

## PERMANENT GENETIC RESOURCES

Isolation and characterization of polymorphic microsatellite loci in common evening primrose (*Oenothera biennis*)

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## Abstract

We developed nine polymorphic microsatellite loci for evening primrose (*Oenothera biennis*). These loci have two to 18 alleles per locus and observed heterozygosities ranging from 0 to 0.879 in a sample of 34 individuals. In a pattern consistent with the functionally asexual reproductive system of this species, 17/36 pairs of loci revealed significant linkage disequilibrium and three loci showed significant deviations from Hardy–Weinberg equilibrium. The loci will be informative in identifying genotypes in multigenerational field studies to assess changes in genotype frequencies.

**Keywords:** heterozygosity, microsatellite, *Oenothera biennis*, Onagraceae, permanent translocation heterozygote

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Common evening primrose, *Oenothera biennis* L. (Onagraceae), and many of its congeneric relatives, have a unique genetic system known as permanent translocation heterozygosity (PTH) (Holsinger & Ellstrand 1984). The genome of *O. biennis* is composed of two haploid sets of seven chromosomes which have reciprocal translocations on every chromosome. During meiosis, the chromosomes form a single complete ring that restricts recombination to the chromosome tips. Subsequent balanced lethality of gametes and self-pollination results in the production of clonally related offspring and the maintenance of heterozygosity (Cleland 1972).

*Oenothera biennis* grows in recently disturbed habitats where it forms discrete patches often comprised of a single genotype. Genetic differentiation between patches is high (Levin 1975), and clonal families show quantitative genetic variation for many morphological, phenological and insect-resistance traits (Johnson & Agrawal 2005). Genetic markers would make it possible to test predicted evolutionary consequences of the PTH genetic system (Holsinger & Ellstrand 1984) and are needed to differentiate genotypes in ongoing multigenerational field studies of natural selection imposed by insect herbivores. Here, we describe nine polymorphic microsatellite loci that were isolated from *O. biennis* genomic DNA.

Plant material was collected in Tompkins County, New York and stored at  $-80^{\circ}\text{C}$ . Whole genomic DNA was extracted with the DNeasy Plant Mini Kit (QIAGEN) from 80 to 100 mg of leaf material with the following modification to the DNeasy Plant Mini Kit Handbook (January 2004, QIAGEN): genomic DNA was eluted with 175  $\mu\text{L}$  of buffer AE. Extracted genomic DNA was digested with *Bsa*I and *Hinc*II and ligated to SNX linkers using T4 DNA ligase (Hamilton *et al.* 1999). Ligated fragments were enriched by hybridization with biotinylated dimeric, trimeric and tetrameric nucleotide repeats (dimers:  $\text{GT}_8$  and  $\text{TC}_{9.5}$ ; trimers:  $\text{TTA}_{12}$ ,  $\text{GAT}_7$ ,  $\text{GTT}_{6.33}$ ,  $\text{GTA}_{8.33}$ ,  $\text{TTC}_7$ ,  $\text{GCT}_{4.33}$ ,  $\text{GTG}_{4.67}$ ,  $\text{GTC}_{4.67}$ ,  $\text{TCC}_5$ ; tetramers:  $\text{TTTA}_{8.5}$ ,  $\text{GAAT}_{5.5}$ ,  $\text{GATA}_7$ ,  $\text{GATT}_{5.5}$ ,  $\text{GTAT}_{6.25}$ ,  $\text{GTTA}_{6.25}$ ,  $\text{GTTT}_{5.25}$ ,  $\text{TTAC}_{6.75}$ ,  $\text{TTTC}_6$ ,  $\text{GATG}_{4.25}$ ,  $\text{GGTT}_4$ ,  $\text{GCTT}_{3.75}$ ,  $\text{GTAG}_{4.5}$ ,  $\text{GTCA}_{4.25}$ ,  $\text{GTCT}_{4.25}$ ,  $\text{GTTT}_4$ ,  $\text{TCAC}_{4.25}$ ,  $\text{TTCC}_{4.25}$ ) in three reactions with incubations for 10 min at  $97^{\circ}\text{C}$  followed by 30 min at  $56^{\circ}\text{C}$ . Single-stranded DNA was recovered using streptavidin-coated magnetic beads (New England Biolabs). Enriched DNA fragments were made double-stranded with the polymerase chain reaction (PCR) using SNX primers. PCR products were cut with the restriction enzyme *Nhe*I (New England Biolabs) and ligated to pUC19 plasmids (previously digested with *Xba*I and dephosphorylated). These ligations were used to transform *Escherichia coli* DH5- $\alpha$  cells and were plated on Luria-Bertani (LB) agar/ampicillin plates. Colonies were transferred to nylon membranes that were

**Table 1** Primer sequences, amplification conditions and diversity indices of nine microsatellite loci for *Oenothera biennis*

Locus	Repeat	Primer sequence (5'–3')	$T_a$	Allele size range (bp)	$N_a$	$N$	$H_O$	$H_E$	GenBank Accession no.
Oenbi2tri2	(GTT) <sub>8</sub>	F: PET-TTCAGCTCCGATTGCCAAATGATGG R: ATTCGCTGATGGAGCTGGGTTGTTCTT	50	407–423	5	34	0.706	0.651	EF988083
Oenbi2tri3	(CTT) <sub>29</sub>	F: 6-FAM-CCCAATCACACCCCTCCCTCAAT R: GCGACGGTGGATAGTGGATCTTCTCTCT	50	402–485	18	34	<b>0.794</b>	<b>0.894*</b>	EF988084
Oenbi2tri6	(CTT) <sub>9</sub>	F: 6-FAM-CAGGCCCTTGATCCGTTACACTTACCA R: GCGCTGCTGGAAGAGACGATGCT	TD	176–198	7	33	<b>0.879</b>	<b>0.710*</b>	EF988085
Oenbi2tri7	(CTT) <sub>9</sub>	F: VIC-CTTTTGGCCCTTCTTTGACCCCATCC R: GACAGCAGCAGCAGCAAAAGCAAAGA	TD	361–367	3	33	0.242	0.311	EF988086
Oenbi2tet1	(AGAT) <sub>9</sub>	F: PET-GCCGCCCATCAAAATATCGAGTA R: CATATACTATTTTCTGTTAATTTTAGAATTTGGTCCTAA	50	341–364	12	30	<b>0.867</b>	<b>0.882</b>	EF988087
Oenbi39di2	(GT) <sub>10</sub>	F: PET-TAACTTCGTGGAACCCAGACAGACTCTC R: GAGCGACAGGCATACACTTTCTCCTT	50	198–205	4	33	0.697	0.733	EF988088
Oenbi39tri1	(CTT) <sub>6</sub>	F: VIC-AACTCATACCATCTCCTTACCTGCATCATTTA R: GCGAGAAGAGGTAGAGAAGCAGAAGGAGTTAG	50	229–235	2	26	0.615	0.434	EF988089
Oenbi39tri4	(CTT) <sub>16</sub>	F: NED-TTTCCTCTCCTTATCTCCTCAATTCTCTCA R: CTTGCGTTATCTTATGCGTAGCCGTTAGTTTAT	50	226–244	9	27	<b>0.778</b>	<b>0.797</b>	EF988090
Oenbi39tet5	(ATAA) <sub>11</sub>	F: 6-FAM-AAATTAATTTGCTCGACACCTTCTT R: GAGCTATATTTTCTTCGATCATCTTGTCTA	50	277–323	14	31	<b>0.000</b>	<b>0.884*</b>	EF988091

F, forward primer; R, reverse primer;  $T_a$ , annealing temperature (°C);  $N_a$ , number of alleles;  $N$ , number of individuals scored;  $H_O$ , observed heterozygosity;  $H_E$ , expected heterozygosity; TD, touchdown PCR. Numbers in bold indicate significant deviations from Hardy–Weinberg equilibrium; \*indicate significant deviations following Bonferroni correction; †the first 4 nucleotides of 5' end are not insert DNA.

then hybridized to <sup>33</sup>P radiolabelled probes of the same repeat motifs used in the enrichment. Plasmid DNA was isolated for sequencing by transferring individual colonies using pipette tips into 0.5× AE buffer and then incubating for 10 min at 97 °C. We PCR amplified inserts using the M13 primers that flank the *Xba*I cloning site in pUC19 and sequenced on an ABI PRISM 3100 DNA sequencer. We trimmed the pUC19 vector and SNX linker from sequences and aligned all clone sequences containing microsatellites using the program SEQMAN PRO (DNASar, Inc.). We designed unmodified primers for unique sequences with the program PRIMERSELECT (DNASar, Inc.).

PCRs were performed in a total volume of 10 µL. For the majority of loci, the reaction included 1.0 µL genomic DNA diluted 1:5 in water, 2 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 0.2 µM of each forward and reverse primer and 0.1 µL (0.5 U) Platinum *Taq* polymerase (Invitrogen) in 1× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl). Loci Oenbi39tri1 and Oenbi39tri4 amplified best using 2 µL DNA diluted 1:5 in water and locus Oenbi39tri1 amplified best using 3 mM MgCl<sub>2</sub>. PCRs for all primers pairs were performed using an initial denaturation at 95 °C for 2 min followed by 35 cycles of 95 °C for 50 s, 50 °C for 1 min and 72 °C for 1 min. Loci Oenbi2tri6 and Oenbi2tri7 followed a touchdown protocol of 35 cycles of 95 °C for 50 s, 59–50 °C for 1 min (the annealing temperature decreased by 1 °C each cycle for the first 10 cycles) and 72 °C for 1 min. The forward primer of each pair that amplified cleanly (visualized using agarose gel electrophoresis) was labelled with a fluorescent tag (6-FAM, PET, NED, or VIC). Fluorescent PCR products

were diluted 1:15 in water and mixed with formamide and Genescan LIZ-500 size standard (Applied Biosystems). Allele sizes were determined with an ABI PRISM 3100 DNA analyser and GENEMAPPER version 3.5 software (Applied Biosystems).

We chose 34 individuals representative of unique clonal genotypes to quantify variation at these loci. We converted our data from a GENEMAPPER (Applied Biosystems) file format using GMCONVERT (Faircloth 2006) and GENEPOP ON THE WEB version 3.4 (Raymond & Rousset 1995). Tests for linkage disequilibrium and deviations from Hardy–Weinberg equilibrium (HWE) were performed using ARLEQUIN version 3.1 (Schneider *et al.* 2000). The breakdown of recombination in PTH taxa is predicted to cause extensive linkage among genes and deviations from HWE (Holsinger & Ellstrand 1984). Consistent with this, we found significant deviation from random association in 25/36 pairs of loci ( $P < 0.05$ , Fisher's method) and 17/36 pairs following Bonferroni correction. Five loci showed significant deviations from HWE using a Markov chain method (Guo & Thompson 1992) and three loci deviated from HWE following Bonferroni correction. The number of loci that deviated from HWE at the  $P < 0.05$  level was significantly more than expected by random chance (binomial expansion test:  $P = 0.001$ ). Observed heterozygosities ranged from 0.00 to 0.879 (Table 1). The nine loci differentiated all 34 individuals as unique genotypes and will be informative in identifying genotypes in multi-generational field studies to assess changes in genotype frequencies.

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## References

- Cleland RE (1972) *Oenothera: Cytogenetics and Evolution: Experimental Botany*, 1st edn. Academic Press, New York.
- Faircloth BC (2006) GMCONVERT: file conversion for GENEMAPPER output files. *Molecular Ecology Notes*, **6**, 968–970.
- Guo SW, Thompson EA (1992) Performing the exact test of Hardy–Weinberg proportion for multiple alleles. *Biometrics*, **48**, 361–372.
- Hamilton MB, Pincus EL, Di Fiore A, Fleischer RC (1999) Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *BioTechniques*, **27**, 500–507.
- Holsinger KE, Ellstrand NC (1984) The evolution and ecology of permanent translocation heterozygotes. *American Naturalist*, **124**, 48–71.
- Johnson MTJ, Agrawal AA (2005) Plant genotype and environment interact to shape a diverse arthropod community on evening primrose (*Oenothera biennis*). *Ecology*, **86**, 874–885.
- Levin DA (1975) Genic heterozygosity and protein polymorphism among local populations of *Oenothera biennis*. *Genetics*, **79**, 477–491.
- Raymond M, Rousset F (1995) GENEPOP (version 3.4): population-genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.
- Schneider S, Roessli D, Excoffier L (2000) *ARLEQUIN, Version 2.000: A Software for Population Genetics Data Analysis*. Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Switzerland.