Genetic structure and post-pollination selection in biennal plants

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Chapter 2

Characterization of six microsatellite loci in *Echium vulgare* (Boraginaceae)

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ABSTRACT

Echium vulgare is a tetraploid plant with a very low selfing rate in the field. We suspect that cryptic self-incompatibility plays a role in this species. In order to show lower success of self pollen/selfed embryos, after pollination with a mixture of self and outcross pollen, a paternity analysis has to be done. For the purpose of such analysis we developed microsatellites in *E. vulgare*. In this article, we report on six microsatellite loci which are easy to score, polymorphic, with number of alleles per locus ranging from two to eight and, therefore, suitable for paternity analysis.

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Echium vulgare is a tetraploid (2n=4x=32), hermaphroditic species pollinated by bumblebees. It produces one to 20 flowering stems, each with hundreds of flowers. *E. vulgare* is self-compatible and bumblebees can cause self pollination by moving from one flower to another within the same plant. Using RAPD's, Rademaker *et al.* (1999) found that selfing rates of *E. vulgare* in the field vary between 0 and 30 % which is only half or less of the theoretical prediction based on bumblebee behavior and pollen dynamics. On average, single pollen donor pollinations with self pollen resulted in the production of as many seeds as outcrossing (Melser *et al.* 1997). Why then are so few selfed seeds produced in the field? A logical explanation would be that self pollen or selfed embryos lose competition when a flower is pollinated by both self and outcross pollen. This is called *cryptic self-incompatibility* because the lower success of self pollen/selfed embryos can be detected only when mixture of self and outcross pollen is applied. A paternity analysis of seeds, following mixed pollination is essential for testing the hypothesis about the presence of cryptic self-incompatibility.

Microsatellites are the best markers for paternity analysis since they are codominant, highly variable and often allow for distinguishing among individuals from the same population. The aim of this study was to develop microsatellite primers suitable for such an analysis in *Echium vulgare*. To our knowledge, this article will be the first report on microsatellite loci in the family Boraginaceae.

Enrichment was done separately for dinucleotide (GA and CA), trinucleotide (AAG and ATG) and tetranucleotide (GATC and GATA) repeats following the procedure described in Hale *et al*. (2001). Enriched DNA was ligated into BAP (dephosphorylated) BamHI digested "ready-to-go" PUC18 vector (Pharmacia) and cloned using JM 109 competent cells (Promega). Positive colonies with inserts were sequenced using ABI Prism Big Dye Terminator (version 1.0) cycle sequencing ready reaction kits (Applied Biosystems) following manufacturers recommended conditions and detected using an automated sequencer ABI 377 (Applied Biosystems). Thirty-six primer pairs were designed using PRIMER 3 program available on the web (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Nineteen primers gave PCR product of expected size for five tested individuals, subsequently these primers were tested for polymorphism using dCTPs labeled with rhodamine dye: R110 (Applied Biosystems). PCRs were carried out in a volume 10 µL, containing 1 ng DNA, 10 mM Tris HCl, 50 mM KCl, 2.0 mM $MgCl_2$, 0.1 mM each of the dNTPs, 0.2 µM [R110] dCTPs, 4 pmol of each primer, 1 µg bovine serum albumin (BSA) and 0.4 U *Taq* DNA polymerase (Amersham Pharmacia). All PCRs were carried out in a T_3 thermocycler (Biometra). PCR fragments were detected on the ABI 377 along with an internal size standard ROX-500 and analysed using GENESCAN® software (Applied Biosystems). Out of these microsatellites: four microsatellites were not polymorphic, three most probably have null alleles, and for the remaining 12 were ordered fluorescently labeled forward primers. In this article we describe microsatellites with the most clear and easy to interpret patterns.

We tested the developed microsatellites on leaf material from 30 flowering plants of *E. vulgare* collected in the dune area of Meijendel (near The Hague, the Netherlands). The biggest distance between two collected plants was approximately 675 meters. DNA was extracted from 0.1 g of fresh or frozen (–80°C) plant material with a Nucleon Phytopure extraction kit (Amersham). Extraction was followed by PEG precipitation to remove polysaccharides. To 500 µL of DNA in sterile water 250 µL of PEG solution (40% PEG-8000, 30 mM $MgCl_2$) was added. After incubation for 30 min at room temperature and centrifuging at 13 000 rpm the pellet was washed twice with 70% cold ethanol and resuspended in 100 µL of $_{0.1}$TE buffer (10mM Tris, 0.1 mM EDTA).

The PCRs were carried out in a volume 10 µL, containing 0.1-1 ng DNA, 1 µL 10x concentrated PCR buffer supplied with the *Taq* polymerase (containing Tris Cl, KCl, $(NH_4)_2SO_4$, and 15 mM $MgCl_2$), 0.1 mM each of the dNTPs, 4 pmol of each primer, 0.5 mM $MgCl_2$ additional, 1 µg BSA and 0.2 U *Taq* DNA polymerase (Qiagen). The forward primers of microsatellites were fluorescently labeled with one of fluorescent dyes: Fam, Joe, Tamra. Final $MgCl_2$ concentration was 2.0 mM. We used a low template concentration because the reaction with 10 ng of DNA not always gives a PCR product. Moreover, adding BSA improves PCR. This suggests that there are inhibitory compounds present in DNA extract. The seedlings seem to contain more of these compounds than the flowering plants. For that reason, we used 1 ng of DNA from flowering plants and 0.1 ng of DNA from seedlings in the PCR. After denaturation for 2 min at 95°C, PCR's were performed for 20 cycles under the following

conditions: 15s at 95 °C, 15s at annealing temperature (see table 1), 15s at 72 °C, then for 10 cycles under the same conditions but with the annealing temperature lowered by 4 °C and finally there was an extension step of 30 min at 72 °C. PCR program for locus E2-11 differed only with a doubled time (30s) for annealing. Labelled PCR products were detected on the ABI 377 using an internal size standard ROX 500 and analysed using GENESCAN® software (Applied Biosystems).

All the presented microsatellites were highly polymorphic within the population of Meijendel and therefore suitable for paternity analysis. We designed a pollination experiment in which 22 out of the 30 collected plants were combined in pairs of plants that do not share any allele at least in one out of 6 tested microsatellites. Such an experimental design will allow for very rapid paternity analysis of offspring from pollinations with a mixed self and outcross pollen. Allele frequencies in this tetraploid species are not easy measured, as we are not able to determine if a peak was the product of one or more copies of an allele. As a result, no information on expected heterozygosity is available.

Table 1 Characteristics of microsatellite loci in *Echium vulgare*.

Name*	Primer sequence (5'→3')	Repeat	T_a (°C)	Allele size range (bp)	No. of alleles	H_o	GenBank Accession number
E2-11	F: CCAACCATTTTCCATCCAAC R: AGTCTTGCCATTCGATGACC	CTCTCAT	58	242-249	2	0.97	AY185304
E3-40	F: CCATTGTTTCACCCGCTAAT R: CCACAGAAGGGGAGTTTGAG	TCA	58	177 - 195	7	1.00	AY185305
E3-46	F: GGGGCTAACTGAATGCAGAA R: CCTCCCATATCCGTTGTCAT	CA	60	220 - 234	6	0.97	AY185306
E3-56	F: GCTAAGAAAGCGTTGGCAAG R: GATCAAGACGCAAGCGAGTA	CAT	61	260 - 285	5	0.96	AY185309
E3-84	F: CCCCCAGTGCAATGAGATAG R: GGAATGGAGCCTAGTGCTTG	GAT	62	293– 305	5	0.83	AY185307
E3-91	F: AAGAGCAATCCAGCCTTTGA R: GATGTTGTCTGCCCAAATCA	GAT	61	169 - 196	8	0.97	AY185308

*E2, sequenced clone originates from enrichment for dinucelotides; and E3, for tri nucleotides. The following number is a number of a sequenced clone.
T_a, locus specific annealing temperature; H_o, observed heterozygosity.
Forward primers were labeled. For locus *E3-40* – Fam lable was used, for *E3-84* - Tamra label and for the rest of loci – Joe label.

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