.72	p = .127
	Hairy	60 % (9)	100 % (11)	0	
Colour of upper involucral bracts	Pink or carmine	93 % (15)	100 % (11)	22.24	p < .0001
	Cream	7 % (1)	0	0	
Hairs on upper involucral bract tips	Black, only along margin	33 % (5)	0	4.54	p = .209
	Black/white, extend down a few cm from margin	67 % (10)	100 % (11)	0	
Curling of upper bracts of previous season's flowers	Curled	86 % (12)	10 % (1)	13.47	p < .01
	Not curled	14 % (2)	90 % (9)	0	
Colour of stylar band	White	55 % (6)	9 % (1)	5.24	p = .156
	Purple	45 % (5)	91 % (10)	0	
Colour of awn	Brown-yellow	58 % (7)	0	9.22	p < .026
	White	42 % (5)	100 % (11)	0	

Spatial extent of hybridisation

The transects in which morphological characters were surveyed identified hybrids up to 256 m from the source *P. nerifolia* population, and in every transect conducted. This was based on individuals possessing traits characteristic of *P. nerifolia* and indicated widespread introgression of *P. nerifolia* genes into the *P. lepidocarpodendron* population at Silvermine (Fig. 11). Hybrids, being those individuals with posterior probabilities of being either *P. nerifolia* or *P. lepidocarpodendron* of less than 0.8, have spread much further than would be suggested by morphological characters such as capitulum length and colour of upper involucral bracts (Fig. 12).

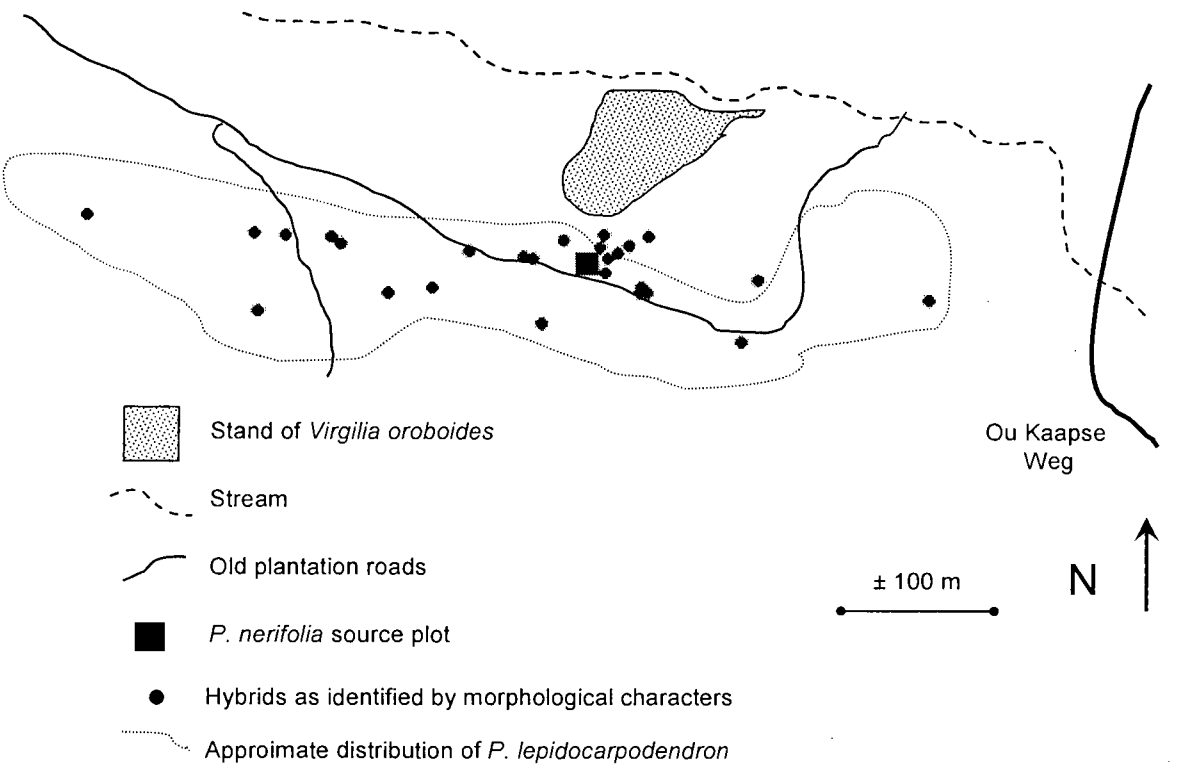

Fig. 11. Spatial extent of hybridisation as determined by identifying 'morphological' hybrids based on qualitative morphological characters found to be the most useful in distinguishing between *P. nerifolia* and *P. lepidocarpodendron*.

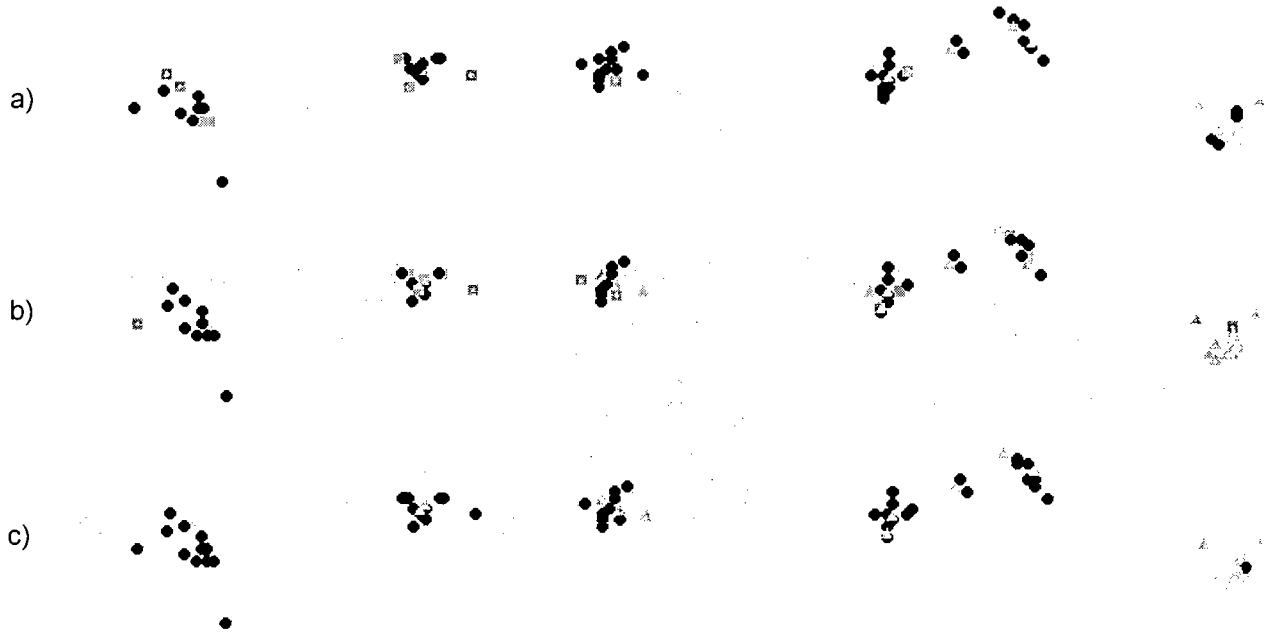

Fig. 12. Comparative spatial distributions of a) individuals identified as having high posterior probabilities of being *P. nerifolia, P. lepidocarpodendron* or a hybrid by NewHybrid (Anderson & Thompson 2002); b) differences in capitulum length; c) upper involucral bract colour. Light grey triangles represent *P. nerifolia*, grey squares represent hybrids and black dots represent *P. lepidocarpodendron* (or their character states).

Discussion

Evidence of hybridisation between naturally allopatric species highlights the permeability of species boundaries to genetic introgression (Rieseberg *et al.* 2004, Mallet 2005) and raises concerns about the translocation of species from a conservation perspective (Levin *et al.* 1996, Rhymer & Simberloff 1996). The Cape Floristic Region has over 9000 species, most of which are thought to have diverged fairly recently (Richardson *et al.* 2001, Linder 2003), which suggests there has not been enough time for most of these species to become reproductively incompatible with other closely related species (Levin 2000, Rieseberg *et al.* 2004). *Protea* is one of the dominant and most characteristic genera in the Cape Floristic Region (Cowling 1992) and the popular use of the genus in horticulture to create cultivars (International Protea Register 2003) draws attention to the ability of species within the genus to

hybridise with one another. The status of this genus as a national symbol, and its popularity in the world flower trade (Leonhardt & Criley1999), stresses the importance of its conservation and the dangers of translocating *Protea* species. This study provides the first evidence of hybridisation in the genus *Protea*, and in particular hybridisation between a locally indigenous species with a formerly allopatric species.

Hybridisation between introduced *Protea nerifolia* and the locally indigenous *Protea lepidocarpodendron* at Silvermine is evidenced by a gradient in morphological and ISSR variation that is spatially structured along a 300 m transect spanning the contact zone between the two species. The correlation between the morphological and genetic data emphasises that the observed spatial variation in morphology is genetically based, and records confidence in the genotypic pattern. These correlations, although significantly positively correlated, show much variance. This, however, is to be expected as Simple Sequence Repeats (SSRs) are present in both coding and non-coding regions of the genome, thus often representing neutral variation and therefore, ISSR markers are not expected to always be directly linked to morphological traits (Zietkiewicz *et al.* 1994, Godwin *et al.* 1994, Scarano *et al.* 2002, Huff & Mazer 2003). The spatial structuring of genetic variation was confirmed through the use of an assignment analysis and suggests a grade in genetic similarity from plot A to plot F. The highly significant correlation between genetic distance and geographic distance then suggests a spatial relationship to the introgression of *P. nerifolia* genes into the population of *P. lepidocarpodendron* at Silvermine. Three individuals from plot A were assigned to plots as far away as D and E, which is interesting as it implies very little genetic difference across the entire transect. That ISSRs were able to detect spatial variation in genetics between two closely related species, and at a scale as fine as 300 m long transect illustrates the utility of ISSR markers in detecting spatial variation in genetics. ISSRs have been used as evidence of hybridisation for a number of putative natural hybrids (Wolfe *et al.* 1998a&b, Archibald *et al.*

2004), and for apparent hybridisation between native and introduced species (Ayres & Strong 2001, Baumel *et al.* 2003). Thus, ISSRs can be used to provide strong support for hybridisation, complementary to morphological data.

Both *P. nerifolia* and *P. lepidocarpodendron* are serotinous species (Rebelo 1995) and therefore experience episodic recruitment after fires. With two fires having passed through Silvermine since the introduction of *P. nerifolia* to the area, it is no longer possible to identify true parent species versus hybrids. The ability of NewHybrid (Anderson & Thompson 2002) to reconstruct the genetic history of an individual is invaluable in this regard. The results of this programme suggest that hybridisation between the two *Protea* species has been very extensive, extending from the site of introduction of *P. nerifolia* all the way to individuals in plot F. It also suggests that genetic introgression is directly related to distance from the site of introduction of *P. nerifolia*. That the programme identifies some individuals within the plot A as having low posterior probabilities of being *P. nerifolia* suggests that some individuals that appear to be *P. nerifolia* morphologically are in fact hybrids.

Since it was not possible to indentify pure *P. nerifolia* or *P. lepidocarpodendron* individuals *a priori* it was thought best to rely on morphological characters, should the removal of hybrids be considered. From a management perspective it is quite useful that only one quantitative character was found to be the best at distinguishing between *P. nerifolia* and *P. lepidocarpodendron* individuals. Similarly, a number of qualitative morphological characters such as the colour of upper and lower involucral bracts, colour of awn, the curling of lower involucral bract tips, and the curling of upper involucral bracts of the previous season's flowers provide easy to use taxonomic characters to distinguish between the two species. Some of these qualitative morphological characters, however, were found not to be 100 % species-specific. It is highly probable that these individuals were given incorrect assignments by the Bayesian analysis. This is because only a few loci were used to distinguish between the two

species (Anderson & Thompson 2002), and only two primers were used for the analysis and not at least three as has been suggested as a minimum needed to genetically fingerprint an individual (Esselman et al 1999). Inherent in the Bayesian analysis is a number of assumptions that may affect the reliability of the results. Firstly, NewHybs assumes that loci are unlinked and this can lead to an overestimation in the certainty with which hybrids are identified. Secondly, it assumes that no ISSR markers are "tightly linked to any loci that are under selection", an assumption they note "will be violated to an extent" (Anderson & Thompson 2002). However, the Bayesian methods employed by NewHybs to identify hybrids have been used successfully, and produce similar results to other assignment methods, in a number of other studies (Cianchi *et al.* 2003, Hanfling *et al.* 2005).

Both morphological and genetic data suggest that penetration of *P. nerifolia* genes has been extensive. from the transect plots seems to suggest that hybridisation has extended to as far away as 270 m from the site of introduction of *P. nerifolia*. Transects conducted to detect hybrids using morphological characters shows the presence of morphological hybrids throughout the distribution of the *P. lepidocarpodendron* population at Silvermine. Extrapolating from the underestimation of the spatial extent of hybridisation in the plot transect, it is highly likely that almost all individuals within this population have experienced introgression of *P. nerifolia* genes. This poses a very real dilemma as to whether only individuals with *P. nerifolia* morphological characters, or the entire population, should be removed. Based on our results, we would predict that even with the removal of all plants showing morphological traits specific to *P. nerifolia*, the next generation at Silvermine should contain individuals with *P. nerifolia* traits.

Allendorf *et al.* (2001) provide a categorisation of hybridisation, and suggest that hybridised populations may be worthy of conservation under certain circumstances. They contend that the number of pure populations remaining, the phenotypic differentiation between

hybrids and pure individuals, and the threat that hybrids pose to remaining pure populations should all be considered when trying to decide on the conservation value of a hybrid population. In the context of this study, there presumably are remaining pure populations of *P. lepidocarpodendron* on the Peninsula as *P. nerifolia* still only has a limited distribution in the region (see Appendix, Fig. 13). However, *P. lepidocarpodendron* has a nearly contiguous through the Peninsula (see Appendix, Fig. 14). Thus, there appears to be little value in trying to conserve these hybrids according to the criteria of Allendorf *et al.* (2001). This, however, ignores the ecological role played by *P. lepidocarpodendron* on the Peninsula. The removal of the entire population at Silvermine might have major negative effects on, for example, food availability for territorial Cape sunbirds at Silvermine (Frost & Frost 1980). On the other hand, the importance of preserving the genetic integrity of *P. lepidocarpodendron* may not be that important if the two species occupy the same niche as has been shown for *Protea obtusifolia* and *Leucadenron meridianum* (Laurie *et al.* 1997).

This study gives direct evidence of how "reproductive barriers rarely protect the entire genome from gene flow in recently diverged species" (Rieseberg *et al.* 2004). *P. nerifolia* has a slightly earlier flowering period than *P. lepidocarpodendron* (Rebelo, 1995), but this phenological barrier is insufficient to prevent reproduction between these two species. The biological species concept argues that species are 'real' and have "special special, species-level qualities: 'isolating mechanisms', 'cohesion', and 'coadapted gene complexes'; species (act) as vessels for the 'storage and protection of genetic variation'" (Mallet 2005). In the case of this study, lack of reproductive barriers between *P. nerifolia* and *P. lepidocarpodendron*, prevents classification of these two entities as separate species if strictly applying the biological species concept (Rieseberg *et al.* 2004). Rieseberg *et al.* (2004) suggested a more encompassing interpretation of the biological species concept, that states that "the amount of reproductive isolation required for species status is simply that necessary to preserve the

evolutionary changes associated with population systems following changes in the location of suitable habitat (e.g. following climatic shifts) or in the proximity of other population." Even under this interpretation of the biological species concept, *P. nerifolia* and *P. lepidocarpodendron* cannot be classified as separate species, even though they are strikingly different morphologically, and possess unique genetic characters. Other approaches to classifying species, such as a cladistic approach, based on defining species using the unique characters they possess, may be practical from conservation viewpoint (Vogler & Desalle 1994).

The implications of this project are enormous both from a theoretical, and conservation perspective. This study highlights the inadequacy of biologically-based species concepts at defining species where there appear to be no reproductive barriers between the two species. From a conservation perspective, this study is the first to give decisive evidence of hybridisation caused by the translocation of an "indigenous" South African species to an area in which it formerly did not occur. At present, the planting of "indigenous" plants is promoted by government and conservation organisations alike, but more often than not, little thought is given to whether the species being planted occur, or used to occur there. Perhaps, because of the risks of planting so-called indigenous species in gardens near wild populations of species, it may be preferable to plant exotic species unlikely to hybridise with local species in these areas. Another important result of this study is that it demonstrates the dangers of establishing protea plantations for the cut flower market in, or near, natural stands of proteas. This study has shown that after just two generations, hybrids have spread up to nearly 300 m away from the site of the initial introduction of the non-local species.

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Appendix

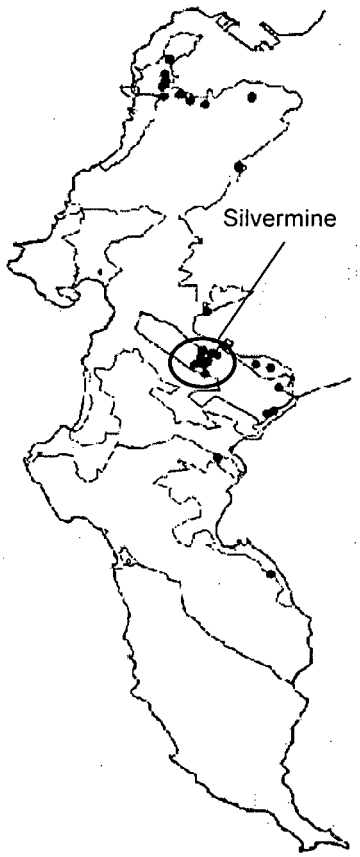

Fig. 13. *Protea nerifolia* introductions on the Cape Peninsula.

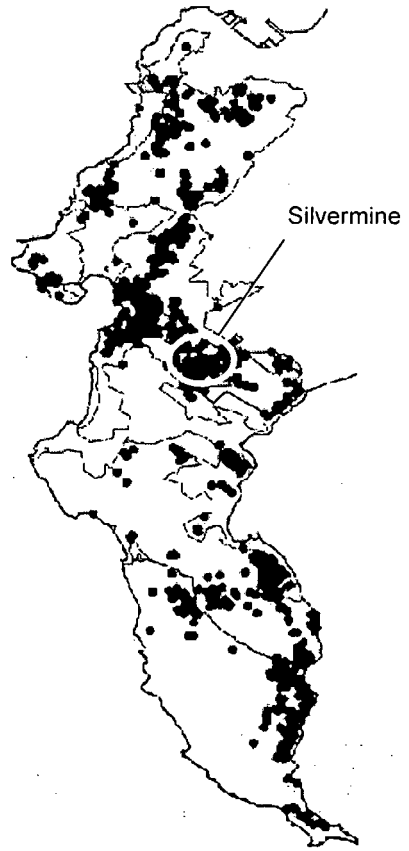

Fig. 14. *Protea lepidocarpodendron* distribution on the Cape Peninsula.

Source: Protea Atlas Project – Interim distribution maps (http://protea.worldonline.co.za/idm_ca.htm).