Intraspecific variation in *Erica coccinea*

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MLNMIC009

Dissertation presented for the degree of Master of Science
Department of Botany
University of Cape Town
February 2013

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Plagiarism declaration

I know the meaning of plagiarism and declare that all of the work in the dissertation, save that for which is properly acknowledged, is my own.

Acknowledgements

The completion of this thesis would not have been possible without the encouragement and support of friends and family too numerous to list, but I owe a special debt of gratitude to my field assistant, friend and editor, Jenny Leonard. We hiked in the hottest sunshine, the coldest rain, thickest fog and howling wind, and survived hours of driving, broken down cars, and fields of gawking ostriches, and still remained friends. Without her help, as well as that of the many other friends I dragged up and down mountains, I would have struggled to do my fieldwork. In addition, this project would not have been possible without the support of Dr Fernando Ojeda, University of Cadiz, who suggested the study, and was kind enough to supply me with the extensive knowledge he has gained working with *Erica coccinea* for over a decade. I greatly appreciate the time he put into helping me with fieldwork, discussing theories and editing drafts, as well as initiating the idea of my travelling to Spain to complete the lab work for this thesis. To my host in Valencia, Spain, Dr Gabi Segarra-Moragues, I am most grateful for his patient tutoring of me in the art of DNA extractions and microsatellite work, as well as cooking up an excellent bobotie for a homesick South African. The scientists at the CIDE in Valencia were incredibly welcoming and patient with me, despite my lack of Spanish and inability to pronounce ‘paella’ properly, and I really enjoyed the time I spent there. I also owe thanks to my co-supervisor, Dr Timo van der Niet, who has always been a source of encouragement and advice, and helped me kick-start my fieldwork. The time I spent working on this thesis was an incredibly rewarding learning experience, though often frustrating, difficult and tiring. I appreciate the faith my supervisor, Dr Jeremy Midgley, seemed to have in me, coughing up ideas and funds whenever they were needed and giving me a lot of freedom to wander along my own path. After over a year of intense fieldwork, the process of writing up is daunting, and I would not have stayed sane without the members of my excellent writing group, Nicholas Zaloumis, Robyn Powell and Nikki Stevens. Finally, the post-grads, academics and staff in the Botany department at UCT were always a great source of help, particularly Dawood Hattas and Sandy Smuts – I would’ve been lost without their technical and administrative help.

SANPARKS and Cape Nature authorities provided permits for collecting plant material. I also thank managers of Vogelgat (Hermanus) nature reserve, and landowners of Brandfontein

(Agulhas), Camphill (Hermanus) and Honingklip (Bot Rivier) farms for allowing me to collect samples on their private property. I was funded for the duration of this project by a Scarce Skills Master's Scholarship from the NRF, and fieldwork expenses were covered by my supervisor, Dr Jeremy Midgley. My trip to Spain was funded by a UCT Postgraduate scholarship for international travel, and lab work costs at the Centro de Investigaciones sobre Desertificacion (CIDE) were covered by Dr Segarra-Moragues and Dr Fernando Ojeda.

Field assistant extraordinaire, Jenny Leonard, at the *Erica coccinea* site at Devil's Peak, Table Mountain National Park.

Abstract

The genus *Erica* is the most specious in the Cape Floristic Region, with a large range of habitats, pollination syndromes and fire survival strategies. *Erica coccinea*, like many other Cape *Erica* species, has high intraspecific variability between populations. In addition to variability in floral characteristics such as colour, this species includes two distinct regeneration forms: a resprouter form which survives fire by resprouting from dormant buds in a swollen lignotuber, and a seeder form which does not survive fire, but whose populations regenerate from fire-triggered seed germination. Previous studies have shown that these two regeneration forms are genetically determined and this dissertation investigates further the differences in floral morphology, phenology, fecundity and genetic relatedness across 29 populations. Results show patterns of seeder individuals investing more effort into nectar and seed production than resprouters and differences in flower colour and flowering phenology between the two fire life history strategies. A P_{ST}-F_{ST} analysis, comparing genetic variability to variability in floral traits shows a strong selective force working on anther length in the seeder form. A complete separation of flowering phenology between seeder and resprouter individuals in 'mixed' populations where the two forms co-occur leads to speculation that this might be a case of incipient speciation.

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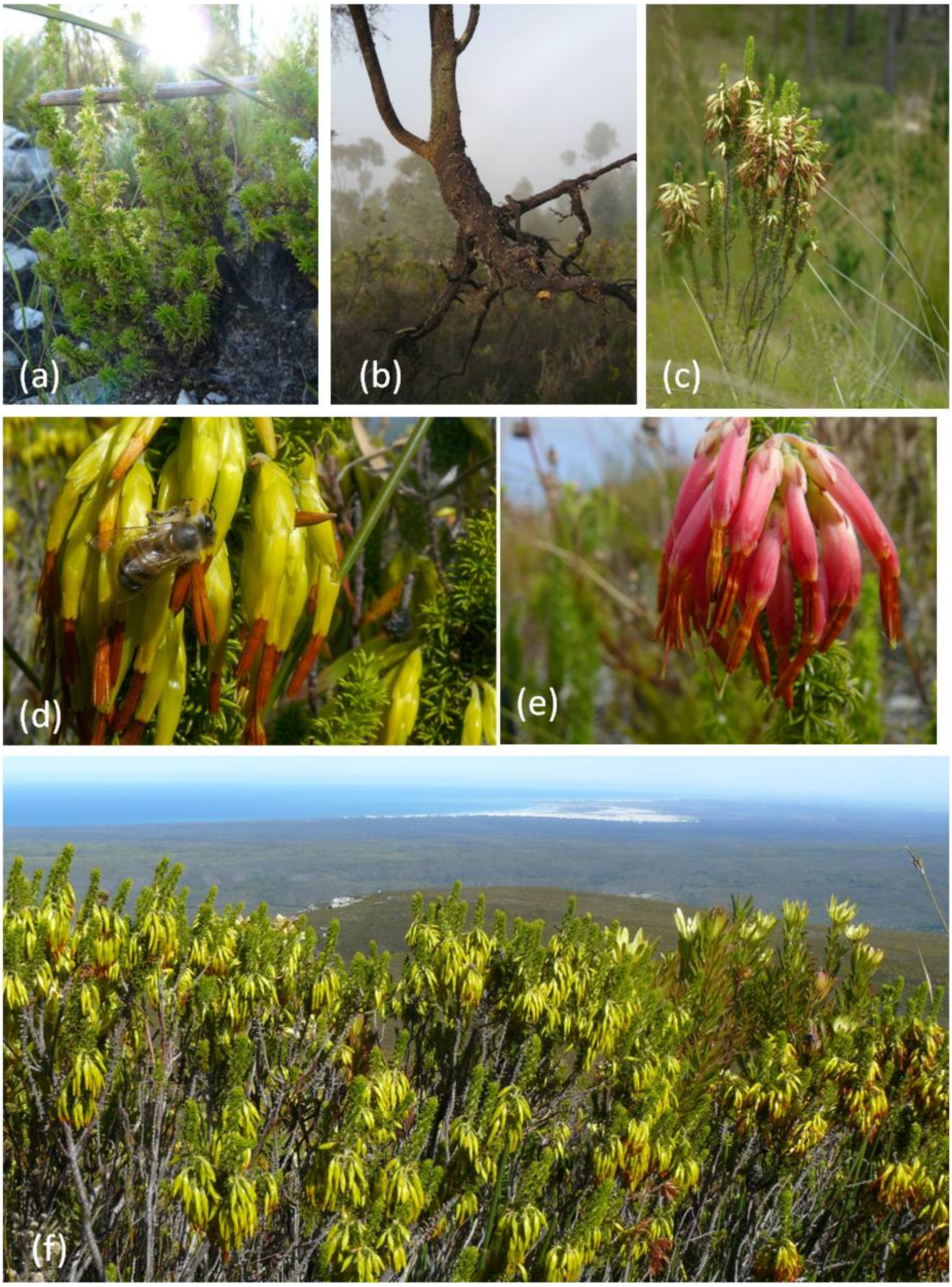

Figure 1: Seeder and resprouter forms of *Erica coccinea subsp. coccinea* from the Cape Floristic region. (a) The base of a resprouter individual showing multiple stems emerging after fire. (b) The base of a seeder individual showing lack of buds and presence of single stems. (c) A yellow flowering resprouter individual at Marloth Nature Reserve, Swellendam. (d) A yellow flowering seeder individual from Potberg Mountain, De Hoop Nature Reserve, being robbed by a bee. (e) A red flowered resprouter individual from Honingklip farm, Botrivier. (f) Yellow flowering seeder plants on Potberg Mountain.

Introduction

The Cape Floristic Region (CFR), also known as the fynbos biome, is an internationally recognised biodiversity hotspot with more than 9000 plant species in a relatively small area of around 90 000 km^2 (Schnitzler et al. 2011). Situated at the southern tip of Africa, in the Western Cape of South Africa, it has a Mediterranean climate with hot, dry summers and mild, wet winters. It is unique amongst other Mediterranean ecosystems in its large diversity, contained in a relatively small geographical area. Much of this diversity originates from a few extremely specious clades (Linder and Hardy 2004) and the radiation in these clades has been attributed to a highly heterogeneous environment, a relatively stable geological history (Cowling et al. 2009), and the influential role of fire as selective force. Pollinator specialization and phenological shifts are also causes of speciation in the region (van der Niet and Johnson 2009, Johnson 2010).

The Western region of the Cape is found to have low phylogenetic diversity, indicating that although the species richness is high, it has mostly originated from a few large radiations, resulting in many closely related species (Forest et al. 2007). This is true for the genus *Erica*, which worldwide has 840 species, with around 680 of those occurring in the CFR (Pirie et al. 2011). What is interesting about the genus *Erica* is that leaf form is relatively conserved across the genus (Oliver 2000) while most of the diversity is in the floral form (Rebelo and Siegfried 1985), suggesting that much of the speciation has been caused by changes in floral traits and not ecological speciation into new climate or soil niches (Johnson 1996).

There are also two main fire adapted life history strategies present in the fynbos and genus *Erica*– both seeder and resprouter forms occur within the genus. The resprouting of woody plants is a strategy for surviving disturbances such as fire in Mediterranean type biomes which are prone to regular burning. Plants are either killed outright (‘nonsprouters’ or ‘seeders’) or are able to recover from fire by resprouting (‘sprouters’, or ‘resprouters’), using energy sources from underground storage organs. Resprouting is advantageous in environments which experience frequent fire because it allows persistence in the landscape of individual plants, through multiple fires and disturbances, and a small amount of recruitment by seed. Seeders adopt a strategy that concentrates resources on seed production, with the result that the parent plant is killed by fire, but they have a large amount of recruitment through seedlings after fire (Bond and Midgley 2003).

Previous studies (Bell and Ojeda 1999, Ojeda et al. 2005) have worked with the assumption that resprouting is the ancestral state in *Erica*, with evidence drawn from the fact that seeder individuals of the dimorphic species *E. coccinea* and *E. calycina* have vestigial signs of the starch-filled lignotubers that aid resprouting after fire. However, this assumption is challenged by the fact that many other Cape lineages with both resprouter and seeder forms seem to have seeders as the ancestral state (Barker et al. 2004). Barker (2004) finds that resprouting evolved multiple times from seeders in the genus *Leucadendron* in the CFR. This is in contrast to Bond and Midgley's (2001) finding that the switch from sprouting to non-sprouting has occurred repeatedly in clades in the Mediterranean biomes. In the genus *Leucadendron*, seeder species are more numerous and basal, indicating that seeding may be the ancestral state. In the genus *Erica*, the majority of species in the CFR are seeder, in contrast to the Mediterranean area where they are mostly resprouters (Ojeda 1998). McGuire and Kron (2005) show that the Cape *Erica* clade originated in the Mediterranean region of Europe and North Africa, *Erica* then dispersed southwards through Africa, where it radiated hugely in the Fynbos biome. The chances of *Erica* radiating as seeders is much higher than if they were resprouters, because of a higher generational turnover and thus mutation rate, creating novel alleles and characteristics to adopt to different niches in the heterogeneous environment of the CFR.

By investigating the cause of diversification in *Erica* we may come closer to understanding the reasons for diversification in other Cape clades. *Erica* is useful to use as a model in understanding the different dynamics of fire life-history strategies because there are several species that have both seeder and resprouter individuals. Three of these, *Erica plukenetii, E. mammosa* and *E. coccinea* are long-tubed bird pollinated species with major variation in floral form, and a fourth, *E. calycina* is an insect pollinated species. Seeder and resprouter populations of *E. calycina* have been identified by Ojeda et al. (2005), but the species lacks the co-variation in floral form typical of the bird-pollinated species of *Erica* making it unsuitable for studying radiation of floral traits. *E. plukenetii* is a widespread species, with variation in floral size and colour across its distribution, but the seeder and resprouter forms do not co-occur – seeder populations are found in the Cape Peninsula and South-Western Cape, whilst resprouters have a more northerly distribution in Namaqualand, making comparisons of life-history forms difficult. *Erica mammosa* has both variation in floral form and populations of seeder and resprouter forms with overlapping ranges, but there is no existing published research for this species, whereas there are several published papers investigating the seeder and resprouter populations and genetic traits of *Erica coccinea,* making it an ideal candidate for this study.

The study species - Erica coccinea

Erica coccinea is an abundant and widespread bushy shrub, common on mountain slopes of the Cape Peninsula, and widespread from the Cedarberg to the Kammanasie Mountains in the Western Cape of South Africa (Oliver and Oliver 2000). There are some differences in geographical distribution (Ojeda et al. 2005) of the two life-history forms. Seeder populations tend to occur close to the coast in the South - Western Cape, where rainfall is more reliable than the inland mountains, where the resprouting form is common (Ojeda et al. 2005, Segarra-Moragues and Ojeda 2010, see Fig. 2). There are, however, several mixed populations where seeder and resprouter individuals co-occur. In addition, there is a high degree of variation in floral form across the range of the species - flower colours ranging from greenish yellow, to orange, deep pinks and dark reds, and there is some variation in flower shape and size that seems to be population specific, creating interesting patterns of diversity in both reproductive and vegetative traits within a single species.

Erica coccinea is an ideal study species for investigating differences between seeders and resprouters in both morphological and genetic traits because through the work of Segarra-Moragues et al. (2009), eight microsatellite loci have been identified for use on both seeder and resprouter morphotypes. These microsatellite loci have been used to conduct a population genetics survey of *E. coccinea*, to investigate patterns of within- and between-population genetic diversity for seeder and resprouter populations across the range of the species (Segarra-Moragues and Ojeda 2010).

Given the prevalence of *Erica* as a typical fynbos element, and its radiation into several different environmental niches, including many different pollination types (Rebelo and Siegfried 1985) , investigating the patterns of diversification in *Erica* may help in understanding the patterns and causes of diversification in other typical Cape genera

Figure 2: Map of the Western Cape of South Africa showing the location of all populations of *Erica coccinea* used in this study. Red markers indicate resprouter populations, blue markers indicate seeder populations, and black and white markers indicate mixed seeder and resprouter populations. *Erica coccinea* subsp. *uniflora* populations are indicated with a yellow marker, and one population of a fire-avoiding form of *E. coccinea* nicknamed the 'dinosaur' form is shown in white (D01).

Structure

This thesis is arranged in three chapters, each intended to be submitted for publication. This requires some unavoidable repetition. Data was collected and analysed by myself, with some collaboration and additional samples from Dr Fernando Ojeda (University of Cadiz) and Dr G. Segarra-Moragues (Centro de Investigaciones sobre Desertificación, Valencia, Spain).

Chapter 1: Quantifying differences in reproductive and floral characteristics of seeder and resprouter populations of Erica coccinea.

The trade-offs between the seeder and resprouter life history strategies involve allocation of resources to either the roots (resprouters) or vegetative and reproductive organs (seeders). This affects fecundity of the two different forms (Lamont and Wiens 2003). Resprouter individuals may persist in the landscape through many generations and fires or disturbance events, but seeder plants are killed after fire, making the attraction of pollinators, production of flowers and total seed output important factors in their survival in a landscape (Bond and Midgley 2003). In this chapter I investigate floral, population genetic and reproductive differences amongst seeder and resprouter populations. I expect seeder populations to invest a greater amount of resources into attracting pollinators and producing seed as they are more reliant on a large seed bank to recruit after fire than resprouters. I also investigate the effect of population size on inbreeding (F_{IS}) and mean number of alleles per locus (A), using the results of a microsatellite analysis (Segarra-Moragues and Ojeda 2010). I hypothesis that seeder populations will show evidence of investing a greater amount of resources into reproductive traits than resprouters.

Chapter 2: Variation in floral traits amongst seeder and resprouter populations of Erica coccinea: inferences from Pst-Fst comparisons.

The second chapter in this thesis explores co-variation in neutral genetic markers (F_{ST}) and morphological traits (P_{ST}). The question in this chapter is whether there are different evolutionary forces acting on seeder and resprouting populations. Using P_{ST}-F_{ST} comparisons the relative importance of selection or genetic drift on different quantitative traits of a population can be determined (Saether et al. 2007). In this chapter I expect to find that selection acts differently on seeder and resprouter populations, as the greater reliance on seeds of the seeder life history should result in a greater selective force acting on traits that are important for reproduction and fecundity. Resprouter individuals persist in a landscape for much longer than seeders and there is an overlapping of generations (Bond and Midgley 2003), with a small amount of recruitment through seed after fire as well as the parent plants surviving the fire. Therefore reproductive traits may not be as strongly selected for in the resprouter populations.

Chapter 3: Is the variability in seeder and resprouter populations of Erica coccinea a sign of incipient speciation or is it merely a polymorphism?

In mixed populations of *E. coccinea*, where both seeder and resprouter individuals occur, I investigate the possible differences in flowering phenologies for the two life-history forms. A seasonal difference in flowering between the seeder and resprouter life-history forms may create a barrier to cross-pollination and reproduction and over time this could lead to a case of incipient speciation (Warren et al. 2011). To maintain the difference between the forms, I expect to find that in mixed populations the flowering periods for seeder and resprouter individuals are completely separated in time. Therefore, I also expect a low genetic similarity between the forms. To test this I build a neighbour joining tree using similarities from neutral markers (microsatellites) to compare the degree of genetic similarity between seeder and resprouter populations in mixed sites. The flowering phenologies of mixed populations in two sites were also monitored over a twelve month period, to investigate whether flowering seasonality of seeder and resprouter individuals in the mixed sites were separated. I hypothesise that if the two life history forms are separated both genetically and by phenology, that this may be case of potential incipient speciation.

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Chapter 1:

Quantifying differences in reproductive and floral characteristics of seeder and resprouter populations of *Erica coccinea.*

Introduction

The contrasting strategies of seeder and resprouter plants are an important dichotomy in fire-prone systems. Woody plants that can resprout from vegetative tissue after fire or other disturbances can persist in a landscape through several generations of nonsprouters (Bell 2001, Bond and Midgley 2003). However, the ability to resprout after fire requires the allocation of resources to storage organs, which may cause a trade-off between resprouting ability and reproductive effort (Bond and Midgley 2001, Verdaguer and Ojeda 2002). This may have an effect on the relative fecundity of the plant, and effort towards attracting pollinators. Nonsprouters, also referred to as 'seeders', mature quickly after fire-stimulated germination of seed, and because adult individuals are killed outright by fire, must rapidly produce large amounts of seed in order to repopulate a burned area and persist in the landscape over time (Carpenter and Recher 1979). The consequences of these contrasting strategies on reproductive and floral characteristics of a species are of particular interest in systems such as the South African Fynbos biome, where fire is an important factor in creating and maintaining the high diversity of the area (Cowling and Pressey 2001).

Lamont and Wiens (2003) predict greater fecundity in seeder than in resprouter species, and find this true in 30 out of 33 pairs of seeders (or nonsprouters) and resprouters. Resprouters are also often found to have a lower overall seed output than seeders (Hansen et. al 1991, Bond and Midgley 2003), possibly due to the fact that seeder plants are generally larger in size than those of closely related resprouters. This suggests that there is a fecundity trade-off where seeder plants, which are completely reliant on seeds to repopulate an area after fire, expend more resources on producing flowers and fruit than resprouters, which direct their energy into creating a fire-proof structure packed with starch (Verdaguer and Ojeda 2002) in order to regenerate after fire. Due to these contrasting fire strategies, individual resprouter plants can persist in the landscape through many fire cycles, whilst seeder populations are replaced by a new cohort of plants after every fire, resulting in resprouter populations being less reliant on seed numbers and viability to survive, and therefore possibly experiencing lower levels of selection on floral traits.

Erica is the most species-rich genus in the Cape (Pirie et. al 2011), occupying a large range of habitats, while the floral types represent several different pollination syndromes, including wind, insect and bird pollination (Rebelo and Siegfried 1985). *Erica coccinea*, like many other Cape *Erica* species, is characterized by high levels of morphological variability at the population level. In addition to variability in floral characteristics such as flower colour, this species includes two distinct regeneration forms: a resprouter form which survives fire by resprouting from dormant buds in a swollen lignotuber, and a seeder form which does not survive fire, but whose populations regenerate from fire-triggered seed germination (Segarra-Moragues and Ojeda 2010). Previous studies have shown that these two regeneration forms are genetically determined (Verdaguer and Ojeda 1995), and the geographic distribution of populations of the two forms may be explained by a geographic-climatic (summer drought strength) gradient (Ojeda et. al 2005). Using microsatellite markers, the genetic structure of seeder and resprouter populations of *E. coccinea* has been investigated, finding both higher within and among population genetic diversity in seeder than in resprouter populations (Segarra-Moragues and Ojeda 2010). These results highlight the potential influence of different population dynamics processes (for example, shorter generation times in seeders) on driving genetic variation. This study aims to further investigate the genetic diversity of these two forms of *E. coccinea*, and link them to floral biology and reproductive traits.

Honnay and Jacquemyn (2007) find that mean gene diversity, percent polymorphic loci, and allelic richness are significantly correlated with population size, which highlights the danger of extinction for small, isolated populations. Small populations tend to have lower genetic diversity (Frankham 1996), which may decrease their fitness and ability to adapt to changing environmental conditions or invade new niches. The relationship between population size and the degree of homozygosity (measured by the inbreeding co-efficient, F_{IS}) in a population is of interest because resprouter populations of *E. coccinea* tend to be far smaller than seeder populations (Segarra-Moragues and Ojeda 2010, see also Table 1.3).

The amount of inbreeding in a population should affect the amount of genetic variability present and therefore help explain the variability in floral traits of *E. coccinea*.

The vegetative characteristics and genetic relationships of the two forms of *E. coccinea* have been well studied (Bell and Ojeda 1999, Segarra-Moragues and Ojeda 2010), but still leave questions about the relationships between the variation in floral morphology within and between populations and the possible correlates between floral variation and life-history form. It is expected that populations with a seeder life history will invest more in the production of flowers and fruit than resprouting populations (Carpenter 1979). In order to gain a greater fruit set and pollinator visitation, seeder flowers are expected to produce greater amounts of nectar as a reward for pollinators. This study aims to describe and quantify the differences in characteristics important to reproduction, and therefore fitness, between the seeder and resprouter forms of *E. coccinea.* As the seeder life-history form is more reliant on reproduction to maintain its position in the landscape, I hypothesise that seeder populations will show evidence of channelling a greater amount of resources into reproductive traits. Seeder populations are expected to attract more pollinators by producing larger amounts of nectar, therefore having a greater fecundity and having less variation in floral morphology, as only the most effective floral shape and colour should survive the strong selective pressure of fire wiping out an entire generation at every pass.

Methods

Study species

Erica coccinea occurs in the Cape Floristic Region in the Western Cape of South Africa. There are two recognised subspecies, *Erica coccinea* subsp. *coccinea* and *E. coccinea* subsp. *uniflora*. *E. coccinea* subsp. *coccinea* is the most common and occurs from the Cedarberg in the North, to the Cape Peninsula and eastwards as far as the Kammanasie mountains (Oliver and Oliver 2002). It is most often found on south facing mountain slopes. *E. coccinea* subsp. *uniflora* differs from subsp. *coccinea* in that the flowers are born singly at ends of short lateral branches (Oliver and Oliver 2002) instead of in triplets. This subspecies is found in lowland areas near the coast in the Cape Peninsula and eastwards towards Mossel Bay, has a seeder life-history and is represented in this study by populations S06 (Sandberg - Agulhas National Park) and S10 (Cape Point - Table Mountain National Park).

Resprouter individuals of *E. coccinea* can be identified by the presence of a swollen rootstock or lignotuber which gives rise to multiple stems, in comparison to seeder individuals which lack a lignotuber and are single stemmed. Resprouters may, however, be single stemmed if they have not yet been through a fire, and in this case they can be identified by the presence of active buds at the base of the stem.

In order to quantify the differences between seeder and resprouter forms of *E. coccinea*, individuals identified as seeder or resprouter forms of the species have been analysed as separate populations, even at sites where the two forms are found growing adjacent to each other. This can be justified in the fact that individuals were identified as seeders or resprouters by checking for presence of active buds at the base of the stem.

Sampled populations – microsatellites

In addition to microsatellite data for ten seeder and ten resprouter populations already collected by Segarra-Moragues and Ojeda (2010), a further three resprouter populations (S01r, S09r and R14), and three seeder populations (R02s, R07s and S11) were sampled in

2011 (Table 1.1). Population codes used correspond with those in Segarra-Moragues and Ojeda (2010), an ‘R’ prefix denotes a resprouter population and ‘S’ indicates a seeder population. All populations were distinctly seeder or resprouter, except for populations S01 (Maclear’s Beacon - TMNP), S09 (Vogelgat Nature Reserve - Hermanus), R02 (Devil’s Peak - TMNP) and R07 (Caledon), which were ‘mixed’ populations where both seeder and resprouter forms occurred at the same site. The seeder and resprouter individuals at these sites were treated as separate populations for this study (identified by and ‘r’ or‘s’ added to the existing population code. One form of the species (S01s, S09s and R02r) had been sampled and genotyped previously by Segarra-Moragues and Ojeda (2010), so this study sampled the remaining form (S01r, S09r and R02s) in order to produce pairs of the two forms occurring in the same populations. Population R07 was previously thought to be an exclusively resprouter population, however, observations made in February 2011 showed it to be a mixed population and consequently both resprouters (R07r) and seeders (R07s) were sampled at this site in July 2011. The remaining populations, S11 and R14, were sampled earlier, in 2010 and genotyped for this study to fill in gaps in the geographical distribution of the species.

In addition, a population of a form of *Erica coccinea* that has been nicknamed the ‘dinosaur’ was sampled for genotyping. DO1 (Chapman’s Peak -TMNP) is a population of *E. coccinea* that appears to be neither seeder nor resprouter. It occurs in rocky fire refugia and, judging by the great thickness of the basal stem, grows much older than the average seeder individual, yet has no thickened rootstock like a resprouter. Although only one population was sampled for this study, several small populations occur around the Cape Peninsula, and it was retained here as an interesting anomaly.

All populations were located in stands of mature fynbos. All individuals sampled were flowering adults, and identified as seeder or resprouter individuals by checking the basal stem for evidence of a swollen rootstock and multiple stems (resprouter) or single stems with no basal buds (seeder).

Table 1.1: *E. coccinea* populations sampled for microsatellite genotyping in 2011.

	Population	**Flower colour**	**latitude**	**longitude**
S01r	Maclears Beacon (TMNP)	Red	33° 58'35''S	18° 25'15''E
S09r	Vogelgat NR (Hermanus)	Red	34° 24'00''S	19° 10'18''E
R07r	Caledon	Mixed	34°13'09''S	19°25'45''E
R07s	Caledon	Mixed	34°13'09''S	19°25'45''E
R02s	Devil's Peak (TMNP)	Yellow	33° 57'13''S	18° 26'21''E
***S11**	Salmonsdam NR	Yellow	34° 25'22''S	19° 38'04''E
***R14**	Sir Lowry's Pass	Red	34°08'58''S	18°55'52''E
D01	Chapman's Peak (TMNP)	Red	34° 04'59''S	18°21'43''E

*Starred populations were sampled by Ojeda in 2010 but processed in 2011.

DNA extraction, PCR amplification and microsatellite genotyping

DNA extraction, PCR amplification and microsatellite genotyping was undertaken at the Centro de Investigaciones sobre Desertificación-CIDE (CSIC-UV-GV) in Valencia, Spain.

DNA extraction (following Segarra-Moragues and Ojeda 2010)

Fresh leaves were sampled from 755 individuals from 29 different populations (Table 1.3) and dried in self-indicating silica gel (Merck, South Africa) until DNA extraction. Approximately 100 mg dry weight per sample was used for DNA extraction. Dry material was reduced to fine powder using 2.3 mm stainless steel beads on a Mini-beadbeater-8 cell disrupter (BioSpec, Bartlesville, OK). DNA was extracted using the SpeedTools plant DNA extraction kit (Biotools, Madrid, Spain) and eluted in 50 µl in Tris-EDTA0.1× buffer. Working dilution for PCR amplification was 1:10 of the eluted DNA solution. Eight dinucleotide microsatellite loci (Table 1.2) were amplified following Segarra-Moragues et al. (2009).

Table 1.2: Locus name, primer sequence, product size of sequenced clone and GenBank accession numbers (GB) for the eight microsatellite loci used on *E. coccinea*, as determined by Segarra-Moragues et al. (2009).

Locus	Primer sequence (5’-3’)	Size (bp)	Size range	GB
Ecoc 108	F:<NED> CGTGCCCTAAACGAAGATAGA R: GATTACCCAGAAGAGATGGATG	165	153-195	FJ543378
Ecoc 115	F:<NED> GTTGAGCTAGTGGAGGGAAGTG R: CTTTCTTCAAATCACACCTAAGCA	168	162-186	FJ543379
Ecoc 117	F:<6-FAM> GGGTCAGTGATAATTTTGAACCT R: TCACTTTGATGGCAATGGACT	157	145-169	FJ543380
Ecoc 122	F:<PET> TCCTTGAACTAGGTTTCATCTCTAA R: GCCATCGGCTGTTATTACTTATC	172	164-206	FJ543381
Ecoc 132	F:<PET> GATGTTAGCAATCCAGGTCCA R: ATCCAGATGCAAAGAACAAAGG	185	177-229	FJ543382
Ecoc 142	F:<PET> TTTGAAGGGTTTCTGCTTCTGG R: AGAGGATGGGAAGTGAGGTGAA	239	217-271	FJ543383
Ecoc 431	F:<VIC> AGTGTATGTGCTACGCCTCTGG R: GAAGGGTTCCCACTCGTCTTC	183	159-193	FJ543384
Ecoc 446	F:<6-FAM> GCGCATTGTGAATTACGCTTT R: AGCGTCTGACACCGCTACAC	235	201-309	FJ543385

PCR conditions (following Segarra-Moragues et al. 2009):

PCRs were performed in a 20µl mix containing 1× Taq buffer (Biotools), 2mM MgCl2, 0.4 mM of each DNTP (Deoxyribonucleotide triphosphate), 5 pmol each of the labelled (forward) and unlabelled (reverse) primers, 1 U of Taq polymerase (Biotools) and 20 ng of template DNA. The PCR program consisted of one step of 4 min at 94°C followed by 35 cycles each of 1 min at 94°C, 1 min at annealing temperature of 56°C and 1 min at 72°C, and a final extension step of 7 min at 72°C. The products were run on an ABI 3730 automated sequencer (Applied Biosystems) using LIZ500 as the internal lane size standard and the amplified fragment lengths assigned to allelic sizes with GENEMARKER v.2.2.0 software (SoftGenetics).

Molecular Analyses

Basic population genetic descriptors including mean number of alleles per locus (*A*), observed (H_O) and unbiased expected (H_E) heterozygosities (Nei 1978) were calculated for each population using GENETIX v. 4.05 (Belkhir et al. 2004). Wright's *F*-statistics were estimated according to Weir and Cockerham (1984) using GENEPOP'007 (Rousset 2008) and tested for significance by Fisher's exact tests. This software was also used to check for departures from Hardy-Weinberg equilibrium. Since multiple tests were carried out, sequential Bonferroni corrections were used to adjust *p*-values.

In order to investigate the relative amount of inbreeding in the two forms of *E. coccinea*, the inbreeding co-efficient, F_{IS} was determined and plotted against estimated population size for each of 10 seeder populations and 13 resprouter populations. The relationship between population size and mean number of alleles per locus (A) was also determined. Regression analyses were performed to test for significance for the relationships between F_{IS} and population size, and population size and mean number of alleles per locus.

Floral traits

Floral morphology

To investigate variation in floral traits, fully developed flowers were collected from 30 to 50 plants in each of 18 populations. Five of these populations were exclusively made up of resprouting individuals, and four were exclusively seeders. In addition, four sites had mixed populations of seeder and resprouter individuals (S01, S09, R02 and R05), often occurring in close proximity to one another(less than two metres apart). Both seeder and resprouter individuals were sampled and treated as separate populations (S01s, S01r,S09s, S09r, R02s, R02r) at the mixed sites except for site R05, where the seeder population had burnt and not yet flowered, so only the resprouter individuals were sampled (represented as population R05r). Two populations of the subspecies *uniflora* (S06 and S10), were also sampled for floral traits.

Four floral trait measurements were identified (length of calyx, corolla, anther exsertion from corolla, and stigma exsertion) and taken for one flower from each plant. Throughout this study the calyx refers to a measurement that includes the bracts, bracteoles and sepals of the flower. The anthers and style of *E. coccinea* are exserted and as the flower develops they continue to extend out of the corolla, so the presence of a broken anther ring (Geerts and Pauw 2011), signalling visitation from a pollinator, was used as an indication of a fully developed flower.

Pollination rate and floral visitors

In order to compare the frequency of visitation by pollinators, and therefore the relative attractiveness of seeder and resprouter flower to pollinators, ten mature flowers from the same branch of each plant were inspected for broken anther rings, as a proxy for visitation. This was scored as number of visited flowers out of ten. Floral visitors (birds and butterflies) were observed opportunistically, to determine whether different species visited the different forms of *E. coccinea*. Minimum estimated time spent at each site during peak flowering was three hours, many sites were visited repeatedly during peak flowering.

Fecundity

To determine variation in seed set, branches of unvisited flowers were marked and number of flowers counted. These branches were collected once seed had developed and the number of pollinated flowers assessed by swollen ovaries (many flowers dropped off, but it was assumed these were unpollinated as observations showed that developing ovaries are retained on plants until dehiscence, while unpollinated flowers drop off). Preliminary investigations showed that *E. coccinea* is capable of geitenogamy, but the occurrence of autogamy was not fully resolved. This was due to the presence of exserted anthers and stigma, which make it difficult to manipulate without damaging the flower.

Nectar volume and concentration

In order to quantify effort made towards attracting and rewarding pollinators, nectar was extracted from mature, unvisited flowers (determined by unbroken anther rings) using a glass capillary tube (1-5 µl), and the sugar concentration was measured using an Eclipse handheld refractometer (Bellingham and Stanley Ltd.). Nectar was sampled for both seeders and resprouters at the ‘mixed’ population R02 (Devil’s Peak – TMNP). Nectar was extracted from between five and ten flowers for eight plants of each life-history form, as early in the day as possible. Flowers that had nectar volume reading of zero were included in the data set as the proportion of flowers without nectar was considered relevant.

Flower colour

Flower colour at all populations sampled for morphology was visually assessed (corolla colour categorised as red, orange or yellow) as well as measured using a spectrophotometer (Jaz PX spectrometer, Ocean Optics). A minimum of three flowers per population were measured, more if the colour variation within the population was significant. In populations of mixed colours, a sample of 30 to 50 plants was visually assessed for colour and a percentage representation of colour in the population was calculated.

Population size

Populations of *Erica coccinea* are usually compact and well defined in the landscape, plants are not scattered over large area, and therefore it was generally easy to see the entire extent of the population from one vantage point. As populations ranged from hundreds to thousands of individuals, counting plants was not practical and population size was estimated by eye at peak flowering.

Data analysis

In order to test for statistical significance of differences in floral morphology, pollination rate, fecundity and nectar volume and concentration between seeder and resprouter forms, t-tests were performed on the data. The difference in variability and colour range of flower colour in seeder and resprouter forms of *E. coccinea* was analysed descriptively.

Results

Inbreeding (F_{IS})

If the inbreeding co-efficient F_{IS} is greater than zero, it indicates that inbreeding or selfing occurs in a population, where F_{IS} is less than zero, outbreeding is the norm in a given population. An F_{IS} value of one implies a completely inbred population, whilst a value of negative one indicates a completely outbred population. Plotting F_{IS} against population size shows the possible effect of population size on the degree of inbreeding (Fig. 1.1a). Neither seeder nor resprouter populations showed a strong relationship between inbreeding co-efficient and population size (with $R^2 = 0.0087$. $p = 0.78$ and $R^2 = 0.2717$, $p = 0.067$ respectively). The seeder populations of *E. coccinea* all had a positive F_{IS} value, indicating inbreeding and heterozygote deficiency in all of these populations, although the highest F_{IS} value was a comparatively low value of 0.155 (population S02) which suggests that although inbreeding can and does occur, it is not the dominant breeding system in the populations. Of the 14 resprouter populations, only two had a negative F_{IS} value, indicating that inbreeding is possible in the resprouter form. Only six of 14 resprouter populations (43%) showed Hardy-Weinberg deviations towards heterozygote deficiency (Table 1.3) that were significantly different from the expected value (H_E), while eight of the 12 seeder populations (67%) showed significant deviations from Hardy-Weinberg. Mean F_{IS} values for seeder and resprouter populations are significantly different ($t = 2.19$, $df = 22$, $p=0.019$).

There is positive correlation of increasing number of alleles per locus (A) with increasing population size for *E. coccinea* (Fig. 1.1b). This is more marked in seeder populations ($R^2 = 0.6395$, $p = 0.0054$) than resprouters ($R^2 = 0.3858$, $p= 0.023$), as seeder population size varies from approximately 200 at S03, S04 and S09 in the Hermanus area, to around 2000 at S07 in Cape Agulhas and over 3000 at population S05 on Potberg Mountain in the De Hoop Nature Reserve. Population S05 was not included in the regression analysis as it appears to be an outlier in the correlation between population size and number of alleles, this may be due to the small sample size (30 individuals) in relation to the size of the entire population (3000). Resprouter population sizes are much smaller and less variable in size than seeder populations, with an average of about 300 plants per population. The mean number of alleles is lower and less variable in resprouter than seeder populations (7.45 and 9.16 respectively, t = 3.3092, df = 22, p = 0.0016).

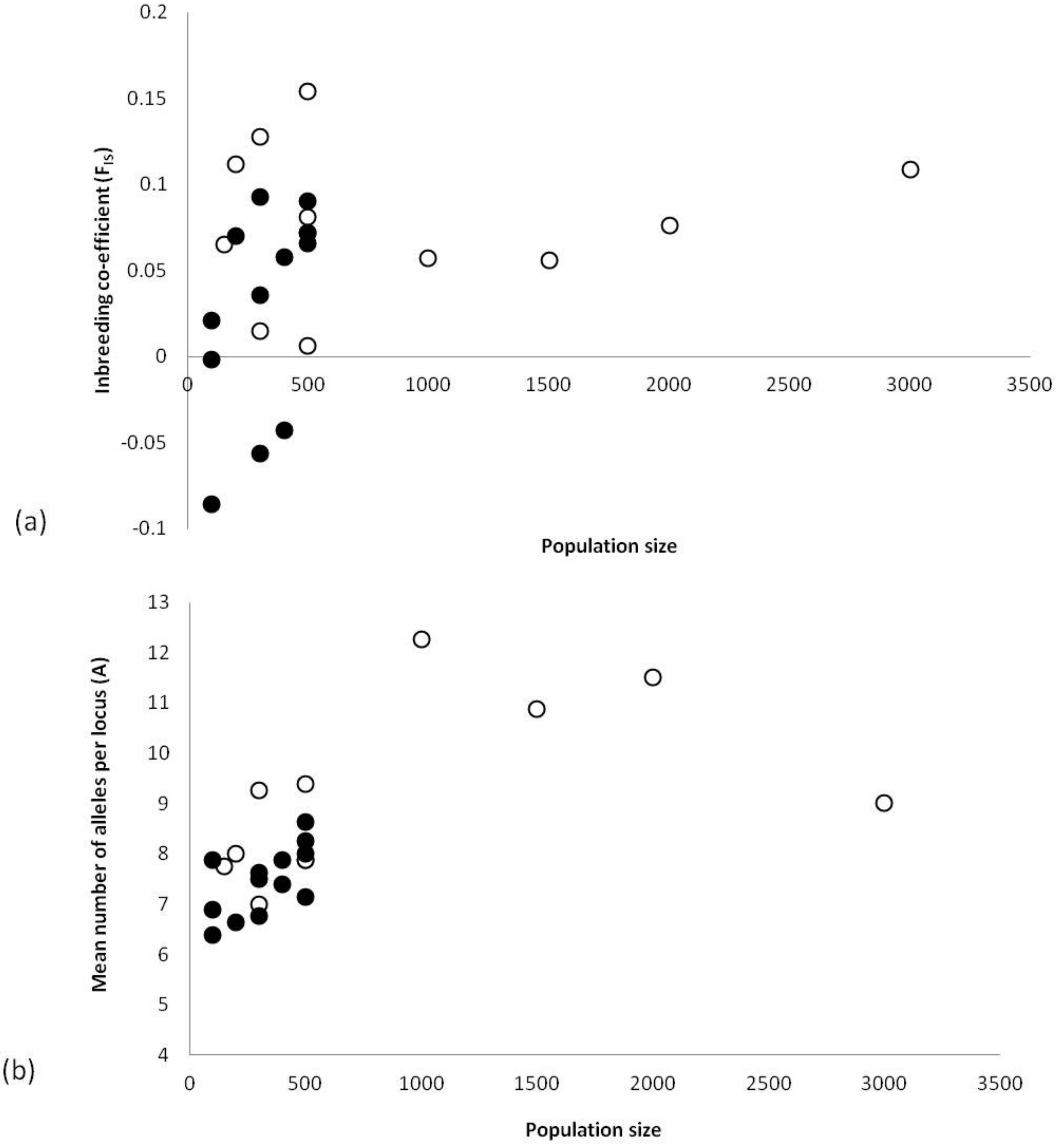


Figure 1.1: (a) The relationship between inbreeding co-efficient (F_{IS}) and population size and (b) Mean number of alleles per locus (a) and population size. Seeder populations are represented by open circles, resprouter populations are represented by solid circles.

Table 1.3: Population data and genetic diversity in 12 seeder, 14 resprouter, two subsp. *uniflora* and one 'dinosaur' population of *Erica coccinea* for eight microsatellite loci. [A = mean number of alleles per locus; H_O and H_E = observed and expected (unbiased) heterozygosity; F_{IS} = inbreeding coefficient].

**data from Segarra Moragues and Ojeda 2010*

Habit and populations	Pop size	N	A	H_O	H_E	F_{IS}
Seeder						
***S01s – TMNP. Below Maclear's Beacon**	>500	30	7.88	0.633	0.637	+0.006^{ns}
***S02 – Kleinmond. Kogelberg Biosphere Reserve**	>500	29	9.38	0.641	0.758	+0.154***
***S03 - Shaw's Pass. Between Caledon & Hermanus**	100-200	19	8.00	0.711	0.800	+0.112***
***S04 – Hermanus. Camphill Village**	150-300	24	7.00	0.703	0.714	+0.015^{ns}
***S05 – De Hoop NR. Potberg**	>3000	30	9.00	0.642	0.720	+0.109***
***S07 – Cape Agulhas. Soetanysberg**	>2000	30	11.50	0.704	0.762	+0.076*
***S08 – Napier. Napierberg FM antenna**	500-1000	31	12.25	0.714	0.757	+0.057^{ns}
***S09 – Hermanus. Vogelgat NR**	200-300	24	9.25	0.646	0.741	+0.128**
S11 – Salmonsdam NR	750-1500	36	10.88	0.760	0.806	+0.056*
R02s – TMNP. Devil's Peak	>500	30	7.88	0.596	0.649	+0.081*
***R05s – Botrivier. Honingklip**	100-150	10	7.75	0.734	0.785	+0.065*
R07s – Caledon. Swartberg		30	8.88	0.758	0.764	+0.008^{ns}
Resprouter						
***R01 – TMNP. Blackburn ravine**	300-500	30	8.63	0.683	0.732	+0.066***
***R02r – TMNP. Devil's Peak**	200-300	20	7.5	0.638	0.661	+0.036^{ns}
***RO3 – Tulbagh Waterval NR**	200-300	31	6.75	0.577	0.636	+0.093*
***RO4 – Franschoek. Mount Rochelle NR**	200-300	19	7.63	0.77	0.729	-0.056^{ns}
***RO5r – Botrivier. Honingklip**	>500	27	8	0.704	0.759	+0.072*
***RO6 – Stellenbosch. Swartboskloof, Jonkershoek NR**	>500	30	8.25	0.654	0.705	+0.072***
R07r – Caledon. Swartberg		27	8.75	0.676	0.748	+0.096*
***R08 – Greyton. Boesmanskloof trail**	300-400	16	7.38	0.719	0.689	-0.043^{ns}
***R09 – Swellendam. Marloth NR**	300-400	30	7.88	0.683	0.726	+0.058*
***R10 – Heidelberg. Near Grootvaderbosch NR**	<100	15	6.38	0.717	0.660	-0.086^{ns}
***R11 – Riversdal. Krystal Kloof trail.**	400-500	27	7.13	0.667	0.732	+0.09^{ns}
R14 – Sir Lowry's Pass.	<100	30	7.88	0.741	0.757	+0.021^{ns}
S01r – TMNP. Below Maclear's Beacon.	100-200	20	6.63	0.600	0.645	+0.07ns
S09r – Hermanus. Vogelgat NR	<100	30	6.88	0.708	0.707	-0.002^{ns}
Subsp. *uniflora*						
***S06 – Cape Agulhas. Sandberg**	500-1000	30	11.00	0.696	0.757	+0.081*
***S10 – TMNP. Cape of Good Hope**	100-200	20	8.00	0.681	0.674	-0.012^{ns}
'Dinosaur' form						
D01 – TMNP. Chapman's Peak.	<100	30	5.75	0.567	0.647	+0.124**

Floral morphology, pollination and fecundity.

Of the four floral morphology traits measured, calyx length and anther exsertion showed significant differences between population means of seeders and resprouters (t=2.88, df=14, p=0.006 and t=1.93, df=14, p=0.037 respectively). Seeders had significantly less variability (measured by co-efficient of variation, CV) in anther exsertion length than resprouters (t= - 2.26, df =14, p=0.02), as well as a greater average exsertion (Fig. 1.2). The two uniflora populations, which also have a seeder life history, had the greatest average anther exsertion at 8.3mm (n=78), although this was not significantly different to the seeder populations (t=1.047, df =396, p=0.14). However, calyx and corolla length in the uniflora subspecies were significantly shorter than the seeder populations (t = 14.21, df=396, p<< 0.05; t=3.44, df=396, p=0.003 respectively).

Nectar volume was, on average, significantly greater in seeders than resprouters (t= 3.05, df=62, p = 0.0016), as was sugar concentration, although this was not highly significant and sample size was small due to the difficulty of extracting enough nectar from the flower to get a concentration reading (t=1.91, df = 29, p=0.03, Table 1.5). There were also a higher number of mature, unvisited flowers that contained no measureable amount of nectar in resprouters (18% of sample) than seeders (8%).

The Orangebreasted sunbird (*Anthobaphes violacea*) was observed as the primary, and often, only visitor to flowers of *E. coccinea* at all sites except the low lying, coastal populations of S06 (Sandberg, Cape Agulhas National Park) and S10 (Cape Point, Table Mountain National Park), where the Southern double-collared sunbird (*Cinnyris chalybeus)* was seen. In February 2012, at the red flowering resprouter population R02 (Devil’s peak, TMNP) two hours of butterfly observations made between midday and 2pm, saw 16 butterfly passes by the Mountain Pride butterfly (*Aeropetes tulbaghia*), which is associated with the pollination of red flowers (Johnson and Bond 1994). On each pass the butterflies bypassed the *Erica coccinea* plants, which were at peak flowering, and landed on the co-occurring red-flowered *Crassula coccinea*, which was also at peak flowering, and fed there for at least a full minute

Contrary to expectations, there was no significant difference in the pollinator visitation rate, quantified by broken anther rings, in seeders than in resprouters (t=-0.7, df =39, p=0.24). However, fecundity of seeders (number of flowers setting seed) was significantly greater in seeders than resprouters (t=-3.19, df=32, p=0.001).

Table 1.4: Floral morphology traits for individual populations for seeder and resprouter populations of *E. coccinea* and two populations of *E. coccinea* subsp. *uniflora.* * indicate traits for which seeder and resprouter populations are significantly different.

			average (mm)				**CV**			
Form	**Pop.**	**n**	**Calyx***	**Corolla**	**Anther exsertion***	**Stigma exsertion**	**Calyx**	**Corolla**	**Anther exsertion***	**Stigma exsertion**
seeder	**S01s**	44	6.94	15.09	7.12	9.65	0.19	0.15	0.18	0.17
	S03	47	9.53	19.16	8.25	10.39	0.09	0.06	0.15	0.13
	S04	49	8.49	18.31	7.85	9.75	0.13	0.08	0.16	0.17
	S05	50	9.14	17.60	7.84	10.07	0.09	0.08	0.14	0.15
	S08	35	9.89	19.18	7.78	9.97	0.10	0.09	0.18	0.23
	S09s	49	9.20	19.19	7.38	9.37	0.08	0.08	0.15	0.14
	R02s	46	7.79	15.53	7.27	10.73	0.09	0.09	0.19	0.15
	mean	**7**	**8.71**	**17.72**	**7.64**	**9.99**	**0.11**	**0.09**	**0.17**	**0.16**
	CV		**0.12**	**0.10**	**0.05**	**0.05**				
resprouter	**R01**	31	7.41	18.31	6.80	8.95	0.11	0.09	0.18	0.19
	R02r	49	8.12	19.50	6.98	9.75	0.12	0.10	0.22	0.20
	R04	50	7.83	17.35	5.70	8.70	0.09	0.08	0.20	0.19
	R05r	50	7.24	17.31	5.88	8.30	0.09	0.10	0.23	0.25
	R06	49	8.32	20.13	7.77	10.43	0.10	0.09	0.21	0.16
	R08	50	7.19	19.36	8.00	11.08	0.10	0.09	0.16	0.17
	R11	50	7.69	19.39	7.59	9.89	0.11	0.12	0.16	0.17
	S01r	33	7.69	18.18	7.69	9.73	0.10	0.11	0.17	0.16
	S09r	45	6.63	15.20	6.11	8.40	0.09	0.09	0.20	0.19
	mean	**9**	**7.57**	**18.30**	**6.95**	**9.47**	**0.10**	**0.09**	**0.19**	**0.19**
	CV		**0.07**	**0.08**	**0.13**	**0.10**				
uniflora	**S06**	50	6.3	15.9	7.8	9.3	**0.10**	**0.07**	**0.15**	**0.17**
	S10	28	7.0	18.4	8.9	10.5	**0.11**	**0.09**	**0.17**	**0.17**
	mean	**2**	**6.7**	**17.1**	**8.3**	**9.9**				

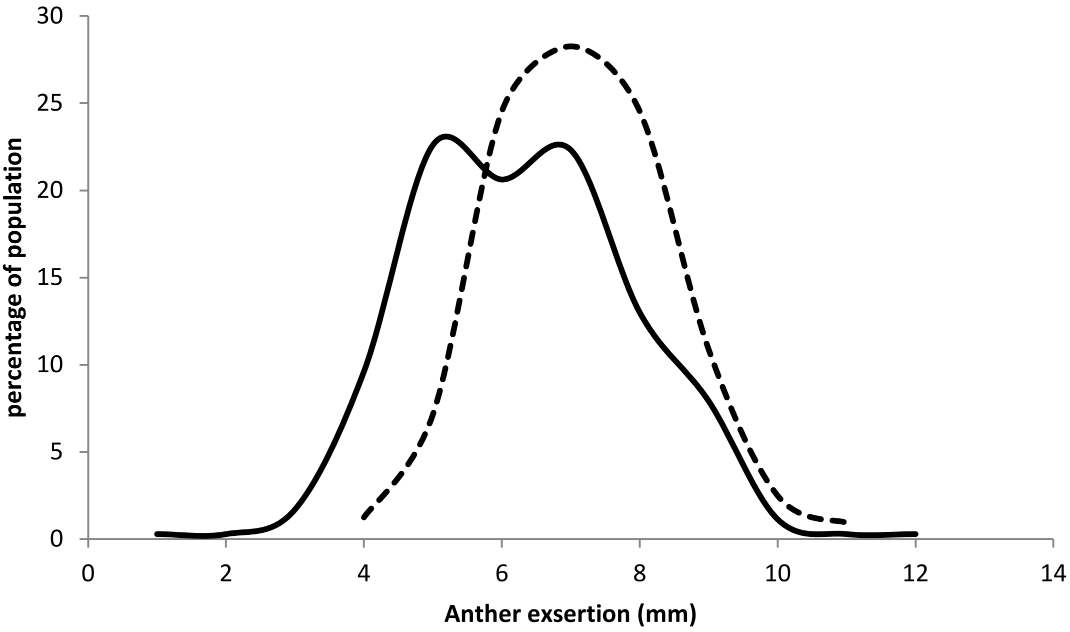


Figure 1.2: Frequencies of anther length in seeder and resprouter populations, controlling for sample size. Solid line represents resprouter populations (n=8), dashed line represents seeder populations (n=7).

Table 1.5: Pollination related traits in seeders and resprouters, averaged from mixed populations.

		Seeder	Resprouter
Nectar (R02: Devil's Peak)	**Volume (µl)**	4.2 (±3.9)	1.7 (±2.1)
	Max volume (µl)	18	11
	sample size	n=36	n=38
	Concentration (%)	14.7 (±3.8)	12.1 (±3.1)
		(range 9-26)	(range 8-20)
	sample size	n=20	n=13
Visitation (S09: Vogelgat)	**average (/10)**	5.7	5.1
	n (plants)	22	19
Fecundity (R02: Devil's Peak)	**% set seed**	41.4 (±26.05)	11.8 (±13.3)
	n (plants)	16	11
	n (flowers)	330	110

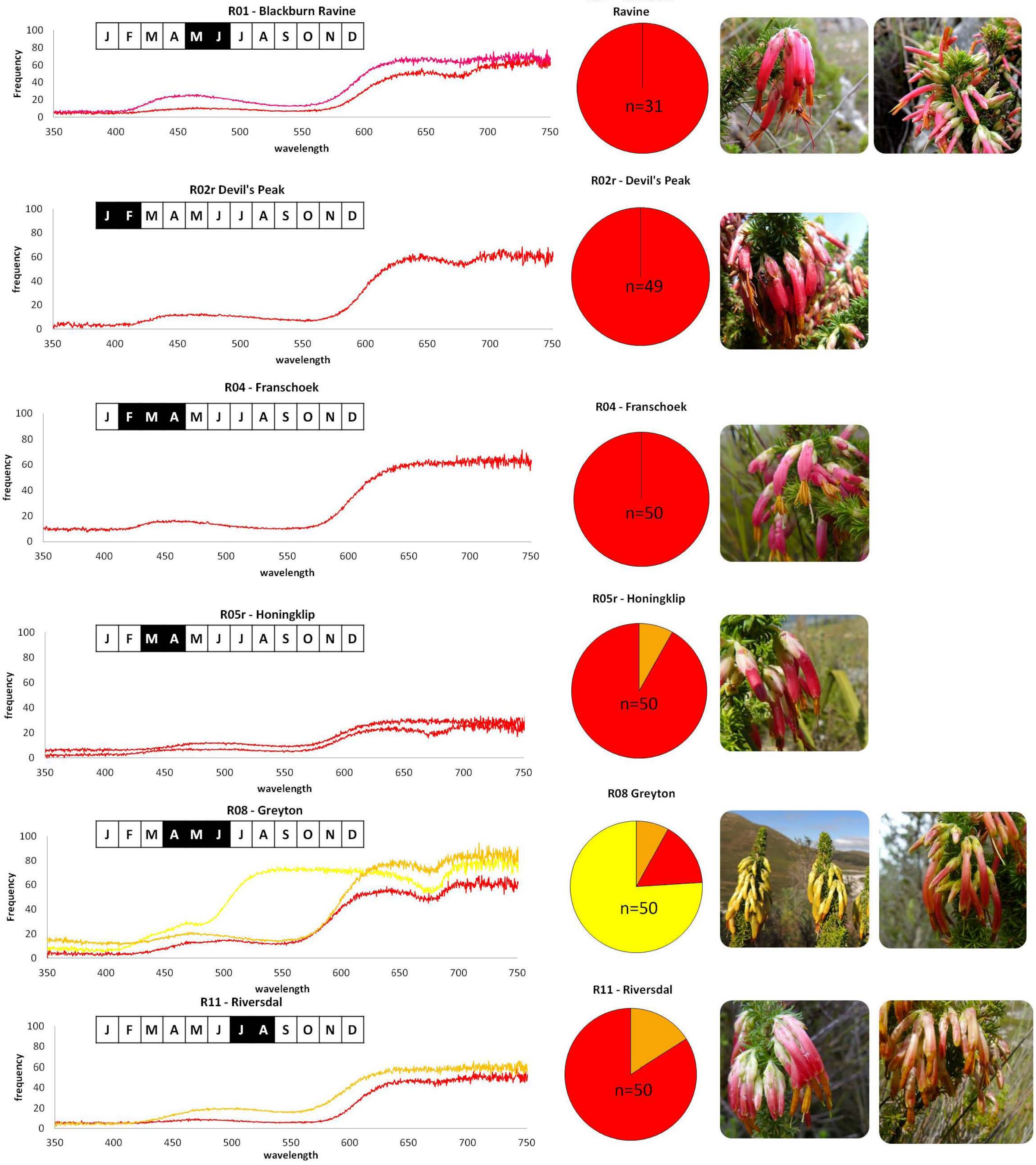
Analysis of corolla colour in eight resprouter and eight seeder (including two uniflora) populations show that colour varied greatly between life history forms and across the geographic range of *E. coccinea.* The majority of resprouter populations were found to have red flowers, whilst seeder populations were almost entirely dominated by yellow individuals. However, this pattern was not true for all populations. The resprouter populations are more variable in colour – for example, the Riversdal population (R11) was dominated by red individuals, but there were also some orange/peach coloured individuals and the later flowering resprouter populations such as R08 (Greyton) and R09 (Marloth), were dominated by yellow flowering individuals. Within mixed colour populations, it was noted that the very rare red individuals came into flower a week or two earlier than the yellow individuals (S01s – Maclears Beacon, TMNP). On a population scale this reflects the general pattern in the species, of red-flowering resprouters coming into flower in mid-summer and yellow-flowering seeders flowering later in the year in autumn and winter (Fig. 1.3).

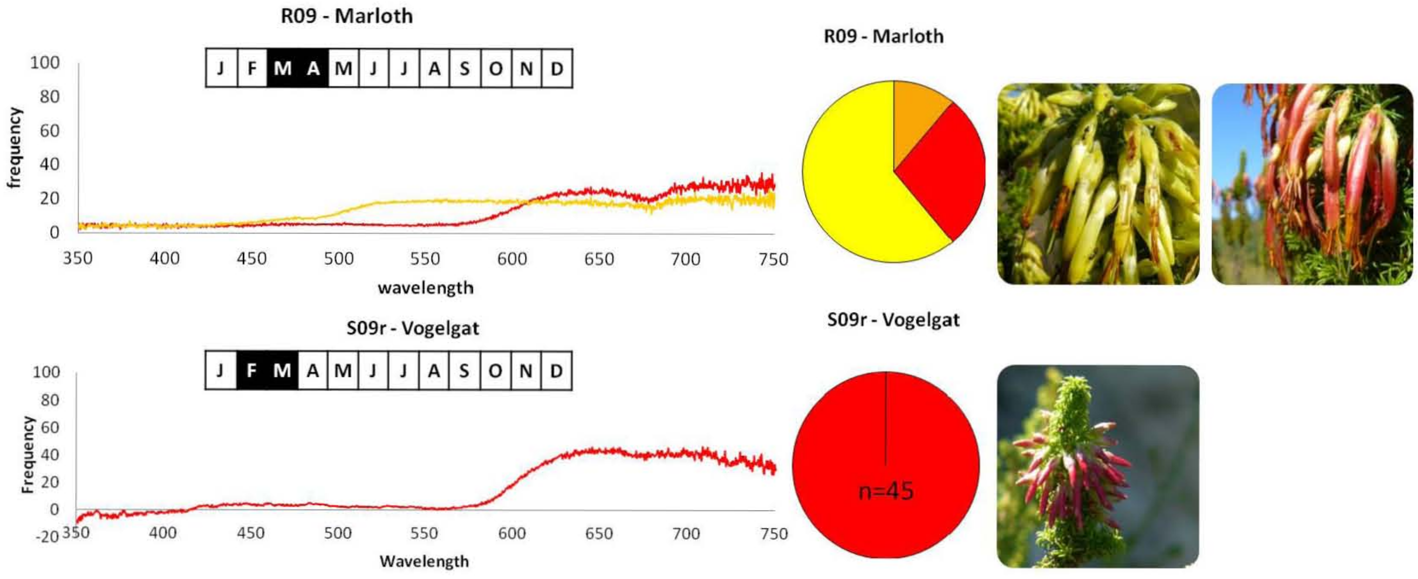
Seeder populations were found to be less variable in colour than resprouters. Only one out of the six seeder populations analysed for colour had variability in colour within the population, whereas four out of the eight resprouter populations had more than one colour represented in the population (Fig. 1.3, a and b). Population S06 (Cape Agulhas), had deep red to orange flowers and was initially assumed to be a seeder population, but on further investigation was found to belong to the subspecies *uniflora,* along with population S10, at Cape Point (Fig. 1.3c).

Resprouter populations which had uniformly red flowers were found to flower mainly in midsummer (peaking in January, tailing off in May), whereas the populations with a greater proportion of yellow or orange flowers (R08, R09, and R11), started flowering later in summer, some even in autumn (peaking in March and tailing off in August). There was less seasonal variation in the flowering of the seeder populations – all observed populations peaked between June and September (late winter/early spring). The recorded flowering period for the Devil's Peak seeder (R02s) population is longer, mainly because it was monitored more frequently than the others. The seeder population at Potberg Mountain (S05) was incredibly large (an estimated 3000 individuals, see Table 3), and may bear flowers all year round, as visits in January/February and June/July of 2011 found plants with buds and mature flowers in all stages of development at this site.

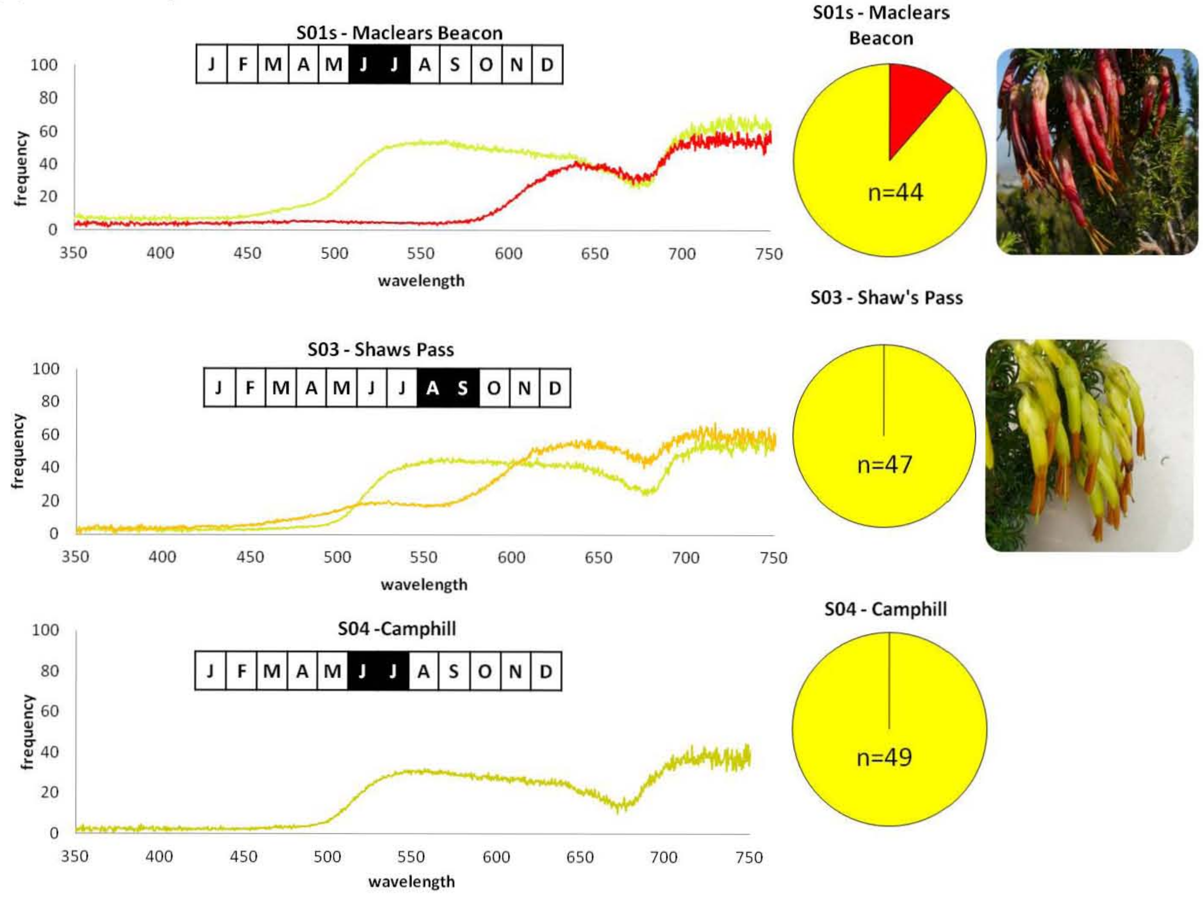
Figure 1.3: Flower colour for populations of *E. coccinea* showing the reflectance of corolla colour, proportion of colour in each population and months of the year peak flowering was observed. (a) Seeder populations, (b) resprouter populations and (c) *E. coccinea subsp. uniflora* populations.

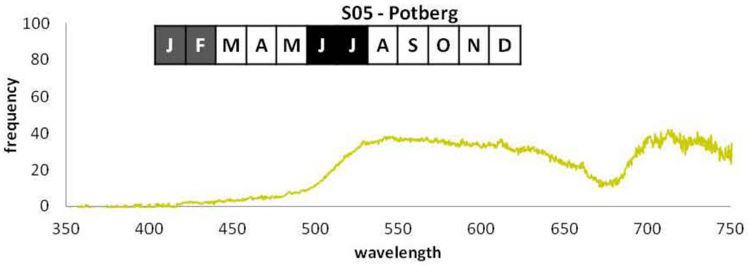
(a) Resprouter populations :

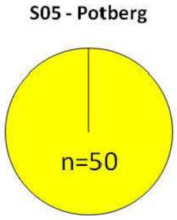


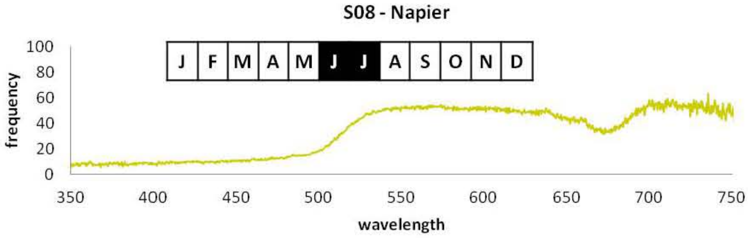


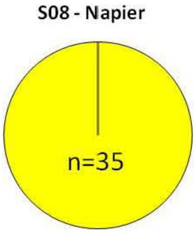
(b) Seeder Populations:

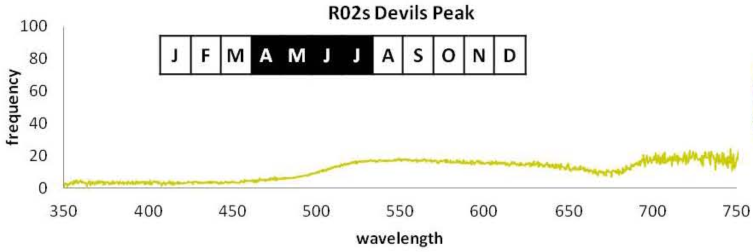


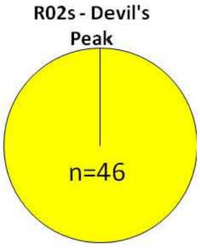




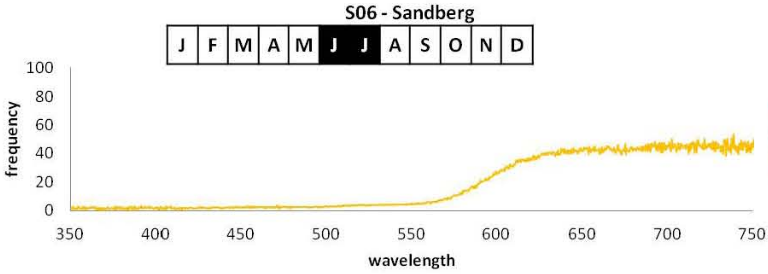


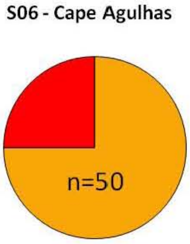


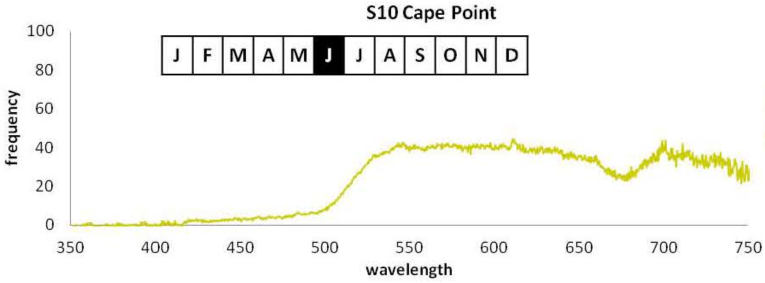


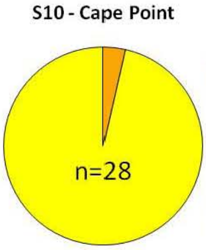


(c) *E. Coccinea* subsp. *uniflora*









Discussion

Segarra-Moragues and Ojeda (2010) find higher within-population genetic diversity and among-population differentiation in seeder populations than in resprouter populations. These results are maintained in this study with the addition of six populations and the resampling of one (Caledon resprouters – R07r). The question of interest was whether this pattern would be reflected in the floral morphology of the two forms. Analysis of morphological traits showed that floral traits are conserved within seeder populations, whilst resprouter populations show much more variation, despite having low genetic variability. This is especially apparent when looking at the range of corolla lengths and anther exsertion, traits which are deemed to be important for pollination. The positive mean F_{IS} values, indicating heterozygote deficiency, found for both seeders and resprouters could be due to small sample size and low allele frequency for the highly variable loci and populations rather than a reflection of inbreeding.

Anther exsertion was found to be the floral trait with the most differentiation between seeders and resprouters (Fig. 1.2); seeder populations had significantly longer anther exsertion than resprouters (Table 1.4). Seeder populations also had less variability in anther length than resprouters, this suggests that seeders may be under stabilising selection, in an environment where greater anther exsertion and therefore perhaps larger anthers and a greater pollen load, may be favourable for reproduction. Seeders may be under a greater selective force because of their life history strategy and shorter generation times, therefore longer anther exsertion selected for. In contrast with microsatellite results which find more within population variation in seeders than resprouters, morphological variation is greater in resprouters than seeders.

Nectar volume is significantly different between forms, seeders have a higher volume, but concentration in seeders and resprouters is not significantly different. Variability in both volume and concentration measurements was high, but this seems to be a common trait in the *Erica* genus - Turner et al. (2012) found a much greater nectar volume in *Erica halicacaba*, also a sunbird pollinated *Erica* species, but this ranged from 1.25 to 100 µl. The

nectar sugar concentration was also higher, with 36.4%, but once again, variability was high, from 19-63.5%.
With respect to flower colour specificity across life-history forms, the seeder populations were dominated by yellow individuals, with only population S01s (Maclears Beacon) containing a few red individuals. This suggests that selection on the yellow colour (or against the red colour) in seeders may be stronger than the necessity for the red colour in resprouters.

Both seeder and resprouter populations are pollinated by the orange-breasted sunbird, and the assumption that the Mountain Pride butterfly has influenced the red colour of summer-flowering resprouter populations has proved invalid, as the butterfly appears to be more interested in other red-flowering species. This argument would be strengthened with further observations, studying proboscises of mountain pride butterflies caught in the area for the presence of *Erica* pollen. It also remains to be seen whether the butterfly proboscis is strong enough to break the anther ring of the *Erica* flower, releasing the pollen, and it's more likely that any observed visits would be of the butterfly robbing the flower by piercing the wall of the corolla (Johnson and Bond 1994), and therefore having no effect on the selection of colour in the species. The question of what influences the pattern of different corolla colours remains unanswered in this study, although a seasonal pattern of red flowers in midsummer, mixed red, orange and yellow flowers in late summer and autumn and yellow flowers in winter and early spring has been identified. This suggests that flower colour is not influenced by pollinator choice, and perhaps not even linked to life-history form, but rather a physiological factor linked to seasonal climate or resources available. Further work on the presence of protective red anthocyanin pigments in the leaves and flowers of *E. coccinea* in the hot summer months may help to answer this question.

Although the two morphotypes of *E. coccinea* have been treated as a single species, it is clear there are differences in population dynamics between the seeder and resprouter forms. Seeder populations invest more resources into attracting pollinators and have higher fecundity than resprouters. Resprouters have much smaller populations with lower genetic diversity and a lower heterozygote deficiency, indicating a lower degree of inbreeding. Although seeder populations have a higher instance of homozygosity, they also have a greater number of alleles per locus, but this may be attributed to larger population sizes and not to a different rate of molecular change to resprouter populations.

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Chapter 2:

Variation in floral traits amongst seeding and resprouting populations of *Erica coccinea*: inferences from P_{ST}-F_{ST} comparisons

M.C. Malan, J.G. Segarra-Moragues, J.J. Midgley, T. van der Niet, F. Ojeda.

Introduction

One of the main aims of evolutionary biology is to understand the processes that drive differentiation in quantitative traits amongst populations of the same species (Merilä and Crnokrak 2001). In order to understand those processes we first have to identify them, hence in this study using P_{ST}-F_{ST} comparisons to identify which morphological traits are selected for or simply due to drift.

A major life-history dichotomy in fire prone ecosystems is that of seeders versus resprouters (Verdu et al. 2007, Clarke et al. 2012). Seeders tend to have shorter generation times than resprouters, as each generation is completely wiped out by fire and the population regenerates entirely from seed. Plants with a resprouter life history survive most fires and may also have some recruitment of seedlings after fire, resulting in a cross-over between generations, and longer generation times. Although it was previously assumed that the high turnover of generations would increase the rate of evolution in seeders, it is now relatively well established that resprouter lineages do not have lower rates of molecular evolution and diversification than nonsprouters (Lamont and Wiens 2003; Verdu et al., 2007). However, higher generational turnover and the heavy reliance of seeders on a high fecundity and seedling recruitment to prevent extinction of the population after fire means that selection on floral biology and reproductive traits may be stronger in seeders than resprouters.

Studies comparing the two strategies of resprouting or seeding after fire often use closely related species pairs, but in the Cape Floristic region the genus *Erica* has several species that contain both seeder and resprouter morphotypes (Ojeda et al 2005). *Erica coccinea* is one of these species and presents us with the ideal opportunity to contrast the patterns of selection within a species which has both morphotypes in distinct populations. In *Erica coccinea*, resprouter plants survive fire by resprouting from an underground lignotuber, whilst seeder plants are killed by fire, but regenerate through a soil-stored seed bank. Both seeder and resprouter populations are pollinated by the same species, the orange-breasted sunbird (*Anthobaphes violacaea*). Seed dispersal is limited as seeds have no morphological traits to help their dispersal in the wind or by other means, field observations show that seeds seem to be released and dropped right at the base of the parent plant, so gene flow across landscapes can only be affected by a pollinator. In taxonomic terms the floral morphology of both seeder and resprouter forms appears to be the same, however, at a microevolutionary scale, there is a greater genetic diversity in seeder populations than those of resprouters (Segarra-Moragues and Ojeda 2010). It is proposed that much of the genetic diversity in seeders is explained by their ability to diversify and adapt to new ecological niches at a fast pace (Wells 1969, Smith and Donoghue 2008, Segarra-Moragues and Ojeda 2010), relative to the slower resprouters, which, however, continue to persist in the landscape.

F_{ST}, the inbreeding co-efficient, defined by Wright (1951), is a measure of population differentiation defined as the ratio of among-deme to total variance at the allelic level (Miller et al. 2008). One way of determining F_{ST} is using neutral markers such as microsatellites. The F_{ST} of neutral market loci is determined by the balance between random genetic drift and migration (Merilä and Crnokrak 2001), therefore we can use F_{ST} to determine the relative contributions of these factors to differentiation in a population. P_{ST} is a measure of variance in quantitative phenotypic traits between populations of an organism. It is also known as phenotypic Q_{ST} (Saether et al. 2007). To estimate quantitative genetic variation (Q_{ST}), phenotypic traits must be measured in F2-generation individuals from experimental crosses under controlled, common-garden conditions. In doing so, non-additive environmental effects (i.e. phenotypic plasticity) are purged and thus only phenotypic variation attributable to additive genetic effects is accounted for. However, depending on the species under study, this is often difficult to achieve (Leinonen et al. 2008). In such instances, phenotypic differentiation (P_{ST}) based on measures of phenotypic traits in wild individuals across natural populations has been used as a surrogate for Q_{ST} (e.g. Leinonen et al. 2006; Sæther et al. 2007; Wojcieszek and Simmons 2011). The limitations of using P_{ST} are that non-additive genetic effects and environmental effects are ignored, which can mask true levels of genetic variation in quantitative traits and may lead to inaccurate conclusions (Pujol et al. 2008). However, it has been shown that studies that used P_{ST} obtained similar results to other studies based on conventional Q_{ST} values (Leinonen et al. 2008; Brommer 2011; Mobley et al. 2011) and, hence they continue to be useful, at least as a first approach to explore the role of selection on a phenotypic trait (or suite of traits).

P_{ST}-F_{ST} comparisons have been used in several studies to determine the extent of, for example, local adaptation in metapopulations of snipe (Saether et. al 2007), divergent selection in stickleback fish (Raeymaekers et al. 2007) and stabilizing selection in millipedes (Wojcieszek and Simmons 2011), but there are few examples of this kind of analysis being used on plant species (Toyama and Yahara 2012), as the more conventional Q_{ST} measure is easily used in plant studies. Although germination/growth time and the difficulty of growing *Erica coccinea* to flowering maturity makes it impractical to measure traits in F2 generations (hence using P_{ST} instead of Q_{ST} to look at morphological variation), this study deals with floral traits, such as corolla length, which are less phenotypically plastic than are vegetative traits (Mal and Lovett-Doust 2007; Pélabon et al. 2011). In addition, and this is very rare in the P_{ST}-F_{ST} studies, we are comparing two different ecological morphs (seeders and resprouters), in order to investigate whether the natural selection signal is stronger in the seeder morph with short generation times, or the resprouter morph, with long-lived plants and overlapping generations. By using P_{ST} instead of Q_{ST}, we may be including some distortion from both non-additive genetic effects (e.g. maternal) and/or from environmental effects, but it is unlikely that the direction of that distortion will be different in seeder and resprouter populations.

Erica coccinea subsp. coccinea is a long tubed, bird pollinated Erica species which occurs mainly on south-facing mountain slopes in the Cape Floristic Region of South Africa (Oliver and Oliver 2002). It is characterised by well-exserted anthers and red or yellow flowers borne in threes along the branches of whorled, ericoid leaves. Both seeding and resprouting strategies are represented in discrete populations spread across the Western Cape, enabling the study of these contrasting life history strategies and their effects on population variation within a species. This study aims to investigate firstly, whether there are differences in floral attributes between seeder and resprouter populations of *E. coccinea*. Here we are presented with a rare opportunity to not only compare seeding and resprouting populations within a species, but also, in some populations, to sample both seeding and resprouting individuals within the same site, cancelling out, as far as possible, the influence of environmental factors *in situ*. Secondly, through the work of Segarra-Moragues et al. (2009) and following the work of Segarra-Moragues and Ojeda (2010) who have identified and analysed microsatellites for this species, we are able to look at morphological variation in the two forms of *E. coccinea* within a framework of neutral genetic markers. Comparing differentiation quantitative traits between pairs of populations (Q_{ST}) against neutral genetic differentiation (F_{ST}) at putatively neutral molecular markers (such as microsatellites) has been accepted as a valid approach to infer an effect of selection for a phenotypic trait (or group of traits) in natural populations (Merilä & Crnokrak 2001; Leinonen et al. 2008; Whitlock 2008). Basically, if differentiation in morphological traits is found to be higher than that of neutral genetic differentiation amongst populations ($Q_{ST} > F_{ST}$), it can be said that divergent directional selection is acting on the species. However, if differentiation in morphological traits is lower than that of the neutral genetic markers, ($Q_{ST} < F_{ST}$), stabilizing or uniform selection is shown to be acting on the populations. Finally, if both estimates are not significantly different to each other ($Q_{ST} \approx F_{ST}$), the hypothesis of neutral differentiation cannot be rejected, which means that observed differentiation of quantitative traits could be explained by genetic drift alone.

This study has analysed the genetic structure of seeder and resprouter populations of *E. coccinea* using microsatellites. In order to compare this to morphological variation, four floral traits (calyx length, corolla length, anther exsertion and stigma exsertion) were measured in seven seeder and nine resprouter populations across the Western Cape of South Africa. To investigate the relative strengths of selection on each of the floral traits, due to the influence of short generation times in seeders and longer, overlapping generations in resprouters, variation in these four floral traits between pairs of populations (P_{ST}) was then compared to the variation in the microsatellites expected under neutrality (F_{ST}). Comparisons were performed separately for the seeder and resprouter groups of populations, in order to contrast the two regeneration forms. The results of this analysis are expected to show that selection acts more strongly on floral traits of the seeder morphotypes than the resprouter.

Methods

Sampled Populations

Four seeder populations (i.e. composed of only seeder individuals - S03-S05, S08) and six resprouter ones (i.e. composed of only resprouter individuals - R01, R04-R06, R08, R11) were sampled across a large part of the species' geographical range (Fig. 2.1). In addition, three mixed populations (S01, S09, and R02) were sampled. Individuals of both morphotypes were considered as separate populations in all subsequent analyses. Therefore, the overall sampling included seven seeder and nine resprouter populations, (Table 2.1, Fig. 2.1).

Morphology

Fully developed flowers were collected from 30 to 50 plants in each of the 16 populations, at peak flowering. Four morphometric measurements (flower length, calyx length, anther exsertion and stigma to anther separation) were identified and measured for one fully mature flower from each plant using digital callipers. The anthers and style of *E. coccinea* are exserted and as the flower develops they continue to extend out of the corolla, so only flowers which had been visited by a pollinator (determined by broken anther ring structure, see Geerts and Pauw 2011) were measured. Anther exsertion emerged as the measurement having most variability (see Fig 1.2, chapter 1). The four traits were all used for the P_{ST}- F_{ST} calculations.

DNA extraction, PCR amplification and Microsatellite genotyping

Fresh leaves were sampled from 407 individuals (between 19 and 30 plants from each of the 16 populations), dried on silica gel (Chase & Hills 1991) and stored until DNA extraction. Approximately 100 mg dry weight per sample was used for DNA extraction. Dry material was reduced to fine powder using 2.3 mm stainless steel beads on a Mini-beadbeater-8 cell disrupter (BioSpec, Bartlesville, OK, USA). DNA was extracted using the SpeedTools plant DNA extraction kit (Biotools, Madrid, Spain) and eluted in 50µl in Tris-EDTA 0.1× buffer. Eight dinucleotide microsatellite loci were amplified following Segarra-Moragues et al. (2009). PCR products were analysed on an ABI3730 automated sequencer (Applied Biosystems) using LIZ500 as internal lane size standard and fragments were assigned to allele classes using Genemarker v. 1.85 software (Softgenetics, State College, PA, USA).

Data analyses:

Molecular Analyses

Basic population genetic descriptors including mean number of alleles per locus (A), and observed (H_O) and unbiased expected (H_E) heterozygosities (Nei 1978) were calculated for each population using GENETIX v. 4.05 (Belkhir et al. 2004). Wright's F-statistics were estimated according to Weir and Cockerham (1984) using GENEPOP'007 (Rousset 2008) and tested for significance by Fisher's exact tests. This software was also used to check for departures from Hardy-Weinberg equilibrium. Since multiple tests were carried out, sequential Bonferroni corrections were used to adjust p-values.

Pairwise genetic distances between populations were calculated using Nei et al's. (1983) D_A, genetic distance index as implemented in POPULATIONS v.1.2.3 beta (Langella 2000). The statistical robustness of the groupings was assessed by bootstrap analysis over populations with 1000 replicates (Felsenstein 1985). The resulting distance matrix was used to construct Neighbour-Joining (NJ) midpoint rooted trees using MEGA 5.0 software (Tamura et al. 2011). Finally, ARLEQUIN v. 3.5 (Excoffier and Lischer 2010) was used to calculate a matrix of pair wise F_{ST} values between populations.

Genetic (F_{ST}) vs. morphological (P_{ST}) differentiation comparison

Pairwise P_{ST} values were calculated using the equation:

$$P_{ST}=\sigma^2_{GB}/\ (\sigma^2_{GB} + 2\ \sigma^2_{GW})$$

Where σ^2_{GB} and σ^2_{Gw} represent the among-population and within-population variance components for a phenotypic trait in the wild respectively (Raeymaekers et al. 2007, Wojciezek and Simmons 2012). Variance for pairs of populations of seeders and resprouters for each morphological trait was calculated separately using one-way ANOVAS in R. Pairwise F_{ST} values (see appended Table A1) were obtained with Arlequin version 3.5, and these were plotted against the P_{ST} values to determine the relative influences of selection and genetic drift on the resprouter and seeder forms of *Erica coccinea.* Wojciezek and Simmons 2012 checked the significant differences between F_{ST} and P_{ST} by calculating bootstrapped means with 95% confident limits for pairwise F_{ST} values and pairwise P_{ST} values, for each trait. P_{ST} overestimates morphological variability (Saerther et. al 2007), hence we have used a 99% confidence interval here instead. The P_{ST}-F_{ST} comparison was approached by bootstrapping 99% confidence intervals of the P_{ST} estimates for each of the four floral traits and comparing them to the bootstrapped 99% confidence intervals of the F_{ST} values, separately for seeder and resprouter populations (Fig. 2.6).

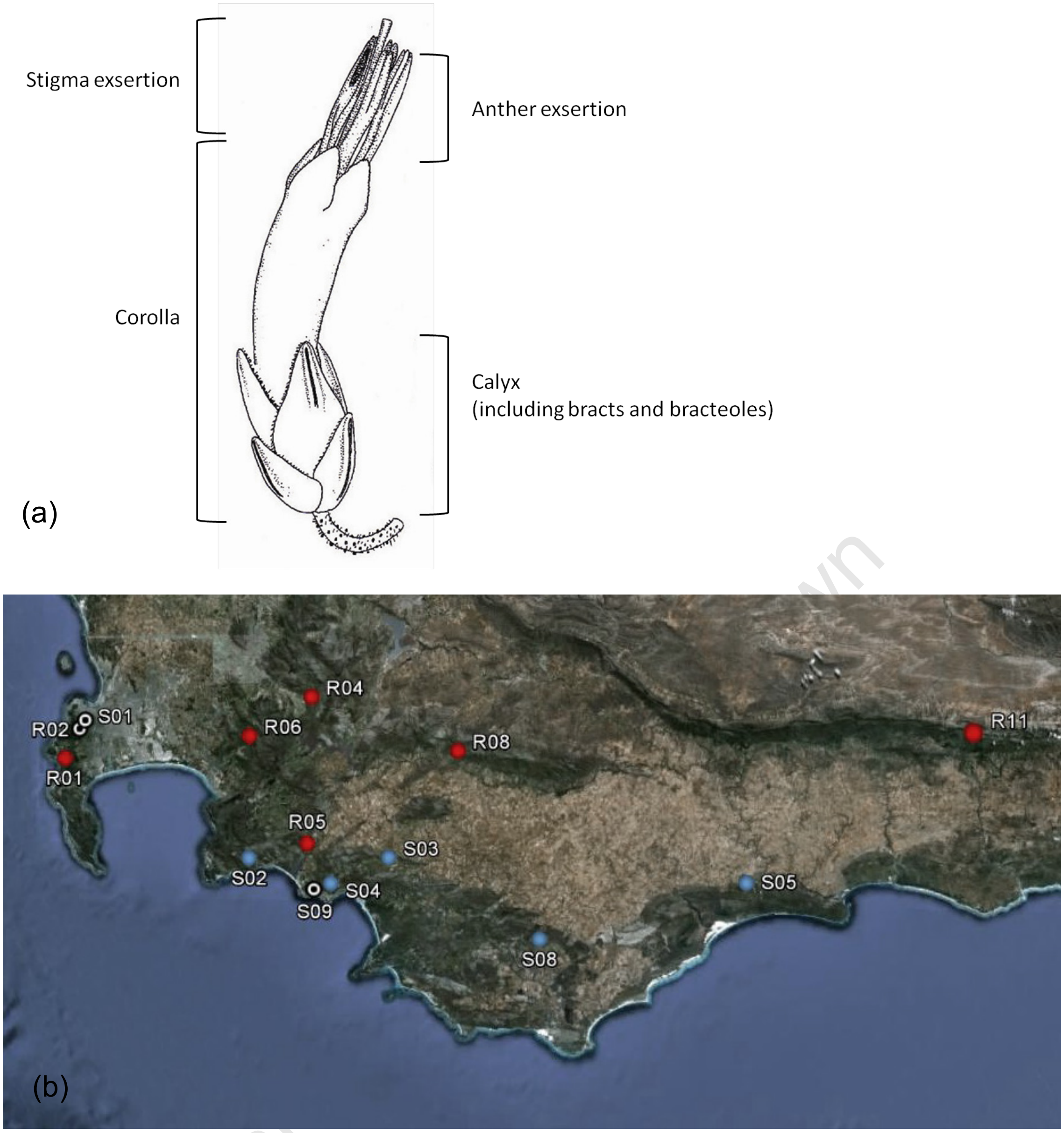


Figure 2.1: a) Diagram of *Erica coccinea subsp. coccinea* flower indicating morphological measurements used (source: Oliver and Oliver 2002) b) Map of the Western Cape of South Africa, showing the location of the 16 populations used in this study (source: Google Earth).

Table 2.1: Population data and genetic diversity indices in seven seeder and nine resprouter populations of *Erica coccinea* for eight microsatellite loci.

Habit and populations	Flower colour	Latitude	Longitude	Altitude (m)	Population size	N^1	A^1	$H_O{}^1$	$H_E{}^1$	$F_{IS}{}^1$
Seeder										
S01s-Maclear's Beacon, TMNP	Yellow	33º 58' 35"S	18º 25' 15"E	800	>500	30	7.88	0.633	0.637	$+0.006^{ns}$
S03-Shaw's Pass, Hermanus	Yellow	34º 19' 03"S	19º 24' 24"E	226	100-200	19	8.00	0.711	0.798	$+0.112^{***}$
S04-Camphill village, Hermanus	Yellow	34º 23' 10"S	19º 13' 22"E	123	150-300	24	7.00	0.703	0.714	$+0.015^{ns}$
S05-De Hoop NR. Potberg	Yellow	34º 22' 19"S	20º 32' 33"E	287	>30000	30	9.00	0.642	0.719	$+0.109^{***}$
S08-Napier	Yellow	34º 31' 41"S	19º 53' 17"E	558	500-1000	31	12.25	0.714	0.756	$+0.057^{ns}$
S09s-Vogelgat NR, Hermanus	Yellow	34º 24' 00"S	19º 10' 18"E	125	200-300	24	9.25	0.646	0.739	$+0.128^{**}$
R02s-Devil's Peak, TMNP	Yellow	33º 57' 13"S	18º 26' 21"E	953	>500	30	7.88	0.596	0.648	$+0.081^{*}$
Resprouter										
S01r-Maclear's Beacon, TMNP	Red	33º 58' 35"S	18º 25' 15"E	800	100-200	20	6.63	0.600	0.644	$+0.070^{ns}$
S09r-Vogelgat NR, Hermanus	Red	34º 24' 00"S	19º 10' 18"E	125	<100	30	6.88	0.708	0.707	-0.002^{ns}
R01-Blackburn Ravine, TMNP	Red	34º 03' 21"S	18º 22' 19"E	205	300-500	30	8.62	0.683	0.731	$+0.066^{***}$
R02r-Devil's Peak, TMNP	Red	33º 57' 13"S	18º 26' 21"E	953	200-300	20	7.50	0.638	0.660	$+0.036^{ns}$
R04-Mont Rochelle NR, Franschoek	Red	33º 53' 46"S	19º 09' 37"E	897	200-300	19	7.62	0.770	0.730	-0.056^{ns}
R05r-Honingklip farm, Bot Rivier	Red	34º 16' 53"S	19º 08' 52"E	145	>500	27	8.00	0.704	0.758	$+0.072^{*}$
R06-Swartboskloof, Jonkershoek NR	Red	33º 59' 56"S	18º 57' 48"E	558	>500	30	8.25	0.654	0.704	$+0.072^{*}$
R08-Bosmanskloof, Greyton	Yellow (mostly)	34º 02' 07"S	19º 37' 23"E	280	300-400	16	7.38	0.719	0.690	-0.043^{ns}
R11-Krystal Kloof, Riversdale	Red (but variable)	33º 57' 43"S	21º 15' 15"E	780	400-500	27	7.13	0.667	0.731	$+0.090^{ns}$

[1] N, sample size, respectively; A, mean number of alleles per locus; H_O, H_E, observed and expected heterozygosity, respectively; F_{IS}, inbreeding coefficient.

Results

A neighbour-joining tree based on D_A genetic distance (Nei et al. 1983) of eight nuclear microsatellite markers shows that the 15 populations of *E. coccinea* used in this study are roughly clustered into two groups – seeders and resprouters (Fig. 2.2).
The relationships between the main floral characters (A) corolla length and anther exsertion, (B) corolla length and stigma exsertion, (C) stigma and anther exsertion in plants, and (D) calyx and corolla length from the seven seeder and nine resprouter populations are shown in Fig 2.3. Regarding (A), there was a very slight, but significant, positive correlation between corolla length and anther exsertion in both seeder and resprouter forms (adjusted-R^2 = 0.038, *p*-value = 0.0002 for seeders; and adjusted-R^2 = 0.11, *p*-value < 10^{-9} for resprouters). Regarding (B), there was no significant correlation between corolla length and stigma exsertion in seeders (adjusted-R^2= 0.0, *p*-value=0.78), whereas in resprouters there was a very slight, but significant, positive correlation (adjusted-R^2= 0.12, *p*-value < 10^{-9}). In (C), there was a significant, positive correlation between stigma and anther exsertion in both regeneration forms (adjusted-R^2 = 0.436, *p*-value < 10^{-9} for seeders; and adjusted-R^2 = 0.479, *p*-value < 10^{-9} for resprouters). (D) shows the slight difference in floral shape between seeders and resprouters.

There was no significant difference in corolla length between regeneration forms (p-value = 0.498; mixed nested ANOVA: population (random factor) nested in regeneration form (fixed factor); Type III sums of squares; Fig. 2.4A). In contrast, mean calyx length was significantly higher in seeders than in resprouters (p-value= 0.016; mixed nested ANOVA; Fig. 2.4B). Also, anther exsertion was significantly more pronounced in seeders (p-value= 0.041; mixed nested ANOVA; Fig. 2.4C). Finally, even though stigma exsertion was slightly more pronounced in seeders (Fig. 2.4D) the analysis failed to find significant differences between regeneration forms (p-value = 0.253; mixed nested ANOVA).

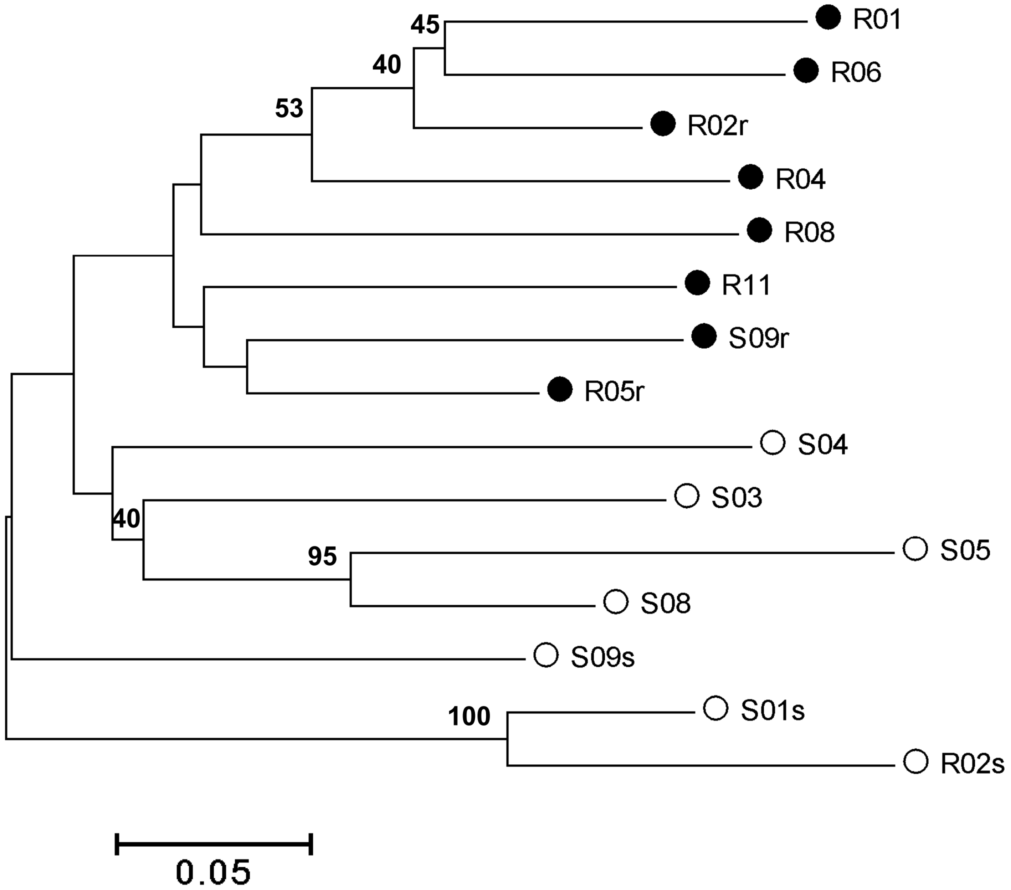


Figure 2.2: Neighbour-joining tree based on D_A genetic distance (Nei et al. 1983) showing the relationships among seeder (open circles) and resprouter (closed circles) populations. Bootstrap values above 40% are indicated above branches.

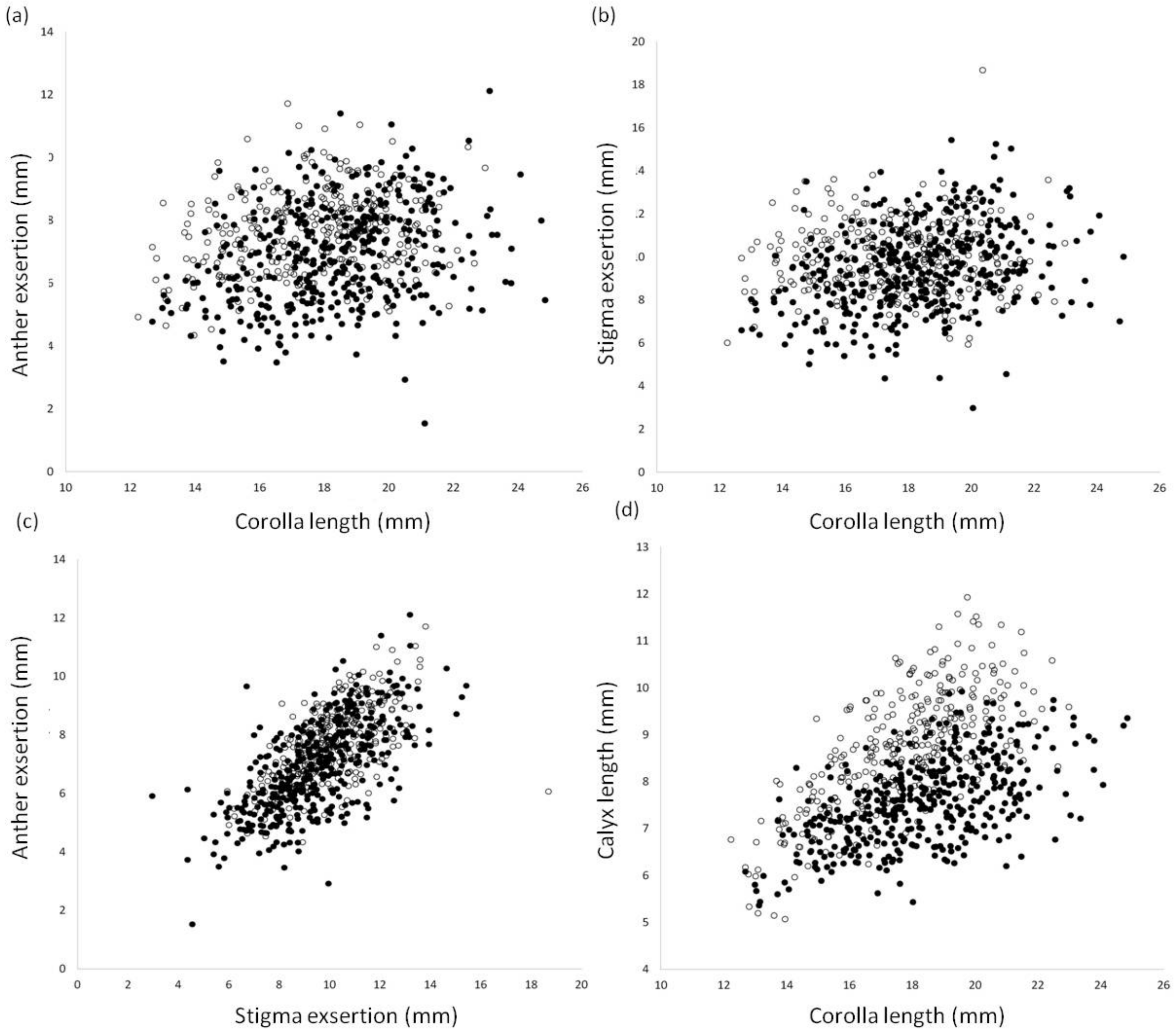


Figure 2.3: Relationship between key floral traits, describing the difference in shape between seeder (open circles) and resprouter (closed circles) forms.

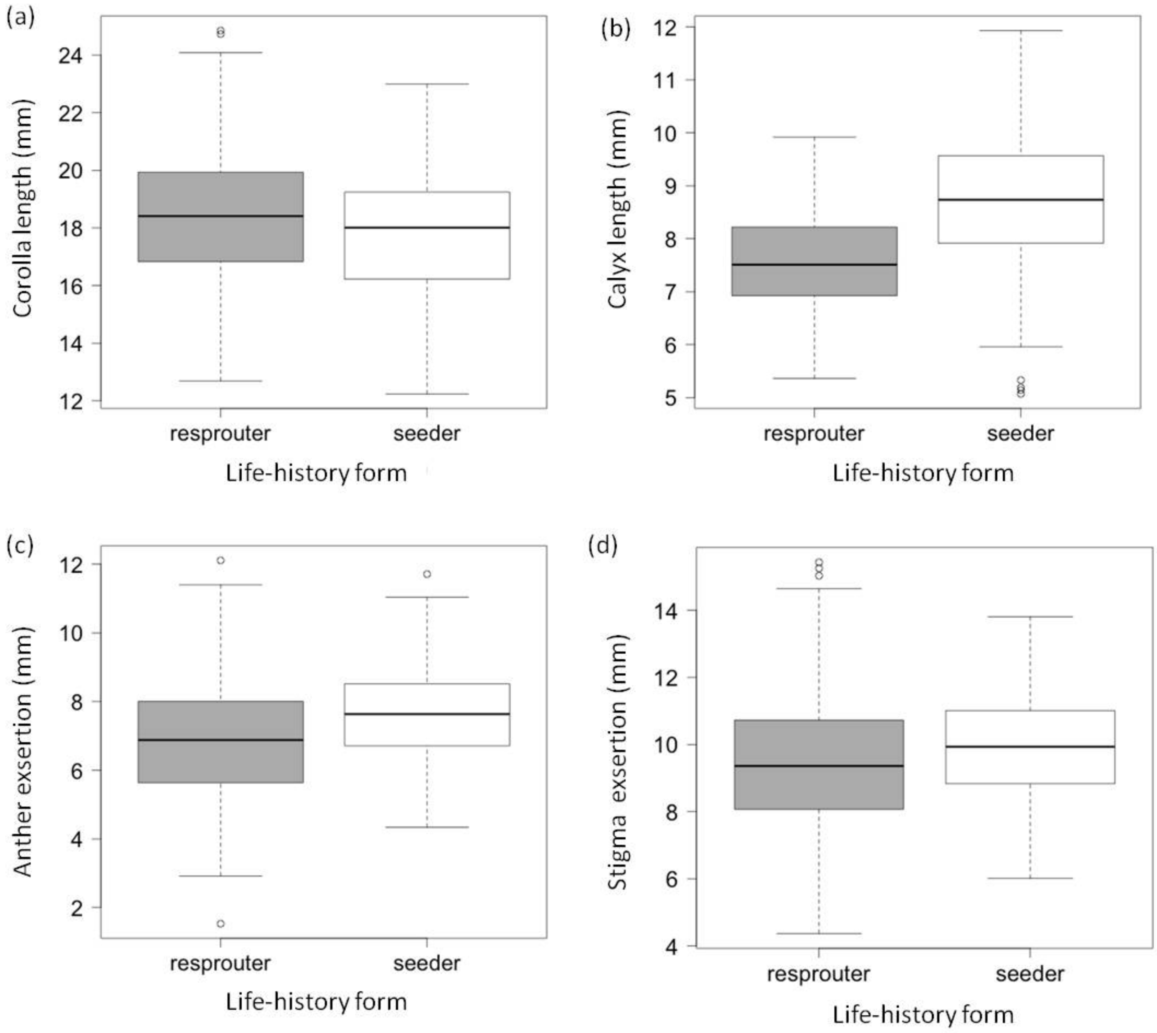


Figure 2.4: Floral trait measurements for seeder and resprouter populations. (a) Corolla length. (b) Calyx length. (c) Anther exsertion. (d) Stigma exsertion.

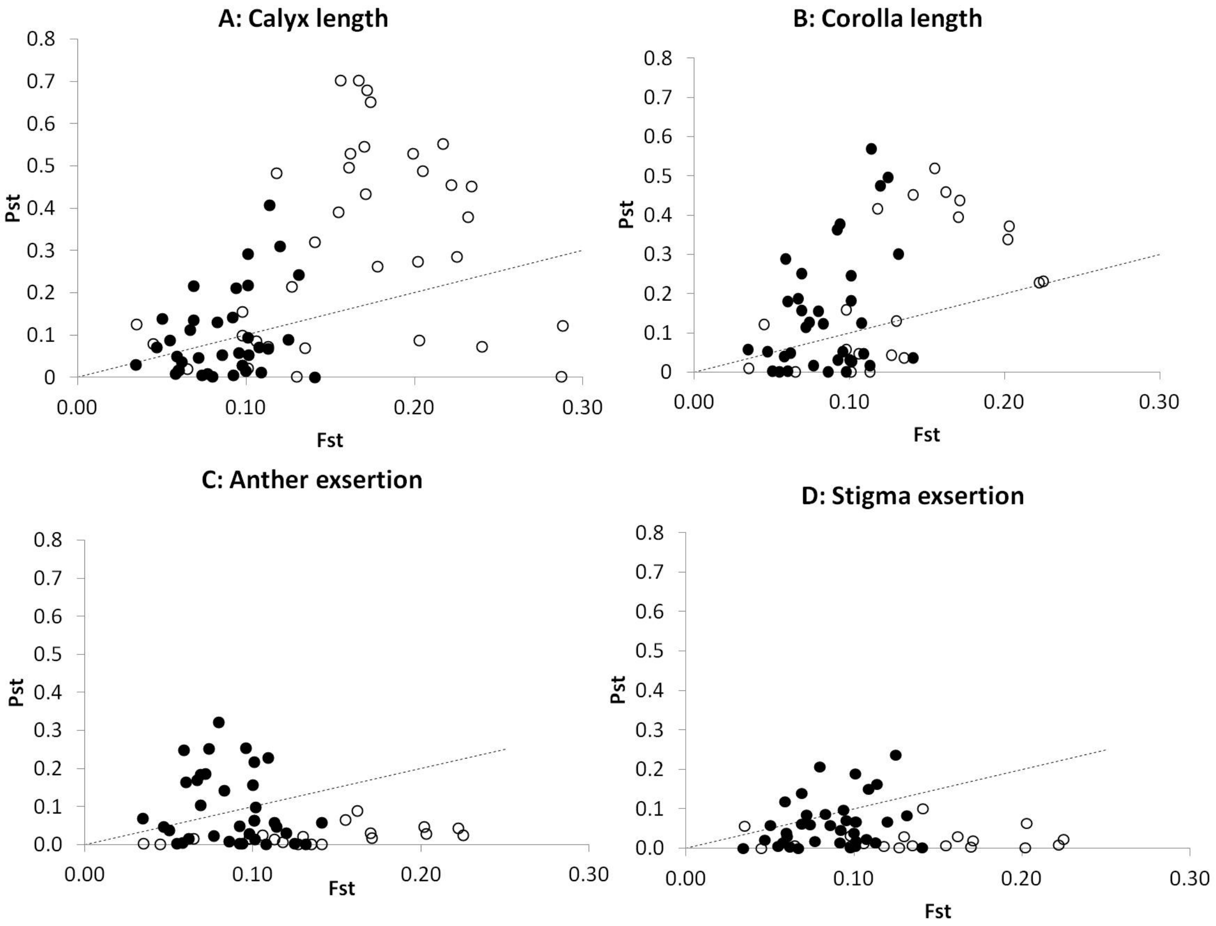
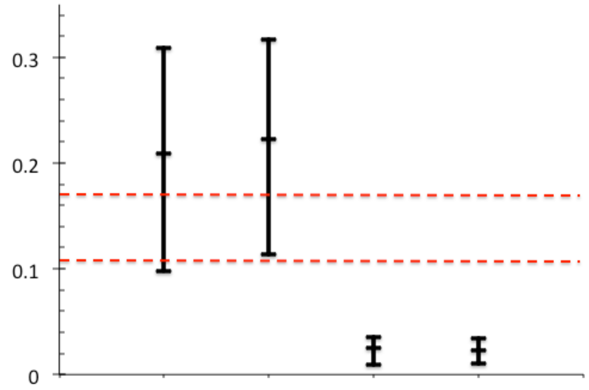


Figure 2.5: P_{ST}-F_{ST} comparisons for each of the four floral traits considered in seeder (open circles) and resprouter (closed circles). The dashed line represents where $P_{ST} = F_{ST}$.

(a) Seeder



(b) Resprouter

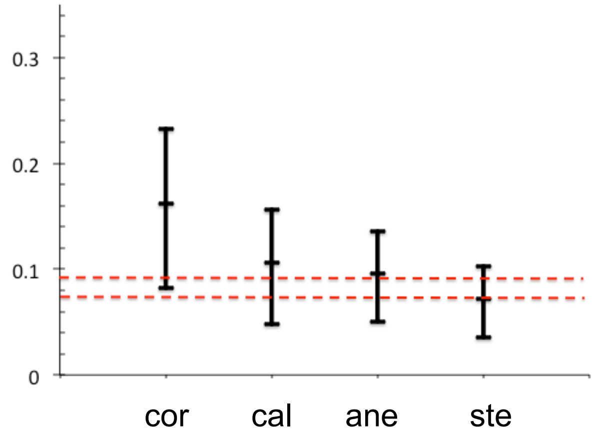


Figure 2.6: Mean P_{ST} estimates (± 99% CI) calculated by bootstrapping (no. of replicates = 10,000) for corolla length (cor), calyx length (cal), anther exsertion (ane) and stigma exsertion (ste) in (a) seeder and (b) resprouter populations. The red dashed lines indicate the 99% CI (also calculated by bootstrapping) of estimated F_{ST}.

Mean anther exsertion was higher in individual seeder populations than the overall mean, (Fig. 2.4C), and in addition, the average within-population coefficient of variation of this trait was significantly lower in seeder (CV= 0.162) than in resprouter populations (CV= 0.195; one-tailed t-test, t = 2.54, df = 12.897, p-value = 0.012). This means that not only is anther exsertion on average higher in seeder populations, but it is also less variable within seeder than within resprouter populations. Fig. 2.5 illustrates the P_{ST}-F_{ST} pairwise comparisons for each individual floral trait in seeder and resprouter populations.

Regarding stigma exsertion, there was significantly lower phenotypic differentiation than expected under neutrality ($P_{ST} < F_{ST}$) in both seeder and resprouter populations (Table 2.2), although 99% confidence intervals overlapped slightly in resprouters (Fig. 2.6). The most interesting result is the comparatively low phenotypic differentiation in anther exsertion among seeder populations compared to resprouter ones, significantly lower than the neutral expectation ($P_{ST} << F_{ST}$) in seeder but not in resprouter populations. This reflects the existence of strong, uniform selection in this trait across seeder populations. A lower P_{ST} than F_{ST} could also be explained by high phenotypic variance within populations owing to divergent selection at the very local scale (Von Wettberg et al. 2008 - read). However, should this be the case, we would also expect a lower P_{ST} than F_{ST} in resprouter populations, owing to their significantly higher within-population variability (i.e. higher CV) in this trait, which is not the case.

Discussion

The strong, uniform selection for increasing anther exsertion (and therefore a possible increase in male function and efficiency) across seeder populations may be explained by the contrasting population dynamics between both regeneration forms (Ojeda et al. 2005; Segarra-Moragues & Ojeda 2010): when a fire occurs, all adult plants in a seeder population are killed and the population regenerates by germination and recruitment from the seed bank (Ojeda et al. 2005). Therefore, selection will favour those phenotypes that contribute more to the seed bank, either by producing more seeds (female component of fitness) or by siring more seeds (male component of fitness). Since resprouter adult plants survive fire and post-fire recruitment is not as important to population persistence (Ojeda et al. 2005) as well as the fact that there is a cross-over of generations in resprouter populations, selection to increase the male component of fitness may be more relaxed, and therefore, the role of random genetic drift could be prevalent to account for higher phenotypic variation.

P_{ST}-F_{ST} analyses clearly show different forces of selection and genetic drift working on the two life-history forms of *E. coccinea*. There appears to be a strong selective force working on anther exsertion of the seeder form. This has resulted in relative uniformity of the anther length within and between the seeder populations, compared to very variable anther exsertion within resprouter populations. Uniformity may be caused by a high degree of inbreeding in the seeder populations – larger floral displays in the seeder form (pers. obs.) give greater opportunities for selfing to occur, whether pollinator mediated or autonomous, leading to more homogenous populations than in the sparser flowered resprouter form. However, genetic diversity is much greater in seeder populations than in resprouter populations (Segarra-Moragues & Ojeda, 2010), which implies that self pollination does not have too strong an effect on the population dynamics, as a high degree of selfing would lead to low genetic diversity. Therefore there must be a strong selective force acting on anther exsertion in seeder populations, resulting in a uniform, optimal size, despite high genetic diversity in the population.

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Appendix to Chapter 2

Table A1: Pairwise Fst values for 16 populations of *E. coccinea* used to create the neighbour-joining tree in Chapter 2 (Fig. 2.2.)

	S01s	S01r	S03	S04	S05	S08	S09s	S09r	R 1	R02r	R02s	R 4	R05r	R 6	R 8	R 11
S01s	0															
S01r	0.1485	0														
S03	0.16214	0.12649	0													
S04	0.20186	0.16989	0.09822	0												
S05	0.22211	0.18241	0.09772	0.13548	0											
S08	0.17023	0.10533	0.06508	0.12671	0.04544	0										
S09s	0.11802	0.16698	0.10096	0.10589	0.13016	0.11339	0									
S09r	0.17474	0.1315	0.11307	0.13814	0.16081	0.11508	0.1347	0								
R 1	0.14212	0.06017	0.10667	0.16453	0.1632	0.09486	0.13467	0.09161	0							
R02r	0.14915	0.0344	0.10397	0.12892	0.16523	0.08846	0.14585	0.12029	0.04698	0						
R02s	0.03472	0.14188	0.15476	0.20256	0.2249	0.17104	0.14092	0.15996	0.13783	0.15194	0					
R 4	0.12786	0.09228	0.09736	0.12722	0.15781	0.10424	0.10262	0.10072	0.06249	0.05973	0.14497	0				
R05r	0.151	0.10151	0.06702	0.11412	0.11923	0.06736	0.07547	0.06704	0.05841	0.06936	0.157	0.05515	0			
R 6	0.15774	0.10777	0.12371	0.1157	0.16179	0.12043	0.12267	0.1142	0.08327	0.07672	0.14881	0.05883	0.06949	0		
R 8	0.19186	0.09579	0.07639	0.09579	0.10876	0.08448	0.13769	0.12494	0.10904	0.05036	0.19227	0.10096	0.07992	0.1012	0	
R 11	0.18159	0.1409	0.08704	0.08388	0.13636	0.10673	0.10259	0.09358	0.10009	0.09831	0.19099	0.07355	0.07201	0.11329	0.08601	0

Chapter 3:

Is the variability in seeder and resprouter populations of *Erica coccinea* a sign of incipient speciation or is merely a polymorphism?

Introduction

It has already been shown that seeding and resprouting is pre-determined in seedlings of *Erica coccinea* (Bell and Ojeda 1999, Verdaguer and Ojeda 2002). Field observations (see chapter one) suggest that the resprouter and seeder forms of *E. coccinea* also have separate flowering times – resprouter populations tend to flower in the summer months, whilst seeder populations flower in winter. This separation of flowering times could be an isolating mechanism between the two forms of *E. coccinea* in populations where both the seeding and resprouting forms occur. Warren et al. (2011) found that shifts in flowering phenology have played a significant role in the radiation of the Cape flora, and in the case of *E. coccinea*, this may be the cause of reproductive isolation of the two life-history forms. Allochronic speciation refers to a mode of sympatric speciation in which the differentiation of populations is primarily due to a phenological shift without habitat or host change (Santos et al. 2007). Where flowering phenologies of adjacent populations are separated, there is a disruption of gene flow between the populations (Levin 2006), leading to reproductive isolation and, over time, differentiation between the populations, or incipient speciation.

In this paper I ask whether seeders and resprouters are genetically isolated or whether they tend to interbreed. Microsatellite markers, also known as Simple Sequence Repeats (SSRs), or Short Tandem Repeats (STRs), are repeating sequences of 1-6 nucleotides found with high frequency in the genomes of most species (Selkoe and Toonen 2006). Microsatellites are a useful tool for determining the relatedness of populations within a species because they are non-coding regions of DNA that mutate rapidly, enabling us to identify individuals and assign individuals to a population. In this way it is possible to track migrations of individuals from one population to another, or to determine if two populations are interbreeding. Segarra-Moragues et al. (2009) have identified eight microsatellite loci for use in *E. coccinea* and in this study I use the results of microsatellite genotyping of 18 populations of *Erica coccinea* to create a Neighbour Joining tree to determine the patterns of relatedness between populations and life history forms.

In this study ‘mixed’ populations of *Erica coccinea* are defined as sites where both seeder and resprouter individuals occur intermingled in the same small area. Previous studies on the population genetics of *E. coccinea* by Segarra-Moragues and Ojeda (2010) included only one of these mixed sites (R05 – Botrivier). My investigation goes further, adding four more mixed sites to the microsatellite analysis (S01- Maclears Beacon; R02- Devils Peak; R07 – Caledon and S09 – Vogelgat). With this increased sample size of mixed populations, the first question for this chapter investigates the genetic similarity of seeder and resprouter individuals in pure and ‘mixed’ populations. I ask whether seeder and resprouter individuals in mixed populations are more closely related to each other, or to seeder/resprouter individuals in other populations. If the two life-history forms in mixed populations are less similar to each other than other seeder/resprouter populations, it suggests limited interbreeding between the forms.

By tracking the flowering phenology of two of these mixed populations I attempt to answer the second question for this chapter: in sites where both seeder and resprouter individuals of *E. coccinea* occur, is the flowering phenology sufficiently separated to consider the forms reproductively isolated from each other? If the two morphotypes are separated genetically and flowering phenology is sufficiently separated to prevent cross-pollination between seeder and resprouter individuals, it can be argued that seeder and resprouter populations in *E. coccinea* may represent a case of incipient speciation. However, if the two forms are intermingled genetically and flowering phenology overlaps, the presence of two different life-history forms within the species may just be a polymorphism.

Methods

DNA extraction, PCR amplification and Microsatellite genotyping

Fresh leaves were sampled from 10 to 30 individuals in each of 29 populations (see Table 1.3 in chapter 1 for populations), the leaves were dried on silica gel and stored until DNA extraction, PCR amplification and genotyping following methods of Segarra-Moragues et al. (2009) and Segarra-Moragues and Ojeda (2010) (see methods in Chapter 1).

Molecular Analyses

Pairwise genetic distances between populations were calculated using Nei et al's. (1983) D_A, genetic distance index as implemented in POPULATIONS v.1.2.3 beta (Langella 2000). The statistical robustness of the groupings was assessed by bootstrap analysis over populations with 1000 replicates (Felsenstein 1985). The resulting distance matrix was used to construct Neighbor-Joining (NJ) midpoint rooted trees using MEGA 5.0 software (Tamura et al. 2011).

Mixed population descriptions

The following populations were chosen because they both contain both the seeder and resprouter forms of *Erica coccinea*, often growing immediately adjacent to each other, and therefore sharing the same soil types, climatic variables, and have access to the same pollinator population. This allowed for the influence of many environmental factors to be ignored in the direct comparison of life-history strategies. Populations R02 and S09 were chosen for the detailed flowering phenology study as they were well separated geographically and relatively easy to access regularly.

R02 – Devil's Peak: This population occurs just below the summit of Devil's Peak, part of the Table Mountain National Park reserve. Seeder and resprouter individuals co-occur on a steep, south-facing slope. Resprouters have red flowers in midsummer, whilst seeders have yellow flowers in late winter/spring.

S09 – Vogelgat Nature Reserve, Hermanus: As at Devil's Peak, seeder and resprouter individuals grow intermingled on a steep, south-facing slope, although at this site the seeder population extended much further than the resprouter one. Resprouter individuals tended to be found in the rockier areas of the slope. Once again, resprouters flowered red in midsummer and seeders flowered yellow in late winter/spring.

S01 – Maclears Beacon, Table Mountain National Park: in this population seeder and resprouter populations are adjacent to each other but not well mixed – the seeder individuals grow higher on the slope than the resprouters, which tend to grow in rockier areas. The resprouters flower red in midsummer and the seeders flower yellow in late winter/spring, but there are some red flowering seeder individuals in this population.

R05 – Honingklip farm, Botrivier: seeder and resprouter populations in this site grew on opposite sides of a small valley. Genetic material for the seeder individuals was collected in 2009, but the population then burned down, and seedlings were not yet large enough to flower when this study returned to the site in 2011. Resprouter individuals, however, had recovered from the fire and were flowering in summer, with mostly red flowers, some tending to orange.

R07 – Caledon: this site was located at a very disturbed site above a reservoir and pine plantation. It was initially thought to contain only resprouter individuals, but subsequent site visits found that there were also seeder individuals present. Flowering at this site was erratic, possibly year-round, and it was often difficult to determine if a plant was seeder or resprouter, leading to the conclusion that there were some hybrids in this population, which looked like it had been protected from fire for a lengthy period.

Phenology

Sixteen to twenty marked plants of each form in two mixed populations (R02-Devil's Peak, TMNP and S09-Vogelgat NR, Hermanus) were monitored monthly over a 12/13 month period. Plants were assessed on what percentage flowers were at the bud (from newly formed buds to mature buds), mature (flower fully formed with anthers exserted), and senescent (flowers mature but visited, anther ring has been disturbed and anthers have released most pollen grains) stage.

Results

Neighbour Joining tree

Populations are roughly separated into two main groups, a resprouter group (group 'A', Fig. 3.1) and a seeder group (group 'D'). The two sampled populations of *E. coccinea subspecies uniflora* separate out into their own branch of the tree (group E). Group B contains the seeder populations that occur in the Table Mountain National Park (R02s and S01s). These two populations seem to be clusterd within the resprouter group, but are not paired with their adjacent resprouter populations (R02r and S01r), suggesting that the seeder form evolved elsewhere and then migrated back into the area. Group C contains two mixed sites of *E. coccinea* – R07 and R05. In these two sites the seeder and resprouter populations appear genetically similar, whereas in the other mixed sites – S01, R02 and S09, the seeder and resprouter populations are not adjacent to each other on the Neighbour Joining tree.

Phenology and floral morphology

At both site R02 (Devil's Peak) and S09 (Vogelgat) peak flowering of seeder and resprouter populations does not overlap (Fig. 3.2 and 3.3), leaving limited opportunity for cross-pollinated between forms. This is particularly clear in the Vogelgat population, where peaks are sharp and distinct between the two forms. At Devil's peak, the seeder form clearly has a longer flowering period than the resprouter form.

Devil's Peak and Maclears Beacon are situated in close proximity to each other in Table Mountain National Park (TMNP), and according to the Neighbour Joining tree (Fig. 3.1), are genetically similar. The Vogelgat site is situated further away in Hermanus in the Overberg District of the Western Cape, in an area where seeder populations traditionally dominate (Ojeda et al. 2005). In the TMNP populations (S01 and R02), the resprouters have, on average, larger flowers than the seeders (Table 3.1), whereas at the Vogelgat population the seeders have larger flowers.

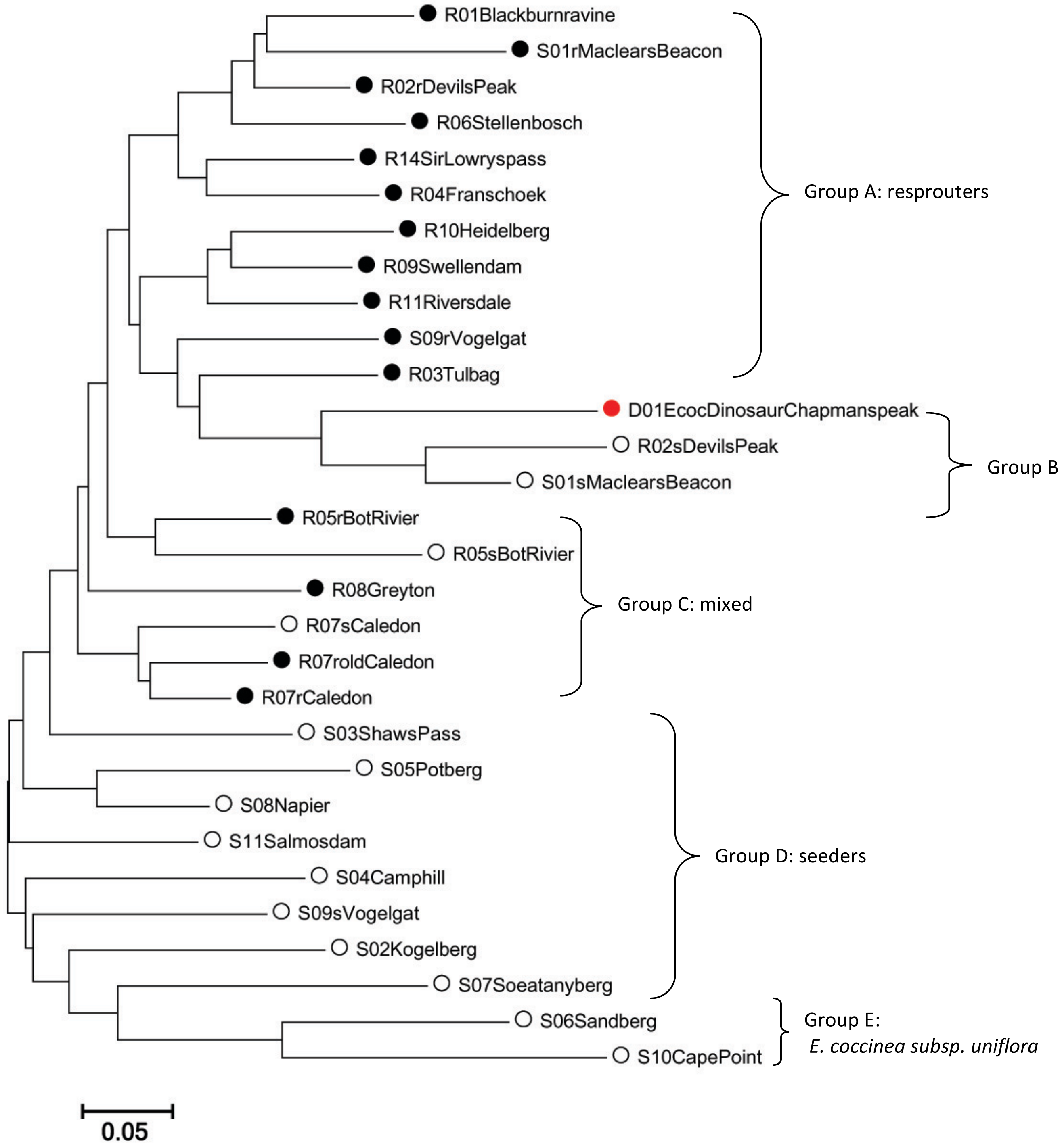

Figure 3.1: Neighbour Joining tree based on genetic distance (Nei et. al 1983) for seeder (open circles) and resprouter (black circles) individuals of *Erica coccinea* in the Cape Floristic Region. Note that populations S06 and S10 belong to the subspecies *uniflora.* Population D01 is neither a seeder nor a resprouter but a form that grows in rocky fire refugia.

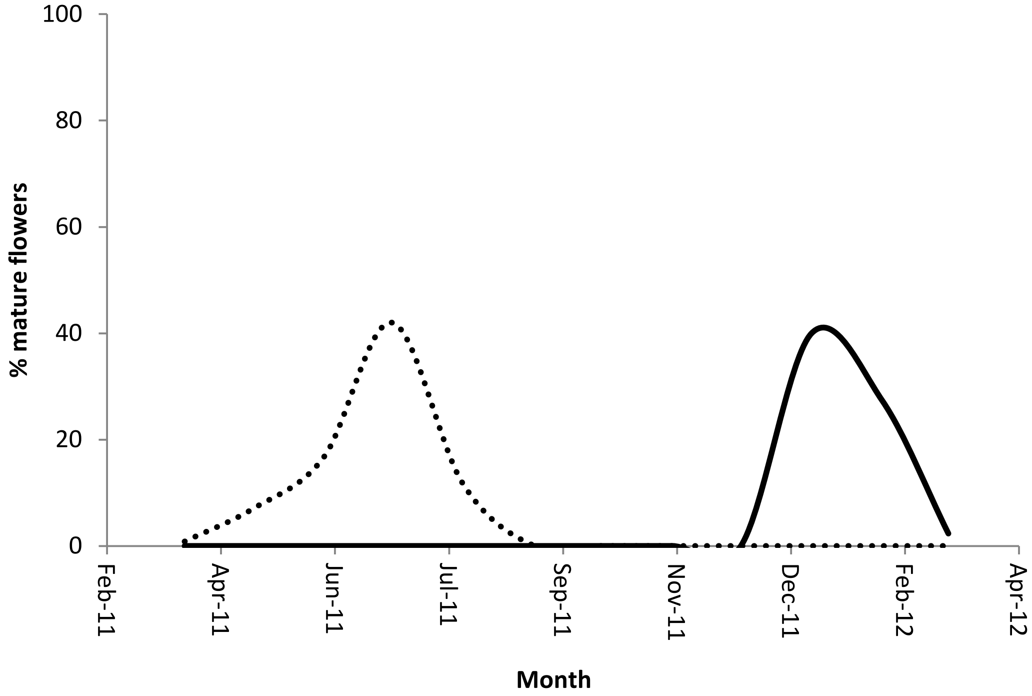

Figure 3.2: Percentage mature flowers present for seeder (dashed line) and resprouter (solid line) populations at Vogelgat Nature Reserve, Hermanus.

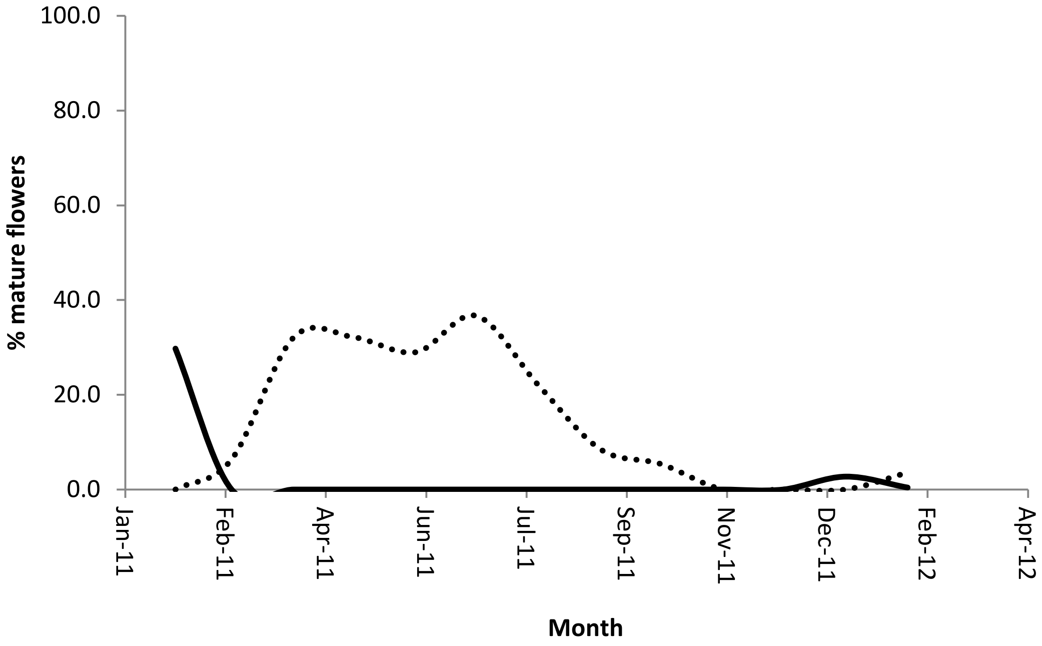

Figure 3.3: Percentage mature flowers present for seeder (dashed line) and resprouter (solid line) populations at Devils Peak on Table Mountain National Park

Table 3.1: A comparison of floral morphology for seeder and resprouter populations in mixed sites at TMNP and Vogelgat Nature Reserve (Hermanus).

site	form	Calyx (mm)	Corolla	Anther length	Anther exsertion
R02 - Devils Peak	resprouter	8.1 (±1.3)	19.5 (±2.9)	26.5 (±3.7)	7.0 (±1.5)
	seeder	7.8 (±0.7)	15.5 (±1.3)	22.8 (±2.3)	7.3 (±1.4)
S01 - Maclears Beacon	resprouter	7.7 (±0.8)	18.2 (±2.0)	25.9 (±2.2)	7.7 (±1.3)
	seeder	6.9 (±0.9)	15.1 (±1.9)	22.2 (±2.4)	7.1 (±1.3)
S09 – Vogelgat NR	resprouter	6.6 (±0.6)	15.2 (±1.3)	21.3 (±2.0)	6.1 (±1.2)
	seeder	9.2 (±0.8)	19.2 (±1.6)	26.6 (±2.0)	7.4 (±1.1)
average	**resprouter**	**7.5 (**±1.0)	**17.6 (**±2.5)	**24.5 (**±3.3)	**6.9 (**±1.5)
range		5.4-9.9	13.0-24.9	18.2-35.2	3.8-12.1
average	**seeder**	**8.0 (**±1.2)	**16.7 (**±2.5)	**23.9 (2.9)**	**7.3 (1.3)**
range		5.1 -10.7	12.2-22.7	17.2-30.4	4.3-11.7

Discussion

The aim of this chapter was to investigate mixed populations of seeder and resprouter forms of *E. coccinea* and, by interpreting their genetic similarities and phenological behaviour, determine whether the two forms are reproductively isolated and also whether this divergence of the two forms would be a case of incipient speciation or rather than a polymorphism within a very variable species.

Using neutral markers, the results of the Neighbour Joining tree show that the *E. coccinea subsp. uniflora* populations cluster separately to the rest of the populations. This introduces some confidence in the use of microsatellite markers as a tool for predicting relatedness of populations within the species. The clustering of some seeder populations within the resprouter group may be caused by a few instances of cross-pollination between the forms, resulting in some shared alleles. Segarra-Moragues and Ojeda (2010) included one mixed population, R05 – Botrivier, in their analysis, and found that the seeder and resprouter individuals in this population are closely related. This suggested that that the derived form, whether it be seeder or resprouter, may be evolving *in situ*, multiple times in mixed sites. Adding four more mixed populations to the data set did not clarify this issue. Population R07 – Caledon has the same pattern as Segarra-Moragues and Ojeda (2010) found for population R05, with seeder and resprouter individuals appearing genetically similar (group C in Fig. 3.1). Visits to the mixed sites from group C revealed that these are both relatively disturbed areas – R05 is situated on a farm that harvests fynbos for the cut flower industry, and may have been ploughed in the past (subsequently to the field season for this study, the seeder population at R05 was completely ploughed up and may have been lost). Population R07 is also a disturbed site – near a recently built reservoir, surrounded by pine plantations. This site may also have been protected from fire for longer than is natural in the fynbos system, resulting in the persistence in the population of many individuals that looked like hybrids between the seeder and resprouter form, which would have been naturally eliminated by fire as they would not function optimally as either seeders or resprouters. The last of the mixed populations, S09 (Vogelgat NR), shows complete separation of the seeder

(S09s) and resprouter (S09r) populations on the Neighbour Joining tree, suggesting separate origins of the two forms and no interbreeding between the forms.

The seeder and resprouter morphotypes of *E. coccinea* are separated genetically and mixed populations are only paired on the Neighbour Joining tree in populations where observations have shown that disturbance and hybridization may occur (R05 and R07). The presence of possible hybrids in population R07 (pers. obs during fieldwork) indicates no genetic barrier to crossing between seeder and resprouter individuals. Mixed populations at S01, S09 and R02 are situated in more pristine environments, where natural fire cycles are more likely to occur, and it is at these three sites that the adjacent seeder and resprouter populations are shown not to be closely related. This suggests that these mixed populations arose due to a migration event of one of these life history forms into the range of another. Where seeders and resprouters in one population appear closely related on the Neighbour Joining tree, one of two situations could be occurring: either the phenology of the two forms is overlapping, allowing hybridization to occur between the forms, or these populations represent sites where the derived form is still evolving from the ancestral form. The former case is more likely, especially in the population at Caledon (R07), where the site appears highly disturbed by both human development and the invasion of alien tree species. A fire going through this mixed population may knock out hybrids which would be intermediate forms between seeder and resprouter and therefore less fit and unable to survive fire as well as either the seeder or resprouting form.

The flowering phenology of two of the mixed populations (R02 and S09) was found to be well separated in time (see Fig. 3.2 and 3.3). Peak flowering in seeder individuals is in late winter and resprouters peak in midsummer. This results in temporal isolation of the two life-history forms, not geographical isolation. In addition, at mixed sites, floral morphology differs between seeder and resprouter individuals. This could be an added mechanism separating the two forms and keeping them separate to prevent hybridization and the introduction of less fit intermediate forms in the population. Both seeder and resprouter morphs are visited and pollinated by the orange-breasted sunbird and a slightly different floral morphology would place pollen on a different part of the bird's head or culmen.

Hansen et al. (1991) found that both seeder and resprouter species of *Bossiaea*, an understory shrub growing in similar Mediterranean type climate in Australia, grew predominantly in spring and summer. They also found that the resprouter species flowered in summer, whilst the seeder flowered in winter. In *E. coccinea*, a change in life history in response to fire and different rainfall regimes (Ojeda et al. 2009) may have led to adjustments to the phenology of growth and seasonal resource allocation, delaying flowering. Over time, this would have separated the flowering times of the two forms when they evolved in allopatry, with the decreased fitness and survival of hybrids maintaining the separation of the two forms. West et al. 2012 found, in an experiment inducing summer drought in a patch of mountain sandstone fynbos, that *Erica* species, which are shallow rooted, were the most severely impacted by the drought. This resulted in seedling mortality and, more importantly for this study, reduced or late flowering. In particular, the flowering of *Erica ericoides*, a seeding species, was delayed by an entire month following the first season of drought treatment. This could explain the change in flowering time for *E. coccinea* if we make the assumption that the seeder life-history is ancestral – a shift from winter/spring flowering to later in spring/summer.

In conclusion, seeder and resprouter forms of *E. coccinea* are separated both genetically and reproductively. Populations of seeder and resprouter forms rarely interbreed, and hybrids, while possible, are not found in areas with a natural fire regime. This suggests that the differentiation between seeder and resprouter life history forms may be a case of incipient speciation and not merely a polymorphism in a highly variable species. I propose the hypothesis that seeder and resprouter life-histories evolved in *E. coccinea* in allopatry, but these were associated with a change in flowering phenology, which then maintained the separation of the two forms when their ranges coincided once again, allowing them to exist in sympatry in the present day.

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Synthesis

I have shown that *Erica coccinea* is a widespread species in the Cape Floristic Region with a large of amount of intraspecific variation in both genetic and floral traits. The primary aim of this thesis was to investigate the patterns of variation in populations of *E. coccinea*, secondarily, to identify the evolutionary forces that have shaped this variation.

The investigation into floral morphology and reproductive traits in chapter one found differences in seeder and resprouter forms of *E. coccinea*. Seeder populations were found to invest more effort into providing rewards for pollinators (for example producing a significantly greater nectar volume), and had a greater fecundity, (percentage flowers setting seed). Flower colour was found to be more variable within resprouter populations than seeders. Whereas seeder populations were almost uniformly yellow, with some anomalous red individuals found in one population (S01s), half of the resprouter populations sampled for colour showed variability within populations. This suggested that either (a) colour was more strongly selected for in seeder populations, but not resprouters, or (b) flower colour was associated with, and co-varies with some other physiological trait, relating to resprouting. It was also found that the two life-history forms differ in flowering phenology. Resprouter populations were found to flower from mid to late summer, whilst seeders flowered in late winter to early spring. The resprouter populations that flowered in midsummer, the hottest, driest time of the year in the CFR, were mostly found to have dark red and deep pink flowers, while those that flowered in the cooler late summer period were more likely to have flower colours varying from red to orange and yellow. Preliminary field observations of co-occurring fynbos species during this study showed that there was a general trend of red-flowering plants of other species at all sites from December to February. This observation should be quantified because it suggests a link between heat stress and flower colour, not only in *Erica*, but possibly in other genera as well. Pigments such as anthocyanins affect flower colour (Streisfeld and Rausher 2009), and also play a role in protecting plants from heat-stress (Steyn et al. 2002), and this relationship deserves further investigation.

Both seeder and resprouter populations of *E. coccinea* were visited by the orange-breasted sunbird, but the more low-lying, coastal dwelling *E. coccinea subsp. uniflora* was observed to be visited by the lesser double-collared sunbird (see Fig. 4.2). In a neighbour joining tree of 29 *E. coccinea* populations (see chapter three) it was shown that the two populations of *E. coccinea subsp. uniflora* sampled were genetically distinct from the rest of the *E. coccinea* populations sampled, but were most similar to the seeder populations. This could suggest that a change in range (from mountain slopes to low-lying coastal areas) in *E. coccinea subsp. uniflora* was accompanied by a change in pollinator (from Orangebreasted sunbird to Lesser Doublecollared sunbird). In order to fully explore the intraspecific variation in both life-history forms and floral variability of *E. coccinea*, it would be interesting to expand the molecular sampling of the subspecies *uniflora* to determine the degree of genetic isolation. However, this isolation may not be complete, as the culmen lengths of these two sunbird species is similar (Geerts and Pauw 2010), therefore a change in pollinator may not have influenced a change in floral morphology of the subspecies *uniflora*. In addition, further genetic sampling of the 'dinosaur' form (Fig. 4.1) should be performed to determine their relationship with the other forms of *E. coccinea*. I predict that it represents a third life history, distinct from seeder and resprouter forms. This fire-avoiding life-history has been observed in other fynbos *Erica* species, such as *Erica nevillei* (Oliver and Oliver 2000).

Figure 4.1: The 'dinosaur' form of Erica coccinea found on Chapman's Peak, Table Mountain National Park. (a) Showing the rocky habitat that protects the plants from fire. (b) The base of the 'dinosaur' plant, showing multiple thickened stems, but no buds at the base.

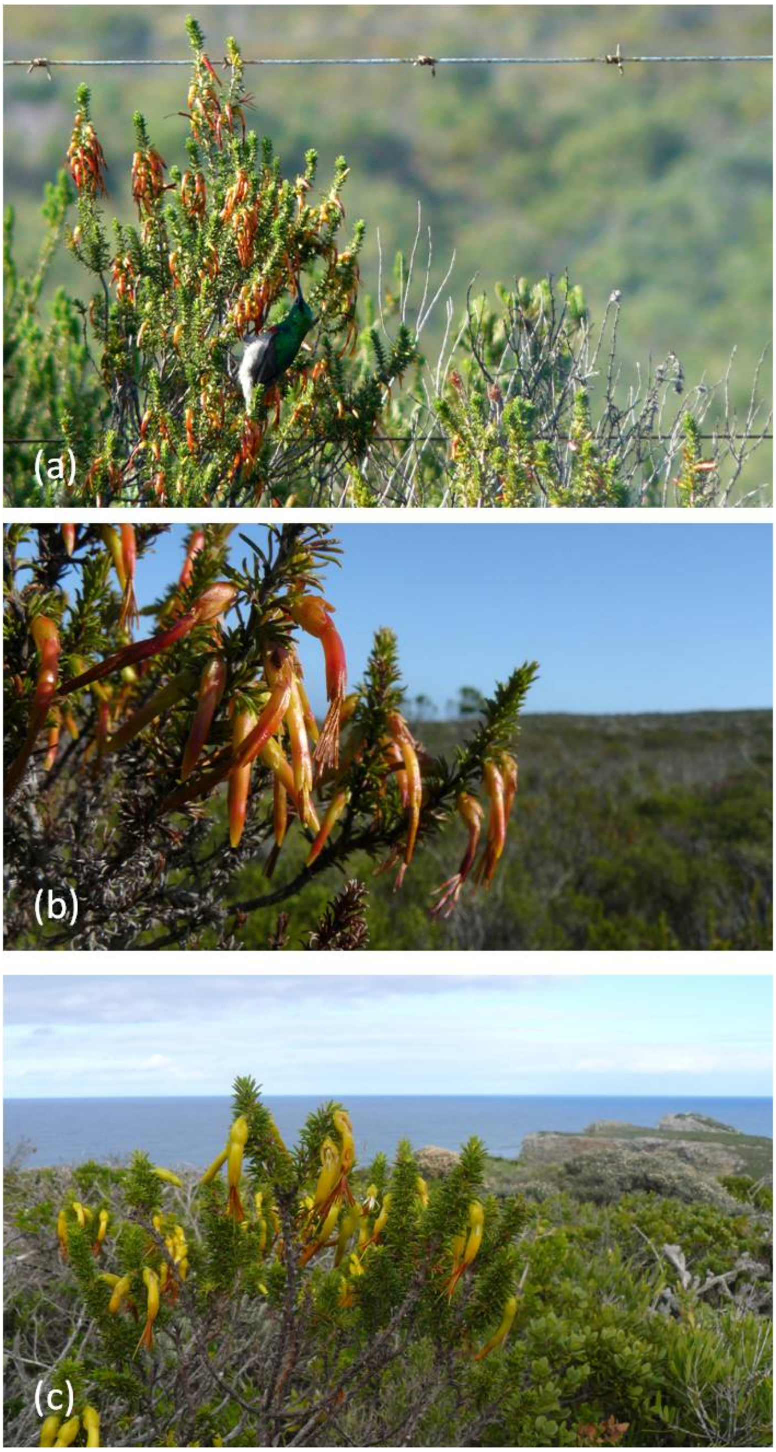

Figure 4.2: *Erica coccinea subsp. uniflora* populations. (a) Being visited by the lesser Double-collared sunbird at a roadside population near Hermanus. (b) The orange flowered form at Sandberg, Cape Agulhas Nature Reserve. (c) The yellow flowered form at Cape Point, Table Mountain National Park.

Using molecular methods, it was found that inbreeding, identified by an excess of homozygous individuals in a population, is very low but does occur to a greater extent in seeder than resprouter populations. However, excess homozygosity was not highly significantly different from the expected level according to the Hardy-Weinberg equilibrium, indicating random mating in the majority of populations sampled. The greater number of populations with excess homozygosity in seeders than resprouters indicates that selection, or assortative mating, could be stronger in seeder populations. This result is justified by the finding of the P_{ST}-F_{ST} analysis in chapter two, where a greater anther exsertion is found to be a trait strongly selected for in seeder populations. Further work is needed to determine the advantage of a longer anther length, my working assumption is that longer anthers bear more pollen grains, resulting in a greater distribution of that individual's pollen, but this needs to be tested. The function of greatly exserted anthers is also of interest for other Cape *Erica* species, such as *E. plukenetii*, which also has exserted anthers and a large amount of floral variability between populations.

The biological species concept (Mayr 1942) states that species are 'groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups'. In the case of *E. coccinea*, populations of both life-history forms appear to have the potential to interbreed, evidence is in the existence of hybrids at some sites. In chapter three I show that where populations of the two life history forms are mixed, the populations are isolated in time. This could lead to reproductive isolation of seeders and resprouters and a possible case of incipient speciation. Alterations in flowering period may allow populations to better exploit different groups of pollinators (Levin 2006) but in this case the two forms of *Erica coccinea* share a pollinator, the Orangebreasted sunbird (see chapter one). Therefore an alternate explanation for this difference in flowering phenology needs to be found. A possible cause is differences in vegetative phenology between the life-history forms, and this deserves further investigation. For example if allocation of photosynthate to lignotubers in the resprouter form alters vegetative phenology, this too may influence flowering phenology.

In large genera that consist of many very closely related species, such as *Erica*, conventional DNA phylogenetic methods may not fully determine the relationships between species (Pirie et al. 2011), and it may be useful to use microsatellite data to tease out finer relationships between spp. For example, using microsatellites, I was able to identify that the *E. coccinea subsp. uniflora* populations were different from the other *E. coccinea* seeder populations.

In summary, key issues for further work are: (1) the cause of seasonal colour variation *in E. coccinea*, and the possible link with the presence/absence of anthocyanins or other protective pigments in response to heat stress. (2) A possible third life-history form of fire-avoiding populations of *E. coccinea* (the 'dinosaur' form) and other *Erica* species such as *E. nevillei*. (3) The function of exserted anthers in *E. coccinea* and other *Erica* species such as *E. plukenetii*, and a possible link with male fitness. (4) The influence of vegetative phenology of seeder and resprouter forms on flowering phenology.

In conclusion, it is hoped that by understanding the processes influencing the variation within a single species, this work can contribute to a greater understanding of the causes of the enormous diversity found in the rest of the *Erica* genus in the CFR. I hope that, by extension, this will add to the growing body of knowledge on the factors influencing species radiations in the fynbos biome.

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