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TECHNICAL NOTE

Isolation and characterization of microsatellite loci in the Cape fynbos heath *Erica coccinea* (Ericaceae)

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Abstract Enriched genomic libraries were used to isolate and characterize dinucleotide microsatellite loci in *Erica coccinea*, a South African Cape fynbos heath species with distinct resprouter and seeder populations. Microsatellites were required to investigate the effect of the contrasting demographic pattern driven by these two post-fire responses in the population genetic structure of seeder and resprouter forms within this species. Eight microsatellite loci were characterised and amplified a total of 106 alleles in 2 samples each of 30 individuals from 1 resprouter and 1 seeder population. Mean allele numbers were 7.88 and 11.0 for the resprouter and seeder population, respectively. Both populations showed similar average observed and expected heterozygosity levels, H_O(resprouter) = 0.683, H_O(seeder) = 0.696; H_E(resprouter) = 0.726, H_E(seeder) = 0.756, and average positive inbreeding coefficients F_{IS}(resprouter) = 0.058, F_{IS}(seeder) = 0.080. This set of microsatellite loci will be used to conduct a population genetic survey of seeder and resprouter populations throughout the range of the species. Cross-species transferability was also assayed in four other South African and four European species of the genus *Erica*, supporting their potential use for population genetic analyses.

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The Cape floristic region is a major hotspot for plant biodiversity. Most diversity is found in fynbos, a Mediterranean or semi-Mediterranean heathland-like vegetation in which recurrent summer wildfires constitute its main selective regime (Goldblatt and Manning 2002). With *ca* 700 species and a high level of endemism, the genus *Erica* L. is the epitome of plant biodiversity in fynbos (Goldblatt and Manning 2002). Species in this genus can be classified into two basic life forms according to their response to fire: resprouter and seeder. Adult plants in resprouter species survive and resprout after fire from dormant axillary buds on the surface of a below-ground lignotuber. By contrast, adult plants in seeder species lack a lignotuber and dormant buds (Verdaguer and Ojeda 2005) and thus, die after fire. Therefore, seeder populations rely solely on seed germination (which is fire-stimulated) from a soil seed-bank for their persistence. Although both seeder and resprouter life forms confer resilience to fire at the population level, their effects on the population dynamics are altogether different (non-overlapping vs. overlapping generation dynamics, respectively; Ojeda et al. 2005).

The seeder life form in *Erica* seems to have emerged from the loss of the resprouting capacity in resprouter lineages (Verdaguer and Ojeda 2005). Such an evolutionary shift is associated with the extraordinary diversification of seeder lineages (Ojeda et al. 2005). There actually exist a few *Erica* species that include distinct seeder and resprouter individuals, nearly always in disjunct populations. One of these species is *Erica coccinea* L., an abundant and widely distributed Cape fynbos species. It is presumably a diploid species with $2n = 24$ chromosomes, like all other

Erica species studied (see Nelson and Oliver 2005, and references therein).

We have conducted a microsatellite characterisation in *E. coccinea* in order to investigate the effects of these two contrasting dynamics in the genetic structure of seeder and resprouter populations. Since seeder populations present a non-overlapping generation dynamics, they are more prone to demographic and genetic bottlenecks, which would emphasize the effects of genetic drift, leading to differentiation or to (local) extinction. The overlapping generation dynamics of resprouter populations, by contrast, would buffer them from bottlenecks, making them more resistant to extinction, but also lowering the between-population differentiation process (Vitalis et al. 2004).

Total DNA was extracted from silica-gel dried young leaves of an individual pool using the DNeasy plant minikit (Qiagen, Barcelona, Spain) following the manufacturer's instructions. Microsatellite isolation followed the protocol of Zane et al. (2002) with some modifications. Total DNA was digested with an excess of *Mse*I (New England biolabs). Digested DNA was purified with the PCR purification kit (Qiagen) that allows for the removal of small fragments and thus, further steps avoid size-selection of fragments. Purified digested products were ligated to *Mse*I adaptors (Zane et al. 2002) in 20 µl volume. The reaction was incubated overnight at 4°C. The ligation mixture was diluted 1:10 and used as template for subsequent PCR amplification. The 50 µl PCR cocktail included: 1× *Taq* buffer (Biotools, Madrid, Spain), 2 mM $MgCl_2$, 0.4 mM of each DNTP, 125 pmol of *Mse*I-N adaptor-specific primer (Zane et al. 2002), 2.5 U of *Taq* polymerase (Biotools) and 5 µl of the 1:10 ligation dilution as template. The PCR program consisted of one cycle of 2 min at 72°C to allow the *Taq* DNA polymerase fill the nicks present in the DNA template, followed by 4 min at 94°C for DNA melting, and then 30 cycles each of 94°C, 30 s; 53°C, 1 min and 72°C 1 min; a final extension step of 7 min at 72°C was added and then samples were kept at 4°C. PCRs were carried out in a PE2720 thermal-cycler (Applied Biosystems, Madrid, Spain). PCRs were systematically repeated in order to obtain several hundred ng of amplified products. PCR amplifications were combined and precipitated with two volumes of Ethanol absolute and 0.5 volumes of Ammonium Acetate 7.5 M, washed with ethanol 70% and resuspended in 50 µl of distilled water.

Separate enrichments were carried out using streptavidin-coated M-280 magnetic beads (Dynal) attached to $(CT)_{14}$ and $(GT)_{14,}$ 5′-biotinylated oligonucleotides. The beads were washed three times with 1× B&W buffer to remove the excess of unbound oligonucleotides and then resuspended in 100 µl of a solution composed of 3× saline sodium citrate (SSC, 20× SSC: 6 M NaCl, 0.6 M Na-citrate, pH 7), 0.1% SDS and 2% PEG-6000 and kept at room temperature in continuous rotation. About 15 µl of the PCR products were denatured 5 min 94°C and immediately cooled on ice for 5 min, afterwards were hybridised to the oligonucleotides at room temperature for 30 min and then washed four times each at 40°C with three decreasing salt-concentration solutions [2× SSC, 1× SSC, 0.5× SSC, respectively, plus 0.1% SDS, and 1.6 µM of *Mse*I-N primer] to remove unbound fragments and unspecific hybridisations. Fragments containing microsatellites were released at 94°C, 5 min in 100 µl 0.2× SSC and desalted with Qiaex II (Qiagen). About 2 µl of enrichment were used for 50 µl PCR with the same cocktail and PCR program as above, but a final elongation step of 12 min at 72°C was added in order to facilitate the generation of 3′-A overhangs required for subsequent cloning. Fragments were purified with the PCR purification kit (Qiagen) and cloned into pGEM-T easy vector system (Promega). About 10 µl of ligation were used to transform 100 µl of XL1-blue *E. coli* competent cells (Stratagene). A total of 288 recombinant colonies were obtained and screened by PCR (Lunt et al. 1999). DNA sequencing was carried out using BigDye Terminator Kit v. 3.1 (Applied Biosystems), followed by electrophoresis on an Applied Biosystems 3730 DNA sequencer.

One hundred and thirty-nine clones were sequenced out of which 113 were unique sequences and of these 72% (81 clones) contained microsatellites. After discarding clones with too short flanking sequences, primers were designed for 54 clones using PRIMER3 (Rozen and Skaletsky 2000). Eleven primer-pairs produced clear amplicons of the expected size in 2% agarose gels and were subsequently selected for analysis on automated sequencers. Forward primers were labelled with fluorescent dyes for automated electrophoresis. PCRs were performed in a 20 µl mix containing 1× *Taq* buffer (Biotools), 2 mM $MgCl_2$, 0.4 mM of each DNTP, 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 (Table 1) 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. 1.71 software (SoftGenetics). After an initial screening of individuals one locus (FJ543388) was monomorphic and two (FJ543386 and FJ43387) showed a multibanding pattern suggesting loci duplications and were subsequently excluded.

Genotypic data were obtained for 30 individuals each of one resprouter population (South Africa: Swellendam, 33°59′56″S-20°26′47″E) and one seeder population (South Africa: Agulhas, 34°48′11″S-19°58′34″E) of *E. coccinea*

Table 1 Locus name, primer sequence, repeat motif, annealing temperature, product size of the sequenced clone and allele range of 8 microsatellite loci characterised in *Erica coccinea*

Locus	Primer sequence (5′-3′)	Repeat motif	T(°C)	Size (bp)	Allele size range	Resprouter ($N = 30$)				Seeder ($N = 30$)				GB
						N_A	H_O	H_E	F_{IS}	N_A	H_O	H_E	F_{IS}	
Ecoc108	F: <NED> CGTGCCCTAAACGAAGATAGA R: GATTACCCAGAAGAGATGGATG	$(CT)_6T(CT)_4$	56	165	157–175	6	0.833	0.816	-0.022^{ns}	9	0.800	0.803	$+0.004^{ns}$	FJ543378
Ecoc115	F: <NED> GTTGAGCTAGTGGAGGGAAGTG R: CTTTCTTCAAATCACACCTAAGCA	$(CA)_8$	56	168	164–182	5	0.500	0.743	$+0.327^{ns}$	10	0.800	0.817	$+0.021^{ns}$	FJ543379
Ecoc117	F: <6-FAM> GGGTCAGTGATAATTTTGAACCT R: TCACTTTGATGGCAATGGACT	$(GA)_5G(GA)_5$	56	157	155–169	3	0.300	0.318	$+0.058^{ns}$	7	0.567	0.648	$+0.125^{ns}$	FJ543380
Ecoc122	F: <PET> TCCTTGAACTAGGTTTCATCTCTAA R: GCCATCGGCTGTTATTACTTATC	$(GA)_{11}$	56	172	166–180	7	0.833	0.785	-0.062^{ns}	5	0.400	0.548	$+0.270^{ns}$	FJ543381
Ecoc132	F: <PET> GATGTTAGCAATCCAGGTCCA R: ATCCAGATGCAAAGAACAAAGG	$(CT)_7$	56	185	179–219	9	0.733	0.714	-0.027^{ns}	12	0.833	0.904	$+0.078^{**}$	FJ543382
Ecoc142	F: <PET> TTTGAAGGGTTTCTGCTTCTGG R: AGAGGATGGGAAGTGAGGTGAA	$(GA)_{16}$	56	239	223–267	14	0.767	0.894	$+0.142^{ns}$	15	0.867	0.877	$+0.012^{ns}$	FJ543383
Ecoc431	F: <VIC> AGTGTATGTGCTACGCCTCTGG R: GAAGGGTTCCCACTCGTCTTC	$(CT)_{14}$	56	183	159–175	4	0.500	0.656	$+0.238^{ns}$	5	0.433	0.505	$+0.142^{ns}$	FJ543384
Ecoc446	F: <6-FAM> GCGCATTGTGAATTACGCTTT R: AGCGTCTGACACCGCTACAC	$(GA)_{10}TA(GA)_2(GT)_{11}$	56	235	205–309	15	1.000	0.885	-0.131^{ns}	25	0.867	0.951	$+0.089^{ns}$	FJ543385
Mean						7.88	0.683 ± 0.230	0.726 ± 0.184	+0.058*	11.0	0.696 ± 0.197	0.756 ± 0.169	+0.080*	

Number of alleles, observed (H_O) and expected (H_E) heterozygosities and F_{IS} values are reported for one resprouter and one seeder populations. 6-FAM, NED, PET and VIC are fluorescent dyes from Applied Biosystems. * $P < 0.05$, ** $P < 0.01$; ns, not significant. GB, GenBank accession numbers

Table 2 Cross transferability of eight microsatellite loci of *E. coccinea* to eight other *Erica* species

Geographical area	Species/locus	Ecoc108	Ecoc115	Ecoc117	Ecoc122	Ecoc132	Ecoc142	Ecoc431	Ecoc446
South Africa	*E. coarctata*	159, 161, 165	166, 168	155, 163	166, 170, 172, 174, 190	189, 191	231, 237, 243, 247	170, 181, 197, 209	213, 229, 239, 255, 259
	E. imbricata	169	158, 170	157, 159	176, 178	191, 199, 211, 213	223, 253, 273	143, 145, 170, 173	211, 213
	E. intermedia	159	168	155	168, 172	203, 209	235, 249	170	227
	E. placentiflora	155, 161	168, 170	163, 165	172, 174, 176	193, 203, 205, 209	231, 235, 257, 259	163, 169, 170, 173	207, 215, 219, 280
Europe	*E. arborea*	155, 157, 163, 165, 167	168	157	–	159, 179, 189	219, 229, 249	159, 167, 173, 179	211, 223, 225, 237, 239, 241, 245, 257, 265
	E. australis	159, 161, 163, 165, 171	–	153, 161, 171, 173, 175, 181	–	173, 179	223, 225, 229, 231, 233, 245	163, 167, 173	191, 211, 217
	E. scoparia	165, 169	–	167, 169, 177, 179	–	173, 175	213, 219, 223, 225, 233, 237, 243	167, 171, 173, 183, 187	157, 159, 189, 201, 209, 219
	E. umbellata	159, 161, 163, 165, 167	–	161	–	185	223, 225, 231, 235, 237, 241, 245, 251	167, 171, 173, 177	207

Allele sizes are indicated in number of bases;—no amplification

for eight microsatellite loci (Table 1). Genetic diversity indices and deviations from Hardy–Weinberg equilibrium and linkage disequilibrium between pairs of microsatellite loci, using 1,000 permutations, were calculated using GENEPOP v. 4.0 software (Rousset 2008). The significance level was adjusted with Bonferroni correction to account for multiple comparisons. The 8 assayed microsatellite loci detected a total of 106 different SSR alleles in the 60 individuals analysed from the two populations. The number of alleles ranged from a minimum of 3 alleles for locus Ecoc117 in the resprouter population to a maximum of 25 alleles for locus Ecoc446 in the seeder population. The mean number of alleles per locus was of 7.88 and 11.0 in the resprouter and seeder population, respectively (Table 1). Out of the 106 different SSR alleles, 19 were exclusive to the resprouter population and 43 to the seeder one and 44 were shared by both populations. Observed heterozygosities ranged from 0.400 (locus Ecoc122 in the seeder population) to 1.000 (locus Ecoc446 in the resprouter population, Table 1). While both resprouter ($F_{IS} = +0.058$) and seeder ($F_{IS} = +0.080$) populations showed mean F_{IS} values indicating slight heterozygote deficiencies, per locus estimates revealed significant ($P < 0.01$) deviation from HW equilibrium towards heterozygote deficiency for just one locus (Ecoc132) in the seeder population ($F_{IS} = +0.078$), which could be attributed to the presence of null alleles, or to population genetic substructuring. None of the 56 pairwise comparisons between loci and populations, showed significant linkage disequilibrium ($P < 0.05$). The seeder population had a higher number of microsatellite alleles than the resprouter population (11.0 vs. 7.88) but similar levels of genetic diversity as measured by observed and expected heterozygosities (0.696 vs. 0.683 and 0.756 vs. 0.726, see Table 1).

Additionally, we tested the cross-species transferability of this set of microsatellite loci in samples of four other South African species of *Erica* (*E. coarctata* J.C. Wendl., $n = 4$; *E. imbricata* L., $n = 2$; *E. intermedia* Klotzsch *ex* Benth., $n = 1$; and, *E. placentiflora* Salisb., $n = 2$) and four European species (*E. arborea* L., $n = 5$; *E. australis* L., $n = 5$; *E. scoparia* L., $n = 5$ and, *E. umbellata* Loefl. *ex* L., $n = 5$) collected from wild populations (Table 2). The different loci amplified alleles in the expected size range of *E. coccinea*. All eight microsatellite loci were transferable to the four South African *Erica* species. In European species six loci were transferable, one locus (Ecoc122) failed to amplify in all taxa and another one (Ecoc115) was transferable only to *E. arborea* (Table 2).

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References

Goldblatt P, Manning JC (2002) Plant diversity of the Cape region of southern Africa. Ann Mo Bot Gard 89:281–302. doi:10.2307/3298566

Lunt DH, Hutchinson F, Carvalho GR (1999) An efficient method for PCR-based isolation of microsatellite arrays (PIMA). Mol Ecol 8:891–894. doi:10.1046/j.1365-294X.1999.00636.x

Nelson EC, Oliver EGH (2005) Chromosome numbers in *Erica*—an updated checklist. Heathers 2:57–58

Ojeda F, Brun FG, Vergara JJ (2005) Fire, rain, and the selection of seeder and resprouter life-histories in fire-recruiting, woody plants. New Phytol 168:155–165. doi:10.1111/j.1469-8137.2005.01486.x

Rousset F (2008) GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. Mol Ecol Res 8:103–106. doi:10.1111/j.1471-8286.2007.01931.x

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, pp 365–386

Verdaguer D, Ojeda F (2005) Evolutionary transition from resprouter to seeder life history in two *Erica* (Ericaceae) species: insights from seedling axillary buds. Ann Bot (Lond) 95:593–599. doi:10.1093/aob/mci061

Vitalis R, Glémin S, Olivieri I (2004) When genes go to sleep: the population genetic consequences of seed dormancy and monocarpic perenniality. Am Nat 163:295–311. doi:10.1086/381041

Zane L, Bargelloni L, Patarnello T (2002) Strategies for microsatellite isolation: a review. Mol Ecol 11:1–16. doi:10.1046/j.0962-1083.2001.01418.x