Genetic structure and post-pollination selection in biennal plants
Korbecka, G.

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Chapter 3

Characterization of nine microsatellite loci in *Cynoglossum officinale* (Boraginaceae)

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ABSTRACT

Cynoglossum officinale is a biennial plant pollinated by bumblebees. We developed microsatelllite loci in order to study the population genetic structure and effects of inbreeding in this species. In this paper, we describe nine polymorphic microsatellites for *C. officinale*. Between two and four alleles per locus were observed in a sample of 20 individuals from one population. Multiplexing allowed the seven most useful loci to be genotyped using three PCR reactions.

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Inbreeding depression is of great interest to both evolutionary biologists and conservation ecologists. Studies of inbreeding depression in plants often only concentrate on the selfing rate, while crosses between related individuals can also intensify inbreeding. Such crosses take place when the pollinators visit neighboring plants and there is a genetic structure in the population (the neighboring plants are related). We intend to study fine scale genetic structure of a population of *Cynoglossum officinale*, a diploid, biennial plant, pollinated by bumblebees. Although, seeds of this species have a clear adaptation to dispersal via animals, large mammals are absent in the studied dune area. Therefore, we suspect that dispersal by gravity plays an important role and we expect to find genetic structure in the population. In this paper, we describe microsatellite loci developed to test this prediction.

Genomic DNA of one individual from the dune area of Meijendel (near The Hague, the Netherlands) was enriched separately for dinucleotide (GA and CA) and trinucleotide (AAG and ATG) repeats, following the procedure described in Hale *et al*. (2001). Enriched DNA was ligated into BAP (dephosphorylated) *Bam*HI digested "ready-to-go" pUC18 vector (Pharmacia) and cloned using JM 109 competent cells (Promega). The plasmid DNA from bacterial colonies were sequenced using ABI Prism Big Dye Terminator (version 1.0) cycle sequencing ready reaction kits (Applied Biosystems) following manufacturer's recommendations and detected using a capillary sequencer ABI 310 (Applied Biosystems). Twenty-two primer pairs were designed using PRIMER 3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). PCRs were carried out in a volume 10 µL, containing 5 ng DNA, PCR buffer (16 mM $(NH)_4SO_4$, 67 mM Tris-HCl, 0.01% Tween-20), 2.0 mM $MgCl_2$, 0.2 mM each of the dNTPs, 2 pmol of each primer and 0.5 U *Taq* DNA polymerase (Bioline). All PCRs were performed using a PTC-100 programmable thermocycler (MJ Research). After denaturation for 12 min at 95°C, PCR's were performed for 20 cycles under the following conditions: 15s at 95 °C, 15s at 55 °C, 15s at 72 °C, then for 10 cycles: 15s at 89 °C, 15 s at 50 °C and 15 s at 72 °C and a final extension of 30 min at 72 °C. Out of twenty-two primers, 17 primers gave PCR product of the expected size for five individuals, when tested on 2% Metaphor-agarose gels (FMC BioProducts). We judged polymorphism based on these gels and ordered fluorescently labelled forward primers for 4 loci. The other 13 loci were further tested for polymorphism using fluorescent dCTPs (FdCTPs), giving us five more polymorphic loci. We used FdCTPs labelled with dyes R110 and R6G (Perkin Elmer) at a concentration of 0.5 µM in the PCRs. The labelled PCR fragments were detected on an ABI 310 using an internal size standard ROX-500 and analysed using GENESCAN® software (Applied Biosystems). In this paper, we characterise all nine loci, although in routine analysis we only use the seven most polymorphic ones (indicated with a fluorescent label in Table 1)

We tested the microsatellites on leaf material from 20 *C. officinale* plants collected from one population in the dune area of Meijendel. The largest distance between two collected plants was 41 meters. The leaves were dried in silica gel and stored at –20°C. Approximately 1 cm^2 of each leaf was homogenized in 1.3mL of 2x CTAB extraction buffer (1% PVP 40, 0.5% v/v βmercapto-ethanol). The

CTAB extraction protocol was adapted for smaller quantities after Doyle and Doyle (1987). After extraction DNA was resuspended in 100 µL of TE buffer (10mM Tris, 1 mM EDTA).

In routine analysis, PCRs and detection were carried out as described above, but we used forward primers fluorescently labeled with 6-FAM, JOE or TAMRA. Six primer pairs were combined in two multiplex sets (Table 1) using the same PCR program and annealing temperature as described above. PCR for locus *C2-42* was carried out separately with 30 sec annealing to increase the intensity of the signal. This locus with 4 alleles: 110, 112, 116 and 124 bp, shows a clear decrease of peak height with increasing size.

We performed a test for Hardy-Weinberg equilibrium for all loci using a program called ARLEQUIN (Schneider *et al.* 2000) and found a significant deviation for two loci: *C2-42* and *C3-79* (Table 1), which showed lower observed than expected heterozygosity. Most other loci also showed a lower than expected heterozygosity, albeit non-significant. We did not find any homozygotes for null alleles among 20 individuals. Moreover, in further 80 individuals tested for 7 loci with fluorescently labeled primers (Table 1) we did not find such homozygotes either. Therefore, we conclude that selfing or mating with related individuals is responsible for lowering heterozygosity. We performed tests for linkage disequilibrium using above-mentioned program ARLEQUIN. No linkage disequilibrium was observed in the population.

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Table 1 Characteristics of microsatellite loci in *Cynoglossum officinale*.

Name †	Primer sequence (5'→3')	Label	Repeat	Size of the cloned allele (bp)	Allele size range in a screened population (bp)	No. of alleles	H_e	H_o
C2-19[1]	`F: CTCCGGTGGTGGTGCTTC` `R: TCCAGGTTAAGAACCCAAGC`	JOE	$(GA)_{26}$	138	115-131	3	0.56	0.40
C2-42	`F: TCAAACCACGTGAGAAAATATAGAA` `R: TGATTCCAATCAATCTTCGTTTT`	6-FAM	$(GA)_{12}$	116	110-124	4	0.50	0.40*
C2-43[2]	`F: ACCCCCCCTTCTCCACTT` `R: GGGAATAGCAGACCATGTCC`	TAMRA	$(CT)_7(CA)_{10}$	133	128-136	3	0.52	0.35
C2-45	`F: TGATGATATTTTCAACCCTATCTCAT` `R: AGCTCAGCAGATATCCAACGA`		$(CT)_6CG(CT)_9$	128	128-140	2	0.35	0.37
C2-62[1]	`F: CCTGTCATACCCGAAACTCG` `R: AGTAGGGAATTGGGCTTTGG`	6-FAM	$(CT)_{12}$	169	167-171	3	0.43	0.40
C2-72[2]	`F: GAATTGAGGAAGGAGATGACG` `R: GATCATGTGGGGGAATCATAA`	JOE	$(GA)_{13}C(GA)_2$	102	91-101	4	0.60	0.45
C3-30	`F: GCTTGCAACAAGCAGACAAC` `R: TTGTGTCTCACTTTGCTGTCG`		$(CAT)_9$	150	137-147	2	0.23	0.20
C3-41[1]	`F: GTGCAAAGGTGCAGGGTAAG` `R: TGTCTATAGGCTCTGCTCTTCTCC`	TAMRA	$(GAT)_7$	134	133-136	2	0.27	0.15
C3-79[2]	`F: GCACCAGGGTTCGTGTTAGT` `R: GCTTTTTGGCTGAGCTGTTT`	JOE	$(GAA)_7$… $(GAA)_{16}$… $(GAT)_6$	220	188-214	4	0.61	0.30**

†C2 means that the sequenced clone originates from enrichment for dinucleotides, and C3 - for trinucleotides, the following number is a number of a sequenced clone and the number in superscript (1 or 2) is the same for those primers, which were taken together for the same multiplex PCR; H_e, expected heterozygosity; H_o, observed heterozygosity; *, ** statistically significant deviation from Hardy-Weinberg equilibrium ($P<0.05$ or $P<0.01$, respectively).

GenBank accession numbers between AY434455 and AY434463 for the described microsatellite loci, in order presented in this table.

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