The Prince or the Depauperate? Population Genetics of the Rare, Closed-flower *Erica occulta*

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Abstract:

Understanding breeding systems and gene flow is important for the conservation of rare species. Despite this, they remain relatively unknown for Fynbos species. *Erica occulta* is a rare, dull, closed-flower *Erica* that is restricted to the few limestone cliffs near Groot Hagelkraal on the Southern Agulhas Plain, South Africa. Its restricted range and small population make it a vulnerable species and determining its breeding system and genetic diversity may inform the best method of conservation. The aims of this study were to determine the genetic diversity of *E. occulta* and how it varies with distance and to make inferences about the breeding system and gene flow based on genetic diversity measures. Due to the population's small, isolated nature, it was predicted that the species would have a low number of alleles and low heterozygosity. Given its floral morphology it was predicted to be a self-fertilising plant which may manifest as a further loss of heterozygosity and strong genetic isolation by distance. Diversity measures including number of alleles, heterozygosity and isolation by distance were determined using six, previously developed microsatellite loci. *E. occulta* was found to be remarkably genetically depauperate and the population was characterised by very few alleles ($mean\ N_a = 2.5$), almost no heterozygosity ($mean\ H_o = 0.07$), high inbreeding ($F_{IS} = 0.37$) and significant isolation by distance ($Mantel\ r = 0.687$), suggesting that it is a self-fertilising species. The findings of this study provide a base line for a naturally small, localised population of *Erica* and may prove useful in decision making about how best to conserve *E. occulta* and other rare endemics.

Introduction:

The Ericaceae is a large, cosmopolitan family made up of 126 genera and approximately 4000 species (Stevens 2001). This adaptable, stress-tolerant family has evolved a variety of life forms to survive in various habitats (Luteyn 1988; Kirsten & Oliver 1992), and is prolific in the temperate regions and cooler montane regions of the tropics. Members of the Ericaceae display a diverse array of morphological features particularly in their floral characteristics, which have coevolved with a variety of pollination strategies. It has been suggested that this coevolution with pollinators and the ability to self-fertilise (Luteyn 1988), as well as their seeder-type life history (Pausas & Keeley 2014), were key factors that drove rapid speciation within this family.

Members of the Ericaceae are particularly well-represented in South Africa's Cape Floristic Region (CFR), which is renowned for its high biodiversity and endemism (Goldblatt & Manning 2002; Linder 2003). *Erica* is the largest clade within the sub-family Ericoideae (Kron et al. 2002), and makes up a large proportion of the highly diverse Cape flora. Of the 840 species of *Erica* currently recognised in the CFR (Pirie et al. 2011), roughly 680 are endemic to the region (Oliver 2000b). The extraordinary species richness of the CFR is thought to be a result of geographic and parapatric radiation which was facilitated by the diversity of niches within the habitat mosaic (Connell & Orias 1964). This mosaic has arisen because edaphic factors combine to produce steep ecological gradients (Goldblatt & Manning 2002; Linder 2005). Other factors that have been suggested as drivers of radiation are fire disturbance regimes and pollinator specialisation (Linder 2005). Fire drives speciation because plants typically employ either a reseeder- or a resprouter-type life history strategy and the traits associated with these strategies are largely mutually exclusive. Interestingly, some species, such as *Erica coccinea*, exhibit both strategies, but these are employed by different morphs rather than by the same plant (Segarra-Moragues & Ojeda 2010). Pollinator specialisation has resulted in the evolution of a wide variety of floral morphologies (Johnson & Steiner 2003).

While Europe has only 23 species of *Erica* (Oliver 2000a), all of which exhibit a bell-shaped morphology, congenerics in South Africa sport a wide variety of floral forms and colours (Schumann & Kirsten 1992). The phylogeny of *Erica* was initially based on these morphological characteristics, but it has subsequently been established, by sampling internal transcribed spacers (ITS), that there have been numerous macro-morphological shifts resulting in converging morphologies (Pirie et al. 2011). Despite this new technique the phylogeny of many species remains unresolved. South African *Erica* species employ several different modes of pollination (anemophily, entomophily or zoophily), (Rebelo & Siegfried 1985). These modes drive the evolution of species' floral morphology, which means that their pollination modes can often be surmised from their corolla morphology (Oliver 1991). However, there are very few studies to confirm the link between morphology and pollination syndrome, such that Pirie et al. (2011) had to speculate on this. Likewise, the breeding systems that *Erica* species employ, that is whether they are self-fertilising (hereafter referred to as "selfing") or out-crossing, have received very little attention in the literature (Turner 2012). The small ranges of the Cape *Erica* species, along with their highly specific pollination strategies, can leave populations at risk of extinction.

Small, localised populations are more vulnerable to Allee effects than larger, wide ranging ones which have a greater buffering capacity (Lande 1993; Szucs et al. 2014). Larger populations typically exhibit more genetic variation (Szucs et al. 2014), while small populations exhibit little variation and can therefore suffer decreased fitness due to inbreeding depression and genetic drift (Hansson & Westerberg 2002). Smaller populations generally lose heterozygosity faster than larger populations because the rate of loss is approximately equal to half of the effective number of individuals in each generation (Futuyma 1986). Genetic drift influences genetic variation in two ways: 1) it causes a decline in heterozygosity, leading to the fixation of alleles within populations, and 2) it causes an increase in differentiation between populations. The above-mentioned phenomena will be more apparent if a population has gone through a bottleneck or is the result of a founder event (Ellstrand & Elam 1993).

Small, localised populations are also more vulnerable to stochastic environmental events (Caughley 1994). In conservation, focus is often placed on the effects of habitat fragmentation and degradation on the genetics of small populations because of the problems associated with low genetic diversity and inbreeding. This focus may be misguided however, as the success of any conservation effort depends on the identification of the real threats the species is experiencing (Ellstrand & Elam 1993). In an influential article, Lande (1988) argued that, in most cases, harmful environmental factors will affect small populations before genetic factors can impact them.

Traditionally, inbreeding is considered to be harmful as it can lead to the build up of recessive diseases and decrease fitness. However, while some species risk a heavy investment in mutualistic relationships for pollination and seed dispersal (García et al. 2012), others can evolve to be self-compatible. A strategy of self-fertilisation offers 'reproductive assurance' in situations where other individuals are too distant or pollinators are absent (Charlesworth & Wright 2001). Some species that are adapted to self-fertilising breeding behaviours can continue to function despite the changes to their genomic composition (Zawko et al. 2001). However, the effects of the selfing breeding system will be evident in the genetic diversity. Selfing populations commonly have higher population genetic sub-structuring than out-crossing species due to the decrease in gene flow (Palacious et al. 1999). In a review, Hamrick & Godt (1990) found that selfing species had 51% of their genetic diversity between populations, while out-crossed, wind pollinated species had less than 10% of their total genetic diversity between populations. Variation in diversity between populations was found to be mainly influenced by breeding system and by seed dispersal to a lesser extent.

The most common explanation for why selfing evolves is the 'reproductive assurance hypothesis' (Jain 1976). The evolution of self-fertilisation from out-crossing has occurred on many occasions in flowering plants (Takebayashi & Morrell 2001) and their morphology, ecology and genetics can be profoundly influenced by this transition (Barrett et al. 2014). The transition is particularly associated with major changes in floral morphology and reproductive traits (Sicard & Lenhard 2011). *Erica* species are predominantly protogynous and this strategy for herkogamy, when coupled with the later maturing stigma, can result in a selfing strategy if there is a slight change in timing.

Along with 'reproductive assurance', selfing allows for a resource allocation shift away from pollen production (Charlesworth & Pannell 2001) and attractiveness, to seed set (Lloyd 1987). Selfing also results in the twofold transmission of genes, when out-crossers can only transmit one set. Despite the advantages of selfing only 20-25% of

plant taxa are predominantly selfers (Barrett & Eckert 1990). It has been suggested that selfing is an evolutionary dead end and thus far no selfers have been found to make the transition back to out-crossing (Takebayashi & Morrell 2001). High rates of selfing restrict recombination and reduce levels of heterozygosity. This increases the probability of the fixation of deleterious mutations by genetic drift (Charlesworth & Wright 2001). Some plants, like *Banksia marginata* can self-fertilise successfully, but their progeny thus produced suffer from inbreeding depression such that they do not survive to maturity (Vaughton & Ramsey 2006). While pollination studies may indicate that a species can self-fertilise successfully they cannot indicate whether the surviving progeny are from self-fertilised or out-crossed seeds. Pollination studies alone are also not sufficient to estimate the contributions of different pollinators to seed production in selfing, resource-limited species (Steenhuisen & Johnson 2012). They should therefore be performed alongside genetic studies.

It is well-known that there is a close association between breeding system and the distribution of genetic variation (Loveless & Hamrick 1984; Hamrick et al. 1991). In small, isolated populations, genetic population sub-structuring and differentiation will develop if gene dispersal is limited due to self-fertilisation and/or the spatially restricted distribution of seed. Determining the population-level genetic structure of species gives us insight into the levels of gene flow and connectivity between populations in different geographic regions (Kyle & Strobeck 1999). This information is useful when trying to conserve or manage these species as it gives insight into their migration routes, their dispersal and population boundaries and breeding systems (Kyle & Strobeck 1999).

In early population genetics studies, diversity was determined using allozymes or random amplified polymorphic DNA (RAPD), but these studies were often hampered by low sensitivity to fine scale genetic variation. With such low variation very little can be inferred about mating systems, population movement or migration (Paetkau et al. 1995). More recently, with the advent of polymerase chain reaction (PCR) technology in the 1980s, population genetic structure could be estimated using highly variable microsatellite markers. These markers allow the detection of variability in highly isolated or severely bottlenecked populations where allozymes or RAPD would normally detect little or no variation (Hughes & Queller 1993).

Microsatellites are small, non-coding regions, also known as simple sequence repeats, that are now widely used to genotype organisms based on naturally occurring variation (Kalia et al. 2010). They are useful because they are prone to deletion and duplication during replication and are therefore hyper-variable (Kalia et al. 2010). While the mechanisms for producing new alleles are not completely understood it has been suggested that they are mostly due to slippage during replication (Tautz & Schlötterer 1994; Innan et al. 1997). Most of these errors change only one repeat unit each time and slippage rates vary for repeat sizes and species (Kruglyak et al. 1998). Because these small changes occur frequently, at a rate of about 2×10^{-4} (Thuillet et al. 2002; Sun et al. 2012), microsatellites can be used to assess allelic variation at a shorter time scale. They can be used to compare levels of observed allelic variation to levels expected at Hardy-Weinberg Equilibrium (HWE). They are also useful because even small, degraded DNA samples can be amplified using PCR to provide reproducible results (Queller et al. 1993; Paetkau et al. 1995; Kyle & Strobeck 1999). Their hyper-variability gives them very strong discriminating power such that they can

be used to detect fine-scale genetic differentiation (Streiff et al. 1998) and to determine parentage (Isagi et al. 2000). It also gives them a high efficiency in distinguishing between populations (Rowe et al. 1998).

The above-mentioned applications have led to microsatellites becoming popular tools in conservation genetics, particularly in the study of rare or isolated species (Kikuchi & Isagi 2002). By providing estimates of genetic distance between populations they have been used to determine gene flow and population boundaries of Blue-listed North American wolverines (Kyle & Strobeck 1999) and to examine the breeding behaviour of arctic grizzly bears (Craighead et al. 1998). In populations of long-distance roaming Canadian polar bears where one might expect their populations to homogenise, microsatellites were used to show that gene flow is still restricted between local populations (Paetkau et al. 1995). Microsatellite DNA markers developed by Paetkau & Strobeck (1998) have been used to trace the origins of black bears, *Ursus americanus*, after translocation projects reintroduced them to Arkansas and Louisiana in the 1950s and 1960s (Csiki et al. 2003). They have also been used to study the level of genetic differentiation among the current and 1980, trans-located populations of natterjack toads (*Bufo calamita*) in Britain (Rowe et al. 1998). These are prime examples of microsatellite-based studies, the majority of which have focused on large, charismatic predators or on endangered animals.

Despite the potential utility of the technique, microsatellite analysis has been used in relatively few studies of plant genetics. Of the existing plant studies, most focus on the disease resistance or productivity of economically important crop species (Kalia et al. 2010). A few focus on assessing genetic diversity to inform conservation (Hogbin & Peakall 1999) and some investigate the genetic consequences of different pollination systems (Schlebusch et al. 2014). Thus, there is still a paucity of data on microsatellite variation in plant populations (Kikuchi & Isagi 2002) and particularly in fynbos species.

Microsatellites have proved to be versatile genetic markers, but they are not without limitations. Four of these limitations are: 1) shorter repeat sections can be complicated by artefacts of PCR stutter or preferential amplification, 2) some markers are not polymorphic and this prevents them from being useful for making inferences (Paetkau et al. 1995), 3) the development of primers can be painstaking and costly and 4) most microsatellites are developed for a particular species and will work on most closely related species, but the percentage of loci that successfully amplify declines with decreasing levels of relatedness.

For microsatellites to be used to describe deviations from HWE, their recombination must not be selected for or against by the environment otherwise this will interfere with allelic ratios. Microsatellites were initially thought to be non-coding regions of DNA, and this is true for the most part (Blair et al. 2003). It has however been found that some microsatellite regions can influence the expression of particular genes (Donaldson & Young 2008) and so care also needs to be taken to ensure that the primers are developed only for selectively neutral, non-coding repeat loci. This then allows the assumption that all variation in allele frequency within and between populations is caused only by the breeding structure which should affect all alleles and loci in the same way (Craighead et al. 1998). We then assume that changes in allelic frequency over time are due to genetic drift and not divergent selection.

For some plant species it is important for conservation and management to concentrate on maintaining high levels of genetic variability through promoting out-crossing and mixing genotypes (Zawko et al. 2001). However, some species develop strategies to overcome a lack of mates due to low numbers and in these cases population genetics may not play an important role in the survival of the species. Ellstrand & Elam (1993) put together a review of population genetics which they hope will serve as an action framework for conservationists looking to effectively conserve a plant species. They state that if factors such as population size and fitness are decreasing, or factors such as degree of isolation are increasing, these serve as warning signs that a species may be vulnerable. They suggest that when a high proportion of the species' genetic variation is distributed between, rather than within, populations, it is advisable to preserve more populations rather than few populations with many individuals. In this way, conservationists may preserve greater allelic and genotypic diversity within the species in question. Ellstrand & Elam (1993) also emphasise that it is important to distinguish between species that might have gone through a recent bottleneck, and those species that have a history of persistent small population size as this will influence the strategy for their conservation.

The IUCN uses factors such as those suggested by Ellstrand & Elam (1993) as criteria to identify which species are under threat of extinction. One such species, *E. occulta*, has recently been upgraded from its red-listing as 'Rare' in 1996 to its current listing as 'Vulnerable' (Raimondo et al. 2009; Turner & Oliver 2009). It is an extremely localised species occupying an area of less than 6km^2 as it is confined to the limestone cliffs on the Southern Agulhas Plain near Groot Hagelkraal (Turner & Oliver 2007). Its range is threatened by more intense fires due to the invasion of *Acacia cyclops* and the area is the potential site for an Eskom nuclear power plant. *E. occulta* flowers from July to October and its flowers are hidden in a mass of hairy leaves giving it its name (from 'occultus' meaning hidden or secret). It is naturally an extremely rare, local endemic and there are approximately 100 individuals (Midgley, *pers. comm.*).

Breeding systems and gene flow are unknown in *E. occulta*, but given its dull, cryptic floral morphology I predict that it is likely to self-fertilise and will have high levels of homozygosity. Due to the small, isolated nature of the *E. occulta* population, I also hypothesised that it would have low genetic diversity and that genetic differentiation will increase with geographical distance. The aims of this study were to determine the genetic diversity of *E. occulta* and how this varies with distance, and to make inferences about this species' breeding system and gene flow from the diversity measures. Eight microsatellite loci, previously developed for *E. coccinea*, were screened of which 6 amplified successfully and these loci were used to characterise the genetic variation within the single population of *E. occulta*.

Very little is known about the population genetics of Fynbos plants and, while conventionally small populations are considered to be vulnerable to extinction due to their low genetic diversity, this study will provide some perspective on the levels of genetic diversity of a naturally small population of *Erica* species. Genetic information has helped to inform conservation efforts for other genetically depauperate species (Kyle & Strobeck 1999), but might indicate that breeding intervention is unnecessary for *E. occulta.* The results will provide a comparison for other small populations of fynbos species and contribute to an area which is thus far relatively unknown.

Materials and Methods:

Study Site and Species

E. occulta samples were collected from individuals growing on cliff faces (100 – 150 m above sea level) in the low-nutrient, alkaline, limestone hills above Groot Hagelkraal in the Kleyn Kloof Private Reserve (34°39'25"S, 19°33'44"E) on the Southern Agulhas Plain, Western Cape, South Africa. These cliffs provide a unique habitat and there are few other areas where the limestone is similarly exposed (Mucina & Rutherford 2006). This is the only known population of *E. occulta* and it consists of approximately 100 individuals (Midgley, *pers. comm.*). The area experiences hot, dry summers and cool, wet winters, and receives between 500 and 1000mm of rain fall annually (Goldblatt 1997). The landscape is dominated by fynbos scrub, which thins out towards the top of the koppies where topsoil is shallow or absent. *E. coccinea* subsp. *uniflora* and *E. nudiflora* are among the few species that grow in the cliff crevices alongside *E. occulta*. The plant is a small (≤ c.30 cm), closed-flower Erica with cream-brown flowers that are approximately 5mm long.

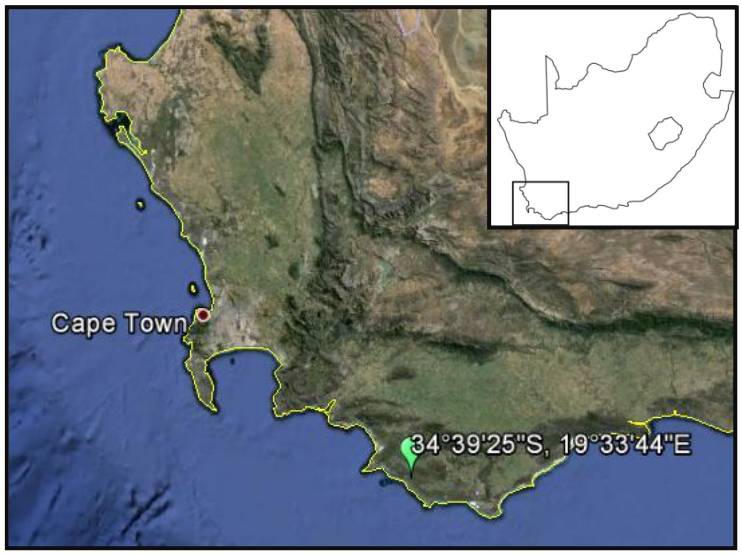

Figure 1 The location of the limestone cliffs in Klein Kloof Private Nature Reserve with an inset indicating the location within South Africa (Google Earth 2014).

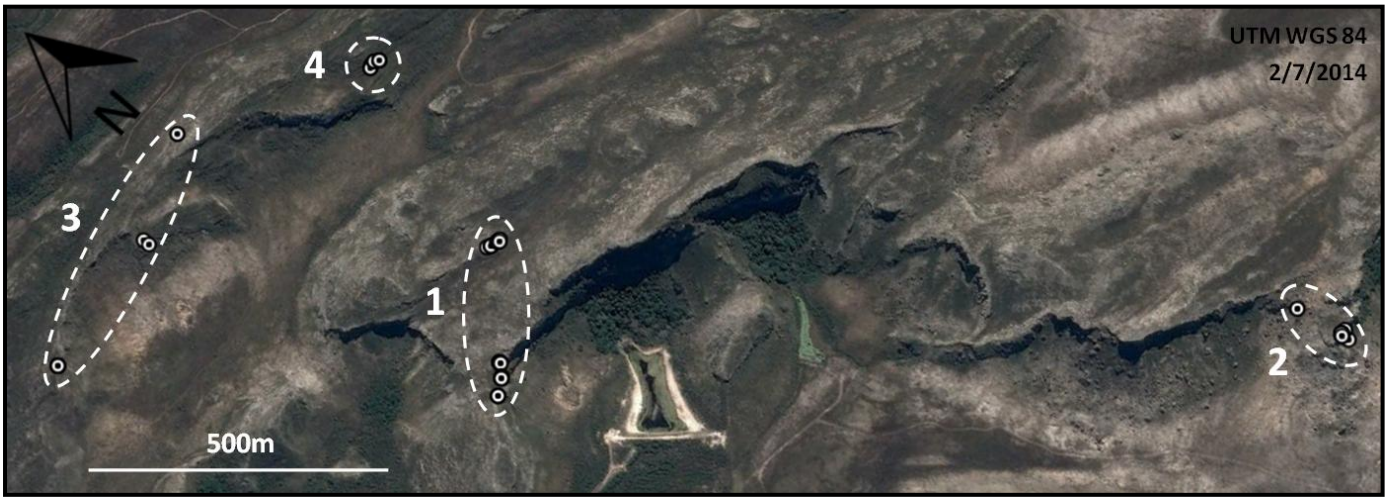

Figure 2 An aerial view of the limestone cliffs where the *Erica occulta* individuals were sampled. Closely clustered individuals are superimposed and the sub populations, determined by geographical proximity, are indicated by dashed lines (Google Earth 2014).

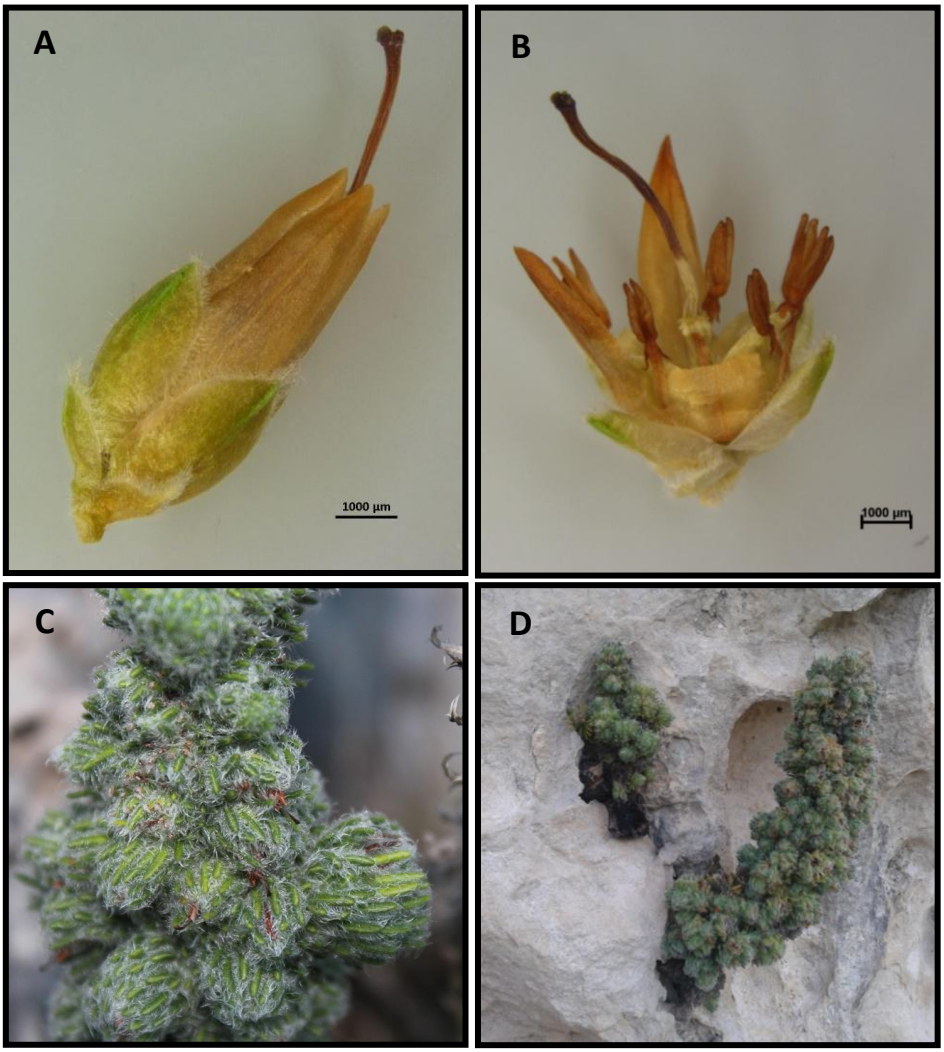

Figure 3 A) a fresh *Erica occulta* flower[1], B) a silica-dried *Erica occulta* flower opened and with three petals removed[2], C) a flowering *Erica occulta* individual *in situ*[3], and D) mature *Erica occulta* individuals growing from crevices in the limestone cliffs of the Southern Agulhas Plain, South Africa[1] ([1]S. Troost; [2]P. Muller; [3]T. Rebelo)

Population Genetics

DNA Extraction and Microsatellite Amplification

Fresh leaves were sampled from each of 50 individuals in June and July of 2014 and their GPS co-ordinates were noted. Samples were stored individually on silica gel in a sealed bag at –80°C until extraction (Chase & Hills 1991). Genomic DNA was extracted from leaves using a modified Dellaporta method (Dellaporta et al. 1983) and compared to that extracted using a modified Bellstedt method (Bellstedt et al. 2010). The DNA extraction quality was compared using a Nanodrop ND1000 Spectrophotometer (Thermo Scientific) and the integrity was checked by comparing the amplified trn-LF region on an ethidium bromide stained 1.7% agarose gel (for the results and comparison see Appendix 1 as it is not further discussed). The following modified Bellstedt method was preferred for its efficiency, and used throughout. Approximately $5mg$ of chopped leaf material was weighed out and placed in a 2ml eppendorf with 3 stainless steel grinding balls and 1ml of grinding buffer (see Appendix 2 for buffer solution contents). The leaf material was macerated for 15 minutes in an oscillating mill (Retsch® MM400) until there were no sizable particles. This was then centrifuged at low speed ($\pm$4000rpm) for 15 seconds and $4\mu l$ of the supernatant was then transferred to a $200\mu l$ PCR tube with $25\mu l$ of GES buffer (see Appendix 2 for buffer solution contents). This tube was then incubated in a thermocycler (GeneAmp® PCR System 9700) for 10 minutes at 95°C and then for 5 minutes at 4°C.

Exactly $1\mu l$ of the extracted DNA was used in PCR amplification. $25\mu l$ reactions were set up for PCR to amplify each of six microsatellite loci. Each $25\mu l$ PCR cocktail included: $12.5\mu l$ of 2xKAPA2G Fast/Hifi HotStart ReadyMix (KAPA Biosystems), $5pmol$ ($1.25\mu l$ of $5\mu M$) of one pair of the eight fluorescently-labelled (forward) and unlabelled (reverse) microsatellite primers developed by Segarra-Moragues et al. (2009) for *E. coccinea* (Table 1), and the remaining volume of nuclease-free water. The PCR conditions consisted of one hold cycle of 4 minutes at 95°C and then 35 cycles of 3 steps: denaturation step at 98°C for 20 seconds, annealing step at 56°C for 15 seconds and an extension step at 72°C for 20 seconds. The final extension was performed at 72°C for 6 minutes. In order to check whether amplification was successful and that there was no contamination between samples, $5\mu l$ of the amplified PCR product was visualised electrophoretically on 1.5% agarose gels, stained with ethidium bromide and run for 40 minutes. The remaining $20\mu l$ of amplified product was then stored at –20°C before PCR clean-up.

PCR clean-up and DNA Analysis

PCR products were sent to the Stellenbosch Central Analytical Facility where a post PCR clean-up was performed using the Nucleofast 96 well PCR plate from Macherey Nagel. The protocols supplied by the manufacturer were implemented on a Tecan EVO150 robotic workstation. After clean-up, the products were run on an Applied Biosystems 3730xl DNA Analyser (Life Technologies) using Lizz75to500-250 as the internal lane size standard. The fluorescent dye in each forward primer allowed for detection and sizing of the amplified fragment lengths and they were assigned allelic sizes using GeneMapper® v4.0 software (Applied Biosystems 2005). Unsuccessful PCRs were input as '0' for statistical analyses (Blyton & Flanagan 2012).

Statistical Analyses

Measures of genetic variability and fit to Hardy-Weinberg equilibrium were calculated per polymorphic locus and for the population as a whole using the Microsoft Excel add-in GenAlEx 6.501 (Peakall & Smouse 2006; Peakall & Smouse 2012). These measures were the number of alleles per locus, observed and expected heterozygosity and the inbreeding coefficient (F_{IS}). Hedrick's (2005) standardised G_{ST} ($G"_{ST}$) which corrects for a small number of populations was calculated as a measure of differentiation among the sub-populations.

Pairwise matrices of genetic distance and geographic distance were calculated using GenAlEx 6.501. The significance of association between genetic distance and the geographic distance was tested using a Mantel test (Mantel 1967) with 9999 permutations. A correlogram was generated to assess the spatial structure of the population using the spatial autocorrelation method using 9999 permutations and 10 000 bootstraps for even size classes. Distances were manually input to achieve approximately even samples per class at 0.06km; 0.45km; 0.762km; 1.485km and 2.25km. To determine the apportionment of the total variation an analysis of molecular variance (AMOVA) was performed using GenAlEx 6.501 using the sub-population classifications (9999 permutations).

Table 1 Summary of the genetic characteristics of eight microsatellite loci developed for *Erica coccinea* (Segarra-Moragues et al. 2009)

Locus	Primer sequence (5'-3')	Repeat Motif	Annealing temperature	Size (bp)
Ecoc108	F:<NED>CGTGCCCTAAACGAAGATAGA R: GATTACCCAGAAGAGATGGATG	$(CT)_6T(CT)_4$	56°C	165
Ecoc115	F:<NED>GTTGAGCTAGTGGAGGGAAGTG R: CTTTCTTCAAATCACACCTAAGCA	$(CA)_8$	56°C	168
Ecoc117	F:<6-FAM>GGGTCAGTGATAATTTTGAACCT R: TCACTTTGATGGCAATGGACT	$(GA)_5G(GA)_5$	56°C	157
Ecoc122	F:<PET>TCCTTGAACTAGGTTTCATCTCTAA R: GCCATCGGCTGTTATTACTTATC	$(GA)_{11}$	56°C	172
Ecoc132	F:<PET>GATGTTAGCAATCCAGGTCCA R: ATCCAGATGCAAAGAACAAAGG	$(CT)_7$	56°C	185
Ecoc142	F:<PET>TTTGAAGGGTTTCTGCTTCTGG R: AGAGGATGGGAAGTGAGGTGAA	$(GA)_{16}$	56°C	239
Ecoc431	F:<VIC>AGTGTATGTGCTACGCCTCTGG R: GAAGGGTTCCCACTCGTCTTC	$(CT)_{14}$	56°C	183
Ecoc446	F:<6-FAM>GCGCATTGTGAATTACGCTTT R: AGCGTCTGACACCGCTACAC	$(GA)_{10}TA(GA)_2(GT)_{11}$	56°C	235

NED, 6-FAM, PET and VIC are the fluorescent dyes from Applied Biosystems.

Results:

Of the eight microsatellite loci, developed for *E. coccinea* (Segarra-Moragues et al. 2009), that were screened for *E. occulta*, Ecoc115 and Ecoc122 failed to amplify. The remaining six amplified successfully and were selected for subsequent analysis. The no-template negative controls were clear, in each case, indicating that there was no contamination (Appendix 3). The cleaned up PCR products showed clear resolution on the ABI analyser. Samples that were amplified using 2xKAPA2G Fast HotStart ReadyMix (KAPA Biosystems) had alleles 1 base pair longer due to the addition of a non-template A on the 3' end and these were accounted for accordingly. Corrected values are indicated in Appendix 4. Genotypic data were obtained for the six microsatellite loci for all 50 individuals, bar 1% which failed to amplify.

Of the six successfully assayed microsatellite loci two were monomorphic (Ecoc108 and Ecoc431) and were excluded from the statistical analyses. Overall, a total of 12 alleles for all 50 individuals were scored and these fell within the expected size range (Table 2). The overall pattern of nuclear genetic diversity in *E. occulta* was characterised by a very low average number of alleles per locus ($N_a = 2.5$) (Table 2). At loci Ecoc132 and Ecoc446 the second allele was so rare such that there was only one heterozygote in the population (Figure 4). Observed heterozygosities range from 0.02 to 0.20. Chi-square heterogeneity testing revealed that the observed heterozygosity (H_o) was significantly lower than the expected heterozygosity (H_e) in loci Ecoc117 and Ecoc142 ($p < 0.001$)(Table 2). Ecoc132 and Ecoc446 did not significantly deviate from HWE as all 50 individuals were homozygous bar a single individual. The population showed an extreme heterozygote deficiency as the overall inbreeding coefficient (F_{IS}) was 0.37 (Table 2).

A Mantel test for the association between geographic distance genetic distance among individuals showed a significant correlation ($Mantel\ r = 0.687; p = 0.0001;\ 9999\ permutations$) indicating that there is strong isolation by distance. Spatial autocorrelation showed that this was due to individuals that were closer than 450 meters from each other being significantly more genetically similar than would be expected by chance alone (Figure 5). Beyond 450 meters there is little evidence for spatial structure of genetic variation by distance. Finer scale genetic structure could not be determined as sample sizes per class distance became too small.

An AMOVA for the four sub-populations indicated that the majority of the variation seen in the population was accounted for among the sub-populations (59%), while 19% and 22% was accounted for within and among individuals respectively. Hedrick's (2005) standardised G_{ST} indicated that, overall, there was strong differentiation among the sub-populations (Table 2).

Table 2 Comparison of *Erica occulta*, a species with a naturally small population from the limestone cliffs of the Southern Agulhas Plain, South Africa, compared to *Erica coccinea*, a wider-ranging species with a reseeder and a resprouter morph, using the genetic characteristics of six microsatellite loci (Segarra-Moragues et al. 2009). [Sample size (n); No of alleles (N_a); Observed- (H_o) and expected (H_e) heterozygosity; chi square (χ^2) tests for Hardy-Weinberg Equilibrium; inbreeding coefficient (F_{IS}) and Hedrick's (2005) standardised G_{ST} ($G"_{ST}$).]

		Erica coccinea								***Erica occulta***								
		Reseeder (n=30)				Resprouter (n=30)				(n=50)								
Locus	**Allele Size Range (bp)**	N_a	H_o	H_e	F_{IS}	N_a	H_o	H_e	F_{IS}	**Allele Size (bp)**	N_a	H_o	H_e	χ^2	p	F_{IS}	$G"_{ST}$	p
Ecoc108	157-175	9	0.80	0.80	0.00	6	0.83	0.82	-0.02	159	1	-	-	-	-	-	-	-
Ecoc117	155-169	7	0.57	0.65	0.13	3	0.30	0.32	0.06	160,161,162	3	0.02	0.38	83.889	<0.001	0.83	0.770	<0.001
Ecoc132	179-219	12	0.83	0.90	0.08	9	0.73	0.71	-0.03	181,183	2	0.02	0.02	0.005	0.943	-0.05	0.007	0.359
Ecoc142	223-267	15	0.87	0.88	0.01	14	0.77	0.89	0.14	225,227,229	3	0.20	0.66	47.467	<0.001	0.08	0.896	<0.001
Ecoc431	159-175	5	0.43	0.51	0.14	4	0.50	0.66	0.24	166	1	-	-	-	-	-	-	-
Ecoc446	205-309	25	0.87	0.95	0.09	15	1.00	0.89	-0.13	186,194	2	0.02	0.02	0.005	0.943	-0.05	0.007	0.359
Overall	-	12.2	0.70	0.76	0.08	8.5	0.68	0.73	0.06	-	2.5	0.07	0.27	-	-	0.37	0.714	<0.001

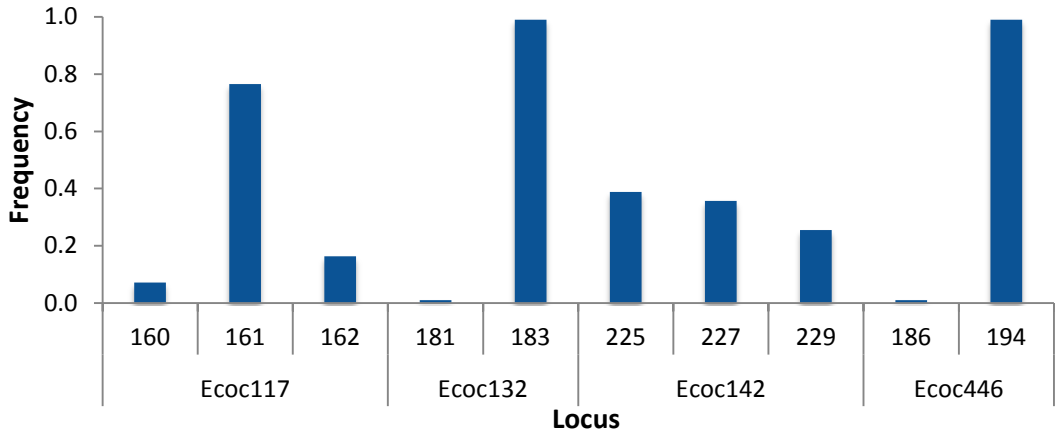

Figure 4 The allelic frequency per locus in 50 individuals from one small population of *Erica occulta* growing on the limestone cliffs of the Southern Agulhas Plain, South Africa.

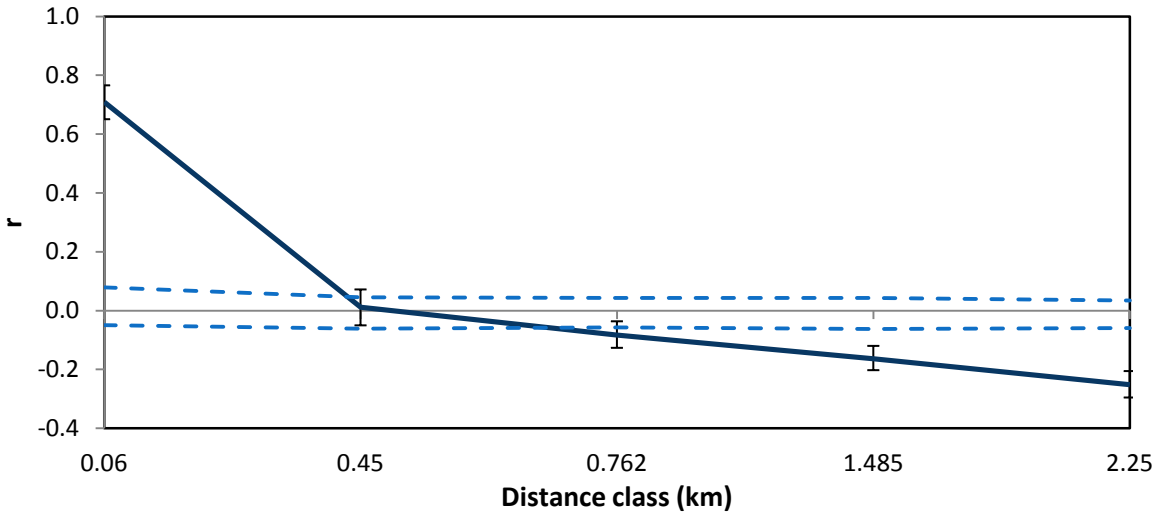

Figure 5 Spatial autocorrelation of *Erica occulta* to investigate the rate at which the genetic autocorrelation coefficient (r) based on 4 nuclear microsatellite markers decreases with distance (km). Upper and lower confidence limits (dashed lines) bound the 95% confidence interval about the null hypothesis of no spatial structure for the population as determined by 9999 permutations.

Discussion:

The allele sizes of the six amplified microsatellite loci (Table 2) fell within the range of published sizes for *E. coccinea* (Segarra-Moragues et al. 2009). Four of the six microsatellite loci showed some variation in allele size across the 50 individuals, but the overall pattern of nuclear genetic diversity in *E. occulta* was characterised by a very low number of alleles (Table 2). Only 12 alleles were scored for all six loci over the 50 individuals. This is exceedingly low when compared to other South African *Erica* species which displayed many more alleles for the same six loci in just a few individuals. A single *Erica intermedia* individual had 8 alleles, two individuals of *Erica imbricata* and *Erica placentiflora* had 19 and 20 alleles respectively, and four individuals of *Erica coarctata* had 19 alleles accumulated over the six loci (Segarra-Moragues et al. 2009). Should a greater sample of these species be taken one would expect this number of alleles to increase further, indicating that they are drastically more gentically diverse than *E. occulta*. The *Erica* phylogeny is not yet sufficiently resolved to be able to attribute the low genetic diversity to *E. occulta* being a young species (Pirie et al. 2011).

E. occulta had, on average, 2.5 alleles per locus (Table 2). This is extremely low when compared to a nearby population (Sandberg, Cape Agulhas) of, the abundant and widely-distributed, *E. coccinea* which had an average of 11 alleles per locus across 30 individuals (Segarra-Moragues & Ojeda 2010). *E. coccinea* consistently had a higher number of alleles per locus and none of its loci were monomorphic, while *E. occulta* displayed complete homozygosity for two loci. While only 50 individuals were sampled for the present study, it was believed that this was approximately half of the existing population and thus, even if every individual were sampled, the number of alleles per locus would be unlikely to equal that of *E. coccinea*. This finding supports the hypothesis that this small, isolated population would have low genetic diversity. It is suspected that this low diversity is due to the amplified effects of genetic drift within this small population.

Direct comparisons of microsatellite variation with other studies can be problematic because different loci generate different levels of variation (Kikuchi & Isagi 2002). However, because *E. occulta* is the second species to be studied using this set of microsatellite loci, the diversity found here is benchmarked against other studies which used different loci. These comparisons place the genetic diversity of *E. occulta* in context.

Population size and breeding system influence the genetic diversity of species. *E. occulta* has a similar average number of alleles per locus to *Magnolia sieboldii* ssp. *japonica* ($N_a = 3.8$ across $c.30$ individuals), which grows in small, isolated populations (Kikuchi & Isagi 2002), but had far fewer than the wide-ranging *E. coccinea* mentioned above. When compared with an out-crossing, bird-pollinated species, *Liparia splendens* ssp. *splendens*, (Schlebusch et al. 2014) ($\mathrm{N_a} = 8$ across $c.100$ individuals) it is not surprising that *E. occulta* has fewer alleles. However, when compared with a self-fertilising species one might expect the levels of genetic diversity in *E. occulta* to be similar, but its displays much less variation than *Arabidopsis thaliana* ($N_a = 10.6$ across $c.40$ individuals), (Innan et al. 1997). This is likely because *A. thaliana* is wide-ranging and Innan et al. (1997) sampled 42 ecotypes from the world over.

This suggests that it is the combination of small population size and a selfing syndrome which leads to such diminished genetic diversity in *E. occulta*.

The level of total observed heterozygosity ($mean\ H_o = 0.07$) was an order of magnitude lower than those reported for both the reseeding ($mean\ H_o = 0.696$) and the resprouting ($mean\ H_o = 0.683$) morphs of *E. coccinea* from the Cape Peninsula (Segarra-Moragues et al. 2009). In comparison to large populations of a predominantly out-crossing Tasmanian tree species, *Eucalyptus pauciflora* ($mean\ H_o = 0.79$) (Gauli et al. 2014), *E. occulta* exhibits low observed heterozygosity which may be due to the small population size. Compared to levels of heterozygosity observed in isolated populations of *Magnolia sieboldii* ssp. *japonica* ($mean\ H_o = 0.366$), which are comprised of only tens of individuals (Kikuchi & Isagi 2002), values reported here suggest that the observed high levels of homozygosity are more than just a relic of a small population.

In a review of allozyme variation, in 449 plant species, Hamrick & Godt (1990) found that endemics contain significantly less genetic diversity than widespread species. This finding was consistent with the results reported here for *E. occulta*. Hamrick & Godt (1990) suggested that this is the case because endemics may consist of small, more ecologically limited populations that are susceptible to loss of variation by drift or bottlenecks. These same authors also found that endemic species had the same levels of genetic differentiation between populations as widespread species, meaning that endemics are experiencing similar levels of gene flow. However, this is not consistent with the findings of the present study. The overall inbreeding coefficient ($F_{IS} = 0.37$) in *E. occulta* indicates far higher levels of homozygosity than one would expect to see if there was random mating. High levels of homozygosity can be a product of selfing or very poor seed dispersal (Nybom & Bartish 2000). Heterozygosity may also decrease if pollination distance was smaller. This was seen in a comparison between a rodent- and a bird-pollinated species. Schlebusch et al. (2014) found that the inbreeding coefficient was higher for *Liparia parva* ($\mathrm{F_{IS}} = 0.233$) than for *L. splendens* ssp. *splendens* ($\mathrm{F_{IS}} = 0.102$) and suggested that this loss of heterozygosity was a consequence of rodent pollination.

In a review of species with different life history traits, Hamrick & Godt (1996) found that, on average, selfing species with gravity-dispersed seeds had five-fold more genetic differentiation than out-crossing, wind dispersed species. The present study supports that finding, as *E. occulta* displays strong genetic isolation by distance ($Mantel\ r = 0.687, p = 0.0001$), which could be due to the limited gene flow by dispersal and pollination. To date, neither the reproductive- nor the pollination biology of *E. occulta* has been investigated. However, *E. occulta's* seeds are not unusual for Ericas (Turner *pers. comm.*) and I suspect that dispersal is by wind, as in other Ericas, where seeds do not travel more than 100m (Bullock & Clarke 2000). I also predict that the individuals self-fertilise as there is no visible attempt to attract pollinators. The flowers are small, closed, dull-coloured and hidden in a mass of hairy fine leaves and no insects were seen to be interacting with the plants or the flowers during the sampling period (*pers. obs.*). Pollination experiments and camera trapping were not attempted due to the difficult terrain on which these species grow, but should be the subject of further study and would ascertain whether *E. occulta* can self-fertilise successfully.

Hamrick & Godt (1996) found that life form and breeding system have a significant influence on between-population diversity. This is evident when comparing the genetic isolation by distance reported here for *E. occulta*, a suspected selfing shrub, with *Eucalyptus pauciflora*, which is a predominantly out-crossing, tree species ($Mantel\, r = -0.12, p = 0.044$). In the case of *E. pauciflora,* genetic distance was found to be correlated up to 93km away, and populations within 38km were significantly more similar in their microsatellite affinities than was expected by chance (Gauli et al. 2014). *E. occulta* individuals appeared to be interacting within only 450 meters. Finer scale genetic structure could not be determined, but if the species is selfing, with a greater sample size, one would expect to see geographic interaction-distance decrease even further.

If *E. occulta* was more common, more individuals could be sampled to make sub-population analyses possible, but the rarity of the species precludes this. This study included a preliminary analysis of population differentiation, but is not statistically strong as the number of individuals per sub-population is low. Nevertheless, strong differentiation between the four sub-populations was found ($G"_{ST} = 0.714$) (Table 2), and along with the high proportion of the diversity explained among populations (59%), this suggests that there is very little gene flow between populations. This is what I would expect to find as, in other selfing species, most of the genetic variability is found among populations (Kamran-Disfani & Agrawal 2014) rather than within populations, as out-crossing taxa display (Nybom & Bartish 2000). Further population genetics studies, using the same microsatellite loci, could be attempted for a number of other small, isolated *Erica* populations for comparison. Further pollination and breeding system experiments could also be carried out to determine whether *E. occulta* is self-compatible, whether it does self-fertilise naturally and whether the progeny are equally as fit as out-crossed individuals, or whether they suffer from inbreeding depression.

Small, isolated populations are more greatly affected by genetic drift and this influences their genetic sub-structure, increasing differentiation between populations (Hogbin & Peakall 1999). The increasing genetic distance with geographic distance between *E. occulta* individuals could be a product of short dispersal distance and the low likelihood of a seed landing in a crevice on a suitable cliff. However, because the genetic isolation by distance is so strong it supports the hypothesis of restricted gene flow by selfing. This cannot be claimed conclusively without further experimentation. The isolation by distance may also be a relic of patchy fires as post-fire recruitment from a single source would enhance differentiation as seen in *E. coccinea* by Segarra-Moragues & Ojeda (2010).

The cliffs provide refugia from fires, but it appears that even if fire occurs on the slopes leading up to the cliffs *E. occulta* cannot survive. This is likely because their crevices do not contain enough soil moisture to compensate for the sudden decline in stem water potential. While the fynbos is fire-adapted and many species need smoke to germinate (Brown & van Staden 1997), an increase in fire frequency or intensity may be the greatest threat to this erica as its range is so limited. With the spread of the alien, invasive *Acacia cyclops* an increase in fire intensity is a possibility. Another threat is that of the predicted temperature increase as a result of global warming. *E. occulta* appear to grow only on the cool, south-facing cliffs. I speculate that this is because they cannot survive great temperature stress as they do not have deep soil to root into and subsequently do not have access to sufficient soil

moisture for evaporative cooling. In the event of the predicted increased temperatures due to anthropogenic greenhouse gas emissions, *E. occulta* has no cliffs further south to escape to. Limestone patches are limited to the small stretch from Groot Hagelkraal west to Gansbaai, with a small patch at Potberg and on the Cape Peninsula (Mucina & Rutherford 2006). Recent research suggests that some *Erica* species, if isolated from fire for a long time, may lose their ability to recognise fire as a cue (Leonard et al. 2014). If this were the case, in *E. occulta,* it would make it further unlikely that they would migrate from their refuge. While some plant species are showing evolutionary and plastic responses to climate change (Franks et al. 2014), it is unclear whether *E. occulta* would be able to make shifts great enough to survive off the cliffs that they currently inhabit. Their adaptability may also be impeded by their low genetic diversity. Many conservation efforts focus around increasing and protecting diversity, but for a highly localised species such as *E. occulta* stochastic effects may cause extinctions before genetic factors do.

E. occulta's population status appears to be stable (Turner & Oliver 2009) and there are recruits growing on most of the sampled cliffs (*pers. obs.*). It does not appear to be suffering from inbreeding depression, but this could be investigated by examining seed set and germination percentages in a future study. It is this study's recommendation that, rather than trying to promote out-crossing and gene flow in a species that is likely adapted to selfing, the alien invasive species be removed and the area is kept protected from anthropogenically related wild-fires. While currently part of the range is protected within the Kleyn Kloof Reserve, a large proportion of the population grows on land owned by Eskom and is the site of a proposed nuclear power plant (Turner & Oliver 2009). While the cliffs are an unlikely place for construction, the increased human activity in the area will pose a greater threat and this should preferably be prevented. A larger reserve system would also protect more of the total genetic variability of the species (Hogbin & Peakall 1999) which, while it may not be important for the direct survival of the species as it is now, may be beneficial for future speciation over evolutionary time.

Conclusion:

As predicted, *E. occulta* is extremely genetically depauperate. The low allelic diversity, high homozygosity and strong isolation by distance are thought to be a product of the small, isolated nature of the population and support the prediction that *E. occulta* employs a selfing strategy. Despite the low genetic diversity, the population appears to be stable and it still occupies all of its available range. It is likely that the greatest threat that *E. occulta* faces is not its low genetic variation, but rather stochastic environmental events. If the alien invasive species in the region are left unchecked, local fires will burn at temperatures that are high enough to negate the protective effects of the cliffs. The results of this population genetics study, of a naturally small population, provide a useful comparison for other small, isolated species and can inform the conservation of *E. occulta*.

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References:

Applied Biosystems, 2005. GeneMapper® v4.0 Software.

Barrett, S.C.H., Arunkumar, R. & Wright, S.I., 2014. The demography and population genomics of evolutionary transitions to self-fertilization in plants. *Philosophical Transactions of the Royal Society of London B*, 369, pp.1–9.

Barrett, S.C.H. & Eckert, C.G., 1990. Variation and evolution of mating systems in seed plants. In S. Kawano, ed. *Biological Approaches and Evolutionary Trends in Plants*. New York, USA: Academic Press, pp. 229–254.

Bellstedt, D.U., Pirie, M.D., Visser, J.C., de Villiers, M.J. & Gehrke, B., 2010. A rapid and inexpensive method for the direct PCR amplification of DNA from plants. *American Journal of Botany*, 97(7), pp.65–68.

Blair, M.W., Pedraza, F., Buendia, H.F., Gaitán-Solís, E., Beebe, S.E., Gepts, P. & Tohme, J., 2003. Development of a genome-wide anchored microsatellite map for common bean (*Phaseolus vulgaris* L.). *Theoretical and Applied Genetics*, 107(8), pp.1362–74.

Blyton, M.D.J. & Flanagan, N.S., 2012. A comprehensive guide to: GenAlEx 6.5. *Journal of the American Animal Hospital Association*, 48(5), pp.1–131.

Brown, N.A.C. & van Staden, J., 1997. Smoke as a germination cue : a review. *Plant Growth Regulation*, 22, pp.115–124.

Bullock, J.M. & Clarke, R.T., 2000. Long distance seed dispersal by wind: measuring and modelling the tail of the curve. *Oecologia*, 124(4), pp.506–521.

Caughley, G., 1994. Directions in conservation biology. *Journal of Animal Ecology*, 63(2), pp.215–244.

Charlesworth, D. & Pannell, J.R., 2001. Mating systems and population genetic structure in the light of coalescent theory. In J. Silvertown & J. Antonovics, eds. *Integrating Ecology and Evolution in a Spatial Context: 14th Special Symposium of the British Ecological Society*. Oxford: Blackwell Science Ltd., pp. 73–95.

Charlesworth, D. & Wright, S.I., 2001. Breeding systems and genome evolution. *Current Opinion in Genetics & Development*, 11(6), pp.685–690.

Chase, M.W. & Hills, H.H., 1991. Silica gel : an ideal material for field preservation of leaf samples for DNA studies. *Taxon*, 40(2), pp.215–220.

Connell, J.H. & Orias, E., 1964. The ecological regulation of species diversity. *The American Naturalist*, 98(903), pp.399–414.

Craighead, F.L., Paetkau, D., Reynolds, H. V., Strobeck, C. & Vyse, E.R., 1998. Use of microsatellite DNA analyses to infer breeding behavior and demographic processes in an Arctic grizzly bear population. *Ursus*, 10, pp.323–327.

Csiki, I., Lam, C., Key, A., Coulter, E., Clark, J.D., Pace, R.M., Smith, K.G. & Rhoads, D.D., 2003. Genetic variation in Black Bears in Arkansas and Louisiana using microsatellite DNA markers. *Journal of Mammalogy*, 84(2), pp.691–701.

Dellaporta, S.L., Wood, J. & Hicks, J.B., 1983. A plant DNA minipreparation: Version II. *Plant Molecular Biology Reporter*, 1(4), pp.19–21.

Donaldson, Z.R. & Young, L.J., 2008. Oxytocin, vasopressin, and the neurogenetics of sociality. *Science*, 322, pp.900–905.

Ellstrand, N.C. & Elam, D.R., 1993. Population genetic consequences of small population size: Implications for plant conservation. *Annual Review of Ecology and Systematics*, 24(1993), pp.217–242.

Franks, S.J., Weber, J.J. & Aitken, S.N., 2014. Evolutionary and plastic responses to climate change in terrestrial plant populations. *Evolutionary Applications*, 7(1), pp.123–39.

Futuyma, D., 1986. *Evolutionary Biology.*, Sunderland, Massachusetts: Sinauer Associates Inc.

García, M.B., Espadaler, X. & Olesen, J.M., 2012. Extreme reproduction and survival of a true cliffhanger: the endangered plant *Borderea chouardii* (Dioscoreaceae). *PloS one*, 7(9), p.e44657.

Gauli, A., Steane, D.A., Vaillancourt, R.E. & Potts, B.M., 2014. Molecular genetic diveristy and population structure in *Eucalyptus pauciflora* subsp. *pauciflora* (Myrtaceae) on the island of Tasmania. *Australian Journal of Botany*, 62, pp.175–188.

Goldblatt, P., 1997. Floristic diversity in the Cape Flora of South Africa. *Biodiversity and Conservation*, 6, pp.359–377.

Goldblatt, P. & Manning, J.C., 2002. Plant Diversity of the Cape Region of Southern Africa. *Annals of the Missouri Botanical Garden*, 89(2), pp.281–302.

Google Earth, 6.0, 2014. 34°39'25"S, 19°33'44"E elevation 100m. Available at: https://www.google.com/maps/place/34%C2%B039'25.0%22S+19%C2%B033'44.0%22E/@-34.6593566,19.5649712,3636m/data=!3m1!1e3!4m2!3m1!1s0x0:0x0 [Accessed September 30, 2014].

Hamrick, J.L. & Godt, M.J.W., 1990. Allozyme diversity in plant species. In A. H. D. Brown, M. T. Clegg, A. L. Kahler, & B. S. Weir, eds. *Plant Population Genetics, Breeding, and Genetic Resources*. Sunderland, Massachusetts: Sinauer Associates Inc., pp. 43–63.

Hamrick, J.L. & Godt, M.J.W., 1996. Effects of life history traits on genetic diversity in plant species. *Philosophical Transactions: Biological Sciences*, 351(1345), pp.1291–1298.

Hamrick, J.L., Godt, M.J.W., Murawski, D.A. & Loveless, M.D., 1991. Correlations between species traits and allozyme diversity: implications for conservation biology. In D. A. Falk & K. E. Holsinger, eds. *Genetics and Conservation of Rare Plants*. Oxford, United Kingdom, pp. 76–86.

Hansson, B. & Westerberg, L., 2002. On the correlation between heterozygosity and fitness in natural populations. *Molecular Ecology*, 11, pp.2467–2474.

Hedrick, P.W., 2005. A standardized genetic differentiation measure. *Evolution*, 59(8), pp.1633–1638.

Hogbin, P.M. & Peakall, R., 1999. Evaluation of the contribution of genetic research to the management of the endangered plant *Zieria prostrata*. *Conservation Biology*, 13(3), pp.514–522.

Hughes, C.R. & Queller, D.C., 1993. Detection of highly polymorphic microsatellite loci in a species with little allozyme polymorphism. *Molecular Ecology*, 2(3), pp.131–137.

Innan, H., Terauch, R. & Miyashita, N.T., 1997. Microsatellite polymorphism in natural populations of the wild plant *Aribidopsis thaliana*. *Genetics*, 146, pp.1441–1452.

Isagi, Y., Kanazashi, T., Suzuki, W., Tanaka, H. & Abe, T., 2000. Microsatellite analysis of the regeneration process of *Magnolia obovata* Thunb. *Heredity*, 84, pp.143–151.

Jain, S.K., 1976. The evolution of inbreeding in plants. *Annual Review of Ecology and Systematics*, 7(1976), pp.469–495.

Johnson, S.D. & Steiner, K.E., 2003. Specialized pollination systems in southern Africa. *South African Journal of Science*, 99, pp.345–348.

Kalia, R.K., Rai, M.K., Kalia, S., Singh, R. & Dhawan, A.K., 2010. Microsatellite markers: an overview of the recent progress in plants. *Euphytica*, 177(3), pp.309–334.

Kamran-Disfani, A. & Agrawal, A.F., 2014. Selfing, adaptation and background selection in finite populations. *Journal of Evolutionary Biology*, 27(7), pp.1360–1371.

Kikuchi, S. & Isagi, Y., 2002. Microsatellite genetic variation in small and isolated populations of *Magnolia sieboldii* ssp. *japonica*. *Heredity*, 88(4), pp.313–321.

Kirsten, G. & Oliver, E.G.H., 1992. *Ericas of Southern Africa* D. Schumann, ed., Vlaeberg: Fernwood Press.

Kron, K.A., Judd, W.S., Stevens, P.F., Crayn, D.M., Anderberg, A.A., Gadek, P.A., Quinn, C.J. & Luteyn, J.L., 2002. Phylogenetic classification of Ericaceae : Molecular and morphological evidence. *Botanical Review*, 68(3), pp.335–423.

Kruglyak, S., Durrett, R.T., Schug, M.D. & Aquadro, C.F., 1998. Equilibrium distributions of microsatellite repeat length resulting from a balance between slippage events and point mutations. *Proceedings of the National Academy of Sciences of the United States of America*, 95, pp.10774–10778.

Kyle, C.J. & Strobeck, C., 1999. Microsatellite analysis of North American wolverines. *Proceedings of a Conference on the Biology and Management of Species and Habitats at Risk*, pp.707–712.

Lande, R., 1988. Genetics and demography in biological conservation. *Science*, 241(4872), pp.1455–1460.

Lande, R., 1993. Risks of population extinction from demographic and environmental stochasticity and random catastrophes. *The American Naturalist*, 142(6), pp.911–927.

Leonard, J., West, A. & Ojeda, F., 2014. The curious case of the “pyrofuge”: resprouters and seeders depend on smoke for germination while fire-avoiders do not. *Paper presented at the UCT Postgraduate Science Symposium*, p.4 September 2014.

Linder, H.P., 2005. Evolution of diversity: the Cape flora. *Trends in Plant Science*, 10(11), pp.536–541.

Linder, H.P., 2003. The radiation of the Cape flora, southern Africa. *Biological reviews of the Cambridge Philosophical Society*, 78(4), pp.597–638.

Lloyd, D.G., 1987. Allocations to pollen, seeds and pollination plants mechanisms in self-fertilizing plants. *Functional Ecology*, 1(2), pp.83–89.

Loveless, M.D. & Hamrick, J.L., 1984. Ecological determinants of genetic structure in plant populations. *Annual Review of Ecology and Systematics*, 15, pp.65–95.

Luteyn, J.L., 1988. Speciation and diversity of Ericaceae in Neotropical Montane Vegetation. In L. B. Holm-Nielson, L. C. Nielson, & H. Balslev, eds. *Tropical Forests: Botanical Dynamics, Speciation & Diversity*. San Diego: Academic Press Limited, pp. 297–310.

Mantel, N., 1967. The detection of disease clustering and a generalized regression approach. *Cancer Research*, 27(2), pp.209–220.

Mucina, L. & Rutherford, M.C., 2006. The vegetation of South Africa, Lesotho and Swaziland. *South African National Biodiversity Institute, Strelitzia 19*, pp.170–174.

Nybom, H. & Bartish, I. V., 2000. Effects of life history traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plants. *Perspectives in Plant Ecology, Evolution and Systematics*, 3(2), pp.93–114.

Oliver, E.G.H., 2000a. Ericaceae. In O. A. Leistner, ed. *Seed Plants of southern Africa: families and genera.* Strelitzia, pp. 246–248.

Oliver, E.G.H., 1991. Ericoideae (Ericaceae) – a review. *Contributions from the Bolus Herbarium*, 13, pp.158–208.

Oliver, E.G.H., 2000b. Systematics of Ericeae (Ericaceae: Ericoideae) species with indehiscent and partially dehiscent fruits. *Contributions from the Bolus Herbarium*, 19, p.10483.

Paetkau, D., Calvert, W., Stirling, I. & Strobeck, C., 1995. Microsatellite analysis of population structure in Canadian polar bears. *Molecular Ecology*, 4(3), pp.347–354.

Paetkau, D. & Strobeck, C., 1998. Ecological genetic studies of bears using microsatellite analysis. *Ursus*, 10, pp.299–306.

Palacious, C., Kresovich, S. & Gonzçlez-Candelas, F., 1999. A population genetic study of the endangered plant species *Limonium dufourii* (Plumbaginaceae) based on amplified fragment length polymorphism (AFLP). *Molecular Ecology*, 8, pp.645–657.

Pausas, J.G. & Keeley, J.E., 2014. Evolutionary ecology of resprouting and seeding in fire-prone ecosystems. *New Phytologist*, 204, pp.55–65.

Peakall, R. & Smouse, P.E., 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. *Bioinformatics*, 28, pp.2537–2539.

Peakall, R. & Smouse, P.E., 2006. GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, 6(1), pp.288–295.

Pirie, M.D., Oliver, E.G.H. & Bellstedt, D.U., 2011. A densely sampled ITS phylogeny of the Cape flagship genus *Erica* L. suggests numerous shifts in floral macro-morphology. *Molecular Phylogenetics and Evolution*, 61, pp.593–601.

Queller, D.C., Strassmann, J.E. & Hughes, C.R., 1993. Microsatellites and kinship. *Trends in Ecology and Evolution*, 8(8), pp.285–288.

Raimondo, D., von Staden, L., Foden, W., Victor, J.E., Helme, N.A., Turner, R.C., Kamundi, D.. & Manyama, P.A., 2009. *Red List of South African Plants* 25th ed., Pretoria: Strelitzia. South African National Biodiversity Institute.

Rebelo, A.G. & Siegfried, W.R., 1985. Colour and size of flowers in relation to pollination of *Erica* species. *Oecologia*, 65, pp.584–590.

Rowe, G., Beebee, T.J.C. & Burke, T., 1998. Phylogeography of the natterjack toad *Bufo calamita* in Britain: Genetic differentiation of native and translocated populations. *Molecular Ecology*, 7, pp.751–760.

Schlebusch, S.A., Midgley, J.J., Turner, R.C. & Illing, N., 2014. Pollinator divergence in *Liparia* (Fabaceae); a population genetics perspective on bird versus small mammal pollination, in press.

Schumann, D. & Kirsten., G., 1992. *Ericas of South Africa*, Portland, OR: Timber Press.

Segarra-Moragues, J.G., Donat-Caerols, S. & Ojeda, F., 2009. Isolation and characterization of microsatellite loci in the Cape fynbos heath *Erica coccinea* (Ericaceae). *Conservation Genetics*, 10(6), pp.1815–1819.

Segarra-Moragues, J.G. & Ojeda, F., 2010. Postfire response and genetic diversity in *Erica coccinea*: connecting population dynamics and diversification in a biodiversity hotspot. *Evolution*, 64(12), pp.3511–3524. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20561051 [Accessed May 9, 2014].

Sicard, A. & Lenhard, M., 2011. The selfing syndrome: a model for studying the genetic and evolutionary basis of morphological adaptation in plants. *Annals of Botany*, 107(9), pp.1433–1443.

Steenhuisen, S.-L. & Johnson, S.D., 2012. The influence of pollinators and seed predation on seed production in dwarf grassland *Protea* "sugarbushes" (Proteaceae). *South African Journal of Botany*, 79, pp.77–83.

Stevens, P.F., 2001. Angiosperm Phylogeny Website. *12*. Available at: http://www.mobot.org/MOBOT/research/APweb/ [Accessed September 30, 2014].

Streiff, R., Labbe, T., Bacilieri, R., Steinkellner, H., Glössl, J. & Kremer, A., 1998. Within-population genetic structure in *Quercus robur* L . and *Quercus petraea* (Matt .) Liebl . assessed with isozymes and microsatellites. *Molecular Ecology*, 7, pp.317–328.

Sun, J.X., Helgason, A., Masson, G., Ebenesersdóttir, S.S., Li, H., Maillick, S., Gnerre, S., Patterson, N., Kong, A., Reich, D. & Stefansson, K., 2012. A direct characterization of human mutation based on microsatellites. *Nature Genetics*, 44, pp.1161–1165.

Szucs, M., Melbourne, B.A., Tuff, T. & Hufbauer, R.A., 2014. The roles of demography and genetics in the early stages of colonization. *Proceedings of The Royal Society B*, 281, pp.1–8.

Takebayashi, N. & Morrell, P.L., 2001. Is self-fertilization an evolutionary dead end? Revisiting an old hypothesis with genetic theories and a macroevolutionary approach. *American Journal of Botany*, 88(7), pp.1143–1150.

Tautz, D. & Schlötterer, C., 1994. Simple sequences. *Current Opinion in Genetics & Development*, 4(6), pp.832–837.

Thuillet, A.-C., Bru, D., David, J., Roumet, P., Santoni, S., Sourdille, P. & Bataillon, T., 2002. Direct estimation of mutation rate for 10 microsatellite loci in Durum Wheat, *Triticum turgidum* (L.) Thell. ssp *durum* desf. *Molecular Biology and Evolution*, 19(1), pp.122–125.

Turner, R.C., 2012. Pollination Biology of Cape *Erica* species (Ericaceae). Thesis, University of Kwazulu-Natal.

Turner, R.C. & Oliver, E.G.H., 2007. *Erica occulta* E.G.H.Oliv. *Red List of South African Plants*. Available at: http://redlist.sanbi.org/species.php?species=1820-627 [Accessed March 10, 2014].

Turner, R.C. & Oliver, E.G.H., 2009. *Erica occulta* E.G.H.Oliv. National Assessment: Red List of South African Plants. Available at: http://redlist.sanbi.org/species.php?species=1820-627 [Accessed March 10, 2014].

Vaughton, G. & Ramsey, M., 2006. Selfed seed set and inbreeding depression in obligate seeding populations of *Banksia marginata*. *Proceedings of the Linnean Society of New South Wales*, 127, pp.19–25.

Zawko, G., Krauss, S.L., Dixon, K.W. & Sivasithamparam, K., 2001. Conservation genetics of the rare and endangered *Leucopogon obtectus* (Ericaceae). *Molecular ecology*, 10(10), pp.2389–2396.

Appendices:

Appendix 1 Genomic DNA Extraction Method Comparison

The Bellstedt method (Bellstedt et al. 2010) for extracting plant genomic DNA was faster than the Dellaporta method (Dellaporta et al. 1983), but produced a more crude extraction with more contaminants (Table 3) and a shorter storage lifespan of approximately two weeks. The Dellaporta method produces a superior quality extraction with fewer contaminants (Table 3), which can be stored for multiple years, it takes approximately 5 hours to complete and there are multiple points at which the DNA can be lost. During this comparison the Dellaporta method was unsuccessful Figure 6), not because it is faulty, but because of the student-method interaction. The Bellstedt method is, therefore, better for novices as there are fewer steps where contamination can occur and less room for error. For the purposes of this study the DNA extraction did not need to preserve well as it was used immediately for amplification and thus the more time efficient method was preferred. The modified method used in this study is highly recommended if leaf samples are tough or not entirely dry.

Table 3 Nanodrop results summary of genomic DNA extraction method quality comparison of *Erica occulta* between a modified Bellstedt et al. (2010) method and a modified Dellaporta et al. (1983) method.

Sample	Extraction Method	Concentration ($ng/\mu l$)	Protein Contamination 260/280	Salts and Phenols Contamination 260/230
S042	Dellaporta	24.2	1.55	0.2
S042	Dellaporta	26.2	1.49	0.19
S042	Bellstedt	10.8	0.05	1.47
S042	Bellstedt	20.0	0.06	1.43

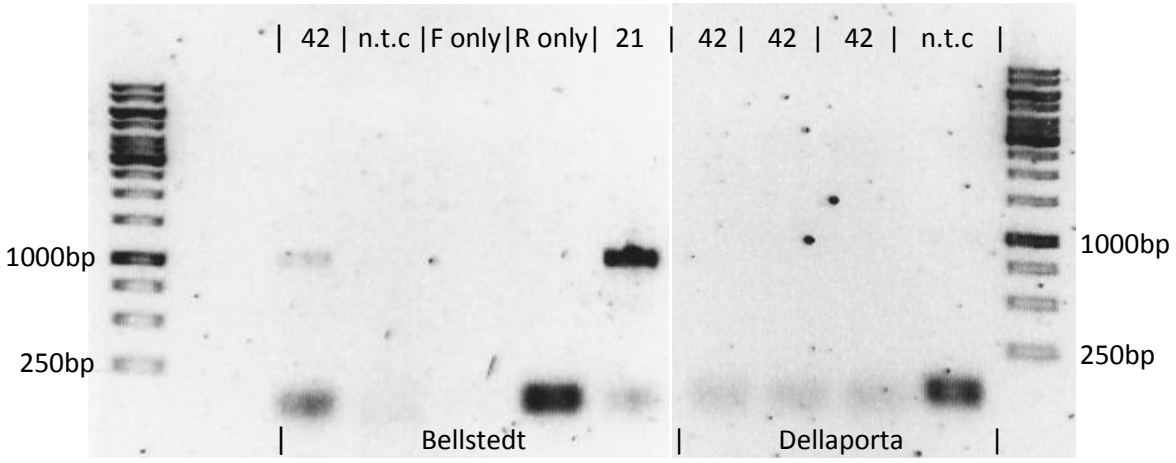


Figure 6 The trn-LF locus amplified at the expected size of 1Kb using a modified Bellstedt DNA extraction method from *Erica occulta*, but the extraction was unsuccessful using a modified Dellaporta Method. Each lane is labelled with its PCR contents where n.t.c is the no-template negative control, F only had only the forward primer (H_1TabF) and R only had only the reverse primer (H_4TabC) and the number represents the individual sampled. A 10Kb DNA ladder SM1163 (KAPA Biosystems) was used to estimate the size of the amplified product.

Appendix 2 Bellstedt Buffer Solution Protocols

Protocols for the preparation of the grinding and GES buffers for the rapid and inexpensive Bellstedt plant DNA extraction method extracted from (Bellstedt et al. 2010).

1) Grinding Buffer

(15 mM Na_2CO_3, 35 mM $NaHCO_3$, 2% (m/v) PVP 40, 0.2% (m/v) BSA, 0.05% (v/v) Tween 20, 1% (m/v) $Na_2S_2O_5$, pH 9.6)

To prepare 100ml of grinding buffer dissolve all components except Tween 20 in double-distilled sterile water. Stir at low speed to avoid excessive foaming. Once all components have dissolved, adjust pH to 9.6 using NaOH solution. Then add Tween 20. Autoclave and store at 4 ° C.

Component	Mass/Volume	[Final]
Na_2CO_3 (Sodium Carbonate)	0.159 g	15 mM
$NaHCO_3$ (Sodium Hydrogen Carbonate)	0.293 g	35 mM
PVP 40 (Polyvinylpyrrolidone)	2.000 g	2% (m/v)
BSA (Bovine Serum Albumin)	0.200 g	0.2% (m/v)
Tween 20	50 μl	0.05% (v/v)
$Na_2S_2O_5$ (Sodium Metabisulphite)	1.000 g	1% (m/v)

2) GES Buffer

(0.1 M glycine-NaOH, pH 9.0, 50 mM NaCl, 1 mM EDTA, pH 8.0, 0.5% (v/v) Triton X-100)

To prepare GES buffer make up the following stock solutions:

1 M Glycine, pH 9.00 (200ml)

Dissolve 15.014 g glycine in 200 ml double-distilled sterile water.
Adjust pH to 9 using NaOH pellets.
Autoclave.
Store at room temperature.
Use 10 ml of 1 M glycine stock per 100 ml GES buffer.

5 M NaCl (200ml)

Dissolve 58.44 g NaCl in 200 ml double-distilled sterile water with mild heating.
Autoclave.
Store at room temperature.
Use 1 ml of 5 M NaCl stock per 100 ml GES buffer.

0.1 M EDTA, pH 8.00 (100 ml)

Dissolve 3.723 g EDTA in 100 ml double-distilled sterile water.
Adjust pH to 8 using NaOH pellets.
Autoclave.
Store at room temperature.
Use 1 ml of 0.1 M EDTA stock per 100 ml GES buffer.

To prepare 100 ml of GES buffer, add the volumes of the above-mentioned components and make up the remaining volume with double-distilled sterile water. Autoclave and pipette aliquots of 500 μl (500 μl of GES is enough to treat 20 samples). Store aliquots and stock at –20 °C.

Appendix 3 Screening 8 microsatellite loci

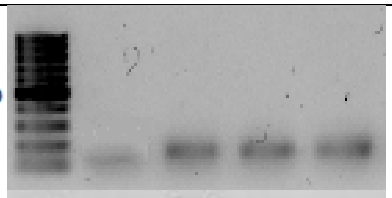
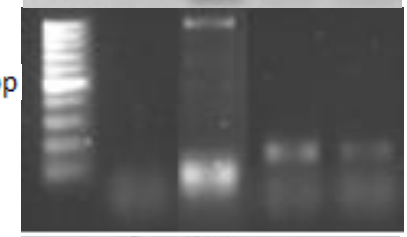
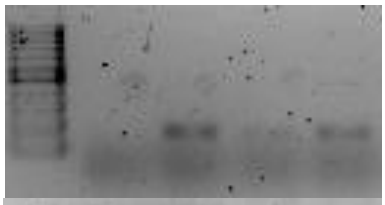
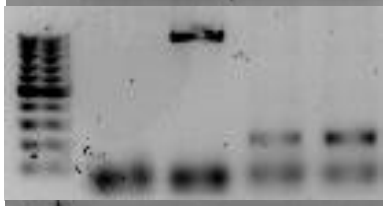
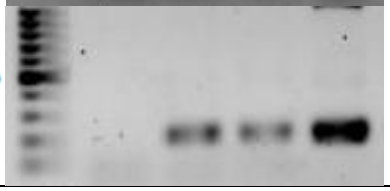
Locus		1 2 3 4 5	Lane Contents
Ecoc108	1000bp		Lane 2 No template -ve control Lane 3 S037 Lane 4 S036 Lane 5 S034
Ecoc115	1000bp		Lane 2 No template -ve control Lane 3 trn-LF +ve control Lane 4 S001 Lane 5 S010
Ecoc117	1000bp		Lane 2 No template -ve control Lane 3 trn-LF +ve control Lane 4 S001 Lane 5 S010
Ecoc122	1000bp		Lane 2 No template -ve control Lane 3 trn-LF +ve control Lane 4 S001 Lane 5 S010
Ecoc132	1000bp		Lane 2 No template -ve control Lane 3 S002 Lane 4 S003 Lane 5 S004
Ecoc142	1000bp		Lane 2 No template -ve control Lane 3 trn-LF +ve control Lane 4 S001 Lane 5 S010
Ecoc431	1000bp		Lane 2 No template -ve control Lane 3 trn-LF +ve control Lane 4 S001 Lane 5: S010
Ecoc446	1000bp		Lane 2 No template -ve control Lane 3 S018 Lane 4 S020 Lane 5 S021

Figure 7 Sections of ethidium bromide stained 1.7% agarose gels to indicate that there was no contamination in the negative controls. The gels show the size of the microsatellite loci produced by PCR amplification of DNA extracted from *Erica occulta*. The negative control was PCR water in place of template DNA in each case. Lane 1 is a 1Kb DNA ladder SM1143 (KAPA Biosystems) in each case.

Appendix 4 Scored Alleles

Table 4 Scored allele sizes (bp) for each microsatellite locus for each of 50 *Erica occulta* individuals sampled from the limestone cliffs of the Southern Agulhas Plain, South Africa. *samples adjusted to account for the additional 3' A added by 2xKAPA2G Fast HotStart ReadyMix (KAPA Biosystems)

Sample	Latitude	Longitude	Pop	Ecoc108		Ecoc117		Ecoc132		Ecoc142		Ecoc431		Ecoc446	
S001	-34.6638000	19.5738670	1	159*	159*	161*	161*	183*	183*	227	229	166	166	194*	194*
S002	-34.6638100	19.5738671	1	159	159	161	161	183	183	229	229	166	166	194	194
S003	-34.6638200	19.5738672	1	159	159	161	161	183	183	229	229	166	166	194	194
S004	-34.6638300	19.5738673	1	159	159	161	161	183	183	229	229	166	166	194	194
S005	-34.6639670	19.5736830	1	159	159	161	161	183	183	229	229	166	166	194	194
S006	-34.6639830	19.5736500	1	159	159	161	161	183	183	229	229	166	166	194	194
S007	-34.6639831	19.5736510	1	159	159	161	161	183	183	229	229	166	166	194	194
S008	-34.6641170	19.5733500	1	159	159	161	161	183	183	229	229	166	166	194	194
S009	-34.6641171	19.5733510	1	159	159	161	161	183	183	229	229	166	166	194	194
S010	-34.6625330	19.5754500	1	159*	159*	160*	160*	183*	183*	227	227	166	166	194*	194*
S011	-34.6625670	19.5755170	1	159	159	161	161	183	183	227	227	166	166	194	194
S012	-34.6625671	19.5755171	1	159	159	161	161	183	183	227	227	166	166	194	194
S013	-34.6625672	19.5755172	1	159	159	161	161	183	183	227	227	166	166	194	194
S014	-34.6626000	19.5756500	1	159	159	161	161	183	183	227	229	166	166	194	194
S015	-34.6626170	19.5756670	1	159	159	161	161	183	183	227	229	166	166	194	194
S016	-34.6626171	19.5757100	1	159	159	161	161	183	183	227	229	166	166	194	194
S017	-34.6626172	19.5757200	1	159	159	161	161	183	183	227	229	166	166	194	194
S018	-34.6626330	19.5756830	1	159	159	161	161	183	183	229	229	166	166	194	194
S019	-34.6742000	19.5838330	2	159*	159*	161*	161*	183*	183*	227	227	166	166	194*	194*
S020	-34.6740670	19.5839330	2	159	159	162	162	183	183	227	227	166	166	194	194
S021	-34.6740830	19.5838670	2	159	159	162	162	183	183	227	227	0	0	186	194
S022	-34.6740830	19.5838000	2	159	159	162	162	183	183	227	229	166	166	194	194
S023	-34.6740831	19.5838100	2	159	159	162	162	181	183	227	227	166	166	194	194
S024	-34.6740500	19.5838330	2	159	159	162	162	183	183	227	227	166	166	194	194
S025	-34.6740510	19.5838331	2	159	159	162	162	183	183	227	227	166	166	194	194
S026	-34.6740520	19.5838332	2	159	159	162	162	183	183	227	227	166	166	194	194
S027	-34.6740530	19.5838333	2	159	159	162	162	183	183	227	227	166	166	194	194
S028	-34.6732830	19.5836830	2	159*	159*	160*	160*	183*	183*	227	227	166	166	194*	194*
S029	-34.6582670	19.5687830	3	159*	159*	160*	160*	183*	183*	225	225	166	166	194*	194*
S030	-34.6582671	19.5687831	3	159	159	161	161	183	183	225	227	166	166	194	194
S031	-34.6582672	19.5687832	3	159	159	160	161	183	183	225	227	166	166	194	194
S032	-34.6582673	19.5687833	3	159	159	161	161	183	183	225	225	166	166	194	194
S033	-34.6580000	19.5716170	3	159*	159*	161*	161*	183*	183*	225	227	166	166	194*	194*
S034	-34.6581000	19.5716171	3	159	159	161	161	183	183	225	225	166	166	194	194
S035	-34.6572670	19.5736330	3	159*	159*	161*	161*	183*	183*	225	225	166	166	194*	194*
S036	-34.6572671	19.5736331	3	159	159	161	161	183	183	225	229	166	166	194	194
S037	-34.6591330	19.5769000	4	159	159	161	161	183	183	0	0	166	166	194	194
S038	-34.6589500	19.5770170	4	159	159	161	161	183	183	225	225	166	166	194	194
S039	-34.6589510	19.5770171	4	159	159	161	161	183	183	225	225	166	166	194	194
S040	-34.6589520	19.5770172	4	159	159	161	161	183	183	225	225	166	166	194	194
S041	-34.6589330	19.5770830	4	159	159	161	161	183	183	225	225	166	166	194	194
S042	-34.6589331	19.5770831	4	159	159	0	0	183	183	225	225	166	166	194	194
S043	-34.6589332	19.5770832	4	159	159	161	161	183	183	225	225	166	166	194	194
S044	-34.6589330	19.5771670	4	159	159	161	161	183	183	225	225	166	166	194	194
S045	-34.6589331	19.5771671	4	159	159	161	161	183	183	225	225	166	166	194	194
S046	-34.6589332	19.5771672	4	159	159	161	161	183	183	225	225	166	166	194	194
S047	-34.6589830	19.5772500	4	159	159	161	161	183	183	225	225	166	166	194	194
S048	-34.6589831	19.5772510	4	159	159	161	161	183	183	225	225	166	166	194	194
S049	-34.6589832	19.5772520	4	159	159	161	161	183	183	225	225	166	166	194	194
S050	-34.6589833	19.5772530	4	159	159	161	161	183	183	225	225	166	166	194	194