

Characterization of fifteen microsatellite loci in the cone and seed-feeding insect, *Pissodes validirostris*, and cross-priming among congeneric species

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Abstract Fifteen microsatellite markers were developed for *Pissodes validirostris*. The number of alleles per locus ranged from 2 to 13 and observed heterozygosity from 0.033 to 0.900. These markers will be useful to confirm the infra-specific phylogeographic patterns of this seed-feeding weevil, potential vector of the pitch canker disease of pine trees, thereby threatening their genetic resources. Most cross-species amplification was successful among the seven congeneric species tested.

Keywords Microsatellites · Genetic diversity · Pine-cone weevil · *Pissodes validirostris*

Seed-feeding insects are responsible for important damages in managed forests or seed orchards (Turgeon et al. 1994), impacting the regeneration dynamics and genetic resources of coniferous trees. The pine-cone weevil, *Pissodes validirostris* (Coleoptera, Curculionidae), has a wide Palearctic distribution from Europe to north eastern China. It completes its larval development only in cones and seeds of Eurasian and Mediterranean *Pinus* species. In addition of limiting the regeneration of pine trees, this seed-feeding insect can transmit the highly virulent fungus, *Fusarium circinatum*, an important pathogen of *Pinus* seedlings recently introduced in Europe (Romon et al. 2007).

Microsatellite markers would be useful for investigating phylogeographic structure and gene flow between populations of *P. validirostris*, in order to adapt pest management strategies.

Genomic DNA was extracted from 20 heads and thorax of adults collected in various localities and hosts using the DNeasy Tissue Kit (Qiagen). Two different procedures were used to develop polymorphic microsatellites. A set of five microsatellites loci were firstly designed following a biotin-enrichment protocol described in Kerdelhué et al. (2003). Out of the 127 positive clones, 40 candidate microsatellites were selected for subsequent PCR from which five were polymorphic. In a second step, we used next generation sequencing combining DNA enrichment procedures with the use of multiplexed microsatellite probes and the update Titanium of the 454 GS-FLX technology (Malausà et al. 2011). Among the initial set of 980 potential microsatellite loci designed for this species, 10 polymorphic loci were finally selected on the 24 primer pairs tested (performed by Genoscreen, Lille, France). The fifteen loci retained were grouped in five multiplex (Table 1) and amplified on 30 individuals of *P. validirostris* collected on *Pinus sylvestris* near Cayrols (France). The amplifications were performed for each individual using a Veriti® 96-Well Fast and a 9800 Fast Thermal Cyclers (Applied Biosystems®) and the Thermo Scientific DreamTaq DNA Polymerase package. Technical details and GenBank accession numbers are given in Tables 1 and 2.

For the first set of five loci, amplifications were performed in a 10 µL volume of reaction containing <40 ng of DNA, following the procedure described in Kerdelhué et al. (2003). For the 10 loci developed by Genoscreen, amplifications were performed in a volume of 25 µL containing <40 ng of DNA, 1 U of DreamTaq DNA Polymerase (Fermentas), 1.875 µL of 10X DreamTaq buffer

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Table 1 Microsatellite data and polymorphism characterization of the *P. validirostris* population from Cayrols

Locus	Primer sequences (5' → 3')	Motif	Multiplex	Expected size (bp)	N_A	Null allele frequency	H_O	H_E	P
PS03	F: VIC-AGATCGACAGATCTCCAACAAG R: AAATGCCTCTCGGACTGATG	(GAA) ₇	M1	109	4	0.0212	0.600	0.632	0.913
PS04	F: PET-TGTTAATCGCCAACGCTCTGC R: CAGCAATTTGCAATGCTTTCAC	(GAA) ₈	M1	129	4	-0.0739	0.733	0.648	0.813
PS05	F: NED-AAGGAGGCTAAATACAACGGAC R: AGAGGTCAGCATGGGGATATAG	(GAA) ₈	M1	129	6	-0.0538	0.833	0.756	0.736
PS06	F: NED-GCTGCCGATGAAGATAAATAG R: TAACCCGATTACTTGAAAACGC	(GAA) ₉	M2	143	5	0.0761	0.433	0.503	0.634
PS08	F: 6-FAM-GTTAAGATGGCGAGATATATAG R: GACGGAAGTGCAGTCTGATC	(GAA) ₁₆	M2	148	11	0.0271	0.767	0.818	0.258
Pis16	F: 6-FAM-AGCGTTGTTCGAAAATCAT R: TGATCACATGTACGGCTGAAA	(TC) ₁₂	M3	160	12	-0.0087	0.900	0.883	0.866
Pis18	F: VIC-AATCAGACCCAGTTTGGTGC R: TTCTTCCCACCTGTAATCAA	(AC) ₁₄	M3	184	8	0.0505	0.667	0.731	0.014*
Pis19	F: NED-GATTGAGGAGGCCAAAGCTC R: TTTGATTGAGAGGGTAGGCA	(GTA) ₁₄	M3	133	9	0.0519	0.733	0.802	0.584
Pis04	F: 6-FAM-ACTCTATTATTGGTTTGTCTATGACG R: AAAGTATAACAACCTGCCTTTCCG	(GAC) ₇	M4	141	2	0.1529	0.200	0.320	0.040*
Pis21	F: VIC-ACAGTTGACGAACAGAGGAA R: CTAAAGCCGCGACACAAAAT	(AG) ₁₇	M4	113	13	0.0537	0.633	0.691	0.003**
Pis10	F: PET-GGCTTTTATGGGAAGTAACCA R: GCAAGGGAGACCTATGTCCA	(TCT) ₉	M4	125	3	0.1111	0.433	0.566	0.155
Pis12	F: NED-GATAATTTGTCATGGCAGCG R: TTCCGAGTAACGTCTTTCCC	(CA) ₉	M4	192	2	0.1487	0.033	0.095	0.000***
Pis23	F: 6-FAM-ACATTGGGTCATAAGGTGC R: TTCTTTGGTCTGAATGAGAGTGA	(GA) ₂₀	M5	104	13	0.2056	0.500	0.861	0.000***
Pis17	F: VIC-ATACCGCAACTTCCAAAGGA R: AAAGTGGACATGTGCTTCC	(TCT) ₁₃	M5	141	6	0.0973	0.467	0.581	0.024*
Pis07	F: NED-CCGCGTATAAATCAAGGGTT R: GGTATAGATTATAACCTGGGTCGG	(GA) ₈	M5	160	4	0.0261	0.500	0.546	0.094

N_A number of alleles; H_O observed heterozygosity; H_E expected heterozygosity; values in bold indicate significant deviation from Hardy–Weinberg equilibrium (*** $P < 0.01$; ** $0.01 < P < 0.05$; * $0.05 < P < 0.1$). Significant linkage disequilibrium was detected between seven pairs of loci ($P < 0.01$), i.e. PS05–Pis07, PS05–Pis17, Pis16–Pis17, Pis07–Pis18, PS05–Pis21, Pis16–Pis23, and between Pis17 and Pis23

including 20 mM $MgCl_2$, 0.60 μL of 20 mM dNTP, 0.10–0.50 μL of 20 μM primers (Table 2) and distilled water. 25 % of the forward primer of each primer set was 5' end-labelled with a fluorescent dye (either 6-FAM, VIC, PET or NED, Applied Biosystems®). The amplified products were detected on an ABI 3500 Genetic Analyzer automatic sequencer using GeneScan™-600LIZ® Size Standard v2.0 (Applied Biosystems®) to size alleles, and then analyzed using the GENEMAPPER® software v4.1 (Applied Biosystems®).

Deviations from Hardy–Weinberg equilibrium (HWE), expected and observed heterozygosity and linkage disequilibrium were calculated using GENEPOP v 4.2

(Raymond and Rousset 1995). The existence of null alleles was tested using MICROCHECKER (<http://www.microchecker.hull.ac.uk/>).

Genetic diversity indices are provided for each locus in Table 1. The number of alleles per locus ranged from 2 to 13 and the expected heterozygosity from 0.095 to 0.883. Five loci revealed a significant excess of homozygotes that could be linked with signs of null alleles for these loci, or could be due to sampling biases associated with the low dispersal rate of this species. Cross amplification was also tested for seven congeneric species, including three out of the five European species, and several of the serious pests of pine plantations in North America, i.e. *P. strobi* and *P. nemorensis* (also

Table 2 Polymerase chain reaction conditions

Locus	10X buffer (μL)	MgCl ₂ (mM)	Each dNTP (pmol)	Forward primer (pmol)	Labelled Forward primer (pmol)	Reverse primer (pmol)	Dream Taq DNA polymerase (units)	Betaine (μL)	Total volume of reaction (μL)	Cycling conditions ^a
PS03	0.75	1.5	20	3	1	4	1	0.2	10	A
PS04	0.75	1.5	20	3	1	4	1	0.2	10	A
PS05	0.75	1.5	20	3	1	4	1	0.2	10	A
PS06	0.75	1.5	20	3	1	4	1	0.2	10	A
PS08	1	2	20	3	1	4	1	0.2	10	A
Pis04	1.875	1.5	12	4		4	1	0.5	25	B
Pis07	1.875	1.5	12	2		2	1	0.5	25	B
Pis10	1.875	1.5	12	2		2	1	0.5	25	B
Pis12	1.875	1.5	12	2.5		2.5	1	0.5	25	B
Pis16	1.875	1.5	12	10		10	1	0.5	25	B
Pis17	1.875	1.5	12	4		4	1	0.5	25	B
Pis18	1.875	1.5	12	2		2	1	0.5	25	B
Pis19	1.875	1.5	12	2		2	1	0.5	25	B
Pis21	1.875	1.5	12	2.5		2.5	1	0.5	25	B
Pis23	1.875	1.5	12	4		4	1	0.5	25	B

^a Cycling conditions used were either A: denaturing at 94 °C for 5 min, followed by 40 cycles at [94 °C for 45 s, primer annealing temperature (Ta) for 45 s, 72 °C for 1 min] and a final elongation at 72 °C for 5 min; or B: denaturing at 95 °C for 10 min, followed by 40 cycles at [95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min] and a final elongation at 72 °C for 10 min

Table 3 Cross–species amplification results for *Pissodes* spp.: number of successful amplifications/number of alleles in the taxon

Species	Host plant	Country	N	PS03	PS04	PS05	PS06	PS08	Pis04	Pis07	Pis10	Pis12	Pis16	Pis17	Pis18	Pis19	Pis21	Pis23
<i>P. castaneus</i>	<i>Pinus sp.</i>	France	2	2/1	2/2	1/1	2/3	2/1	2/1	2/3	0	2/3	0	0	2/4	0	2/1	0
<i>P. piceae</i>	<i>Picea abies</i>	France	2	1/2	0	2/3	2/1	2/1	2/1	2/1	0	2/1	0	0	1/2	0	2/1	0
<i>P. pini</i>	<i>Pinus uncinata</i>	France	2	0	2/1	1/2	2/1	1/1	2/1	2/1	0	1/1	0	0	1/2	0	0	0
<i>P. nemorensis</i>	<i>Pinus patula</i>	South Africa	1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	0	0	1/1	0	0	0	0	0
<i>P. strobi</i>	<i>Pinus sp.</i>	Canada	1	0	1/2	1/1	1/2	1/2	1/1	0	0	0	0	0	0	0	1/1	0
<i>P. punctatus</i>	<i>Pinus armandii</i>	China	1	0	1/2	0	0	0	1/1	1/1	0	0	1/1	0	0	1/2	0	0
<i>P. yunnanensis</i>	<i>Pinus yunnanensis</i>	China	1	0	1/2	0	1/1	0	1/1	1/1	0	0	0	0	1/2	0	0	0

N number of individuals tested

introduced in South Africa), and in China, i.e. *P. punctatus* and *P. yunnanensis*. Amplifications were successful, except for three loci (Pis10, Pis17 and Pis23), which failed for all species but *P. validirostris* (Table 3). These microsatellite loci will facilitate the study of the genetic structure and the dispersal patterns of this seed-feeding insect.

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