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PRIMER NOTE

Characterization of microsatellite loci in the stick insects *Bacillus rossius rossius, Bacillus rossius redtenbacheri* and *Bacillus whitei* (Insecta: Phasmatodea)

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Abstract

Five microsatellite markers were obtained from a dinucleotide enriched genomic library of the stick insect *Bacillus rossius rossius*. The markers were tested in three species of *Bacillus*. All loci were polymorphic when tested across species. The number of alleles at each locus was low (maximum four alleles), but different allelic patters were observed among the species.

Keywords: *Baccillus*, enrichment, isolation, microsatellites

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The holomediterranean genus *Bacillus*, comprises two sharply differentiated bisexual species, namely, the strictly bisexual *Bacillus grandii* found in a very limited area of Sicily and the commonly found *Bacillus rossius* (Scali *et al*. 2003). *Bacillus rossius* spreads over most of the western Mediterranean basin with two Italian subspecies, *Bacillus rossius rossius* and *Bacillus rossius redtenbacheri*. These two subspecies both build up bisexual and facultative parthenogenetic populations, and shifts from bisexual to unisexual reproduction have been directly witnessed (Scali 1996). In Sicily, the interspecific hybrid *Bacillus whitei* (*B. rossius redtenbacheri*/*Bacillus grandii grandii*) is found. This apomictic hybrid reproduces by a process that allows the invariant transmission of fixed heterozygous loci of the maternal genome to the progeny, (Marescalchi *et al*. 1991) and the hybrid species fully reflect the genetic structure of the two ancestral species (Scali *et al*. 2003).

Until now, no highly polymorphic markers have been characterized for any species in the genus *Bacillus*. The development of microsatellite primers could be very useful in the investigation of population dynamics, which is rather complex due to the different modes and shifts in reproduction. Microsatellite markers could also be a valuable tool in studies evaluating the current extent of hybridization in nature, as well as investigating population dynamics of already established hybrid species, since hybridization is a common phenomenon within the genus *Bacillus*.

A dinucleotide-enriched library was obtained from *B. rossius rossius* using the fast isolation by AFLP of sequences containing repeats (FIASCO) protocol (Zane *et al*. 2002). Genomic DNA was extracted from a single individual using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle & Doyle 1987). The DNA was simultaneously digested with *Mse*I and ligated to *Mse*I amplified fragment length polymorphism (AFLP) adaptors (5′-TACTCAG-GACTCAT-3′/5′-GACGATGAGTCCTGAG-3′). Restricted and ligated fragments were amplified with *Mse*I adapter-specific primers and hybridized with a biotinylated probe (AC_{17}) at room temperature for 15 min. DNA molecules hybridized to biotinylated probes were selectively captured by streptavidin-coated beads (Roche). Nonspecific DNA was removed by a series of washes, and dinucleotide repeat containing DNA was eluted from the beads using TLE 1 × buffer at 95 °C for 5 min, precipitated with sodium acetate and ethanol, re-amplified with *Mse*I adapter-specific primers and cloned using the TOPO TA Cloning Kit (Invitrogen). Positive clones were screened by amplification using universal M13 primers, purified with Wizard polymerase chain reaction (PCR) cleaning (Promega) and sequenced in an ABI 310 genetic analyser (Applied Biosystems).

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Table 1 Microsatellite loci in three species of *Bacillus*. Primer sequences, repeat motif, annealing temperature (T_a) and allele size range, which refers to the observed PCR product. Number of alleles (N_A) determined from 76 individuals and observed (H_O) and expected heterozygosities (H_E)

						H_O/H_E			
Locus	Primer sequence (5′–3′)	Motif	T_a (°C)	Allele size range	N_A	*B. r. redte-nbacheri* (n = 27)	B. r. rossius (n = 29)	*B. whitei* (n = 20)	Genbank Accesion no.
B154	F: GATGATACAGGGCGGTTACG R: TTCCAAAAAGTCACCCGAAG	$(CA)_{10}$	55	229–237	3	0.080/0.147	Monomorphic	Monomorphic	AY820824
B67	F: TTGAGGGGTCTCTGACGTTT R: CGCGATGTCACAAATCCATA	$(TA)_4(CA)_5$	55	212–214	2	0.268/0.509	Monomorphic	Monomorphic	AY820826
B101	F: TGTACTCACAGTCGCGGAAC R: TGGCTCAGCACTACAAGCTG	$(AC)_5(AG)$ $(AC)_2(AG)_6$	58	237–241	4	0.107/0.105	0.148/0.201	1.000/0.513	AY820827
B152	F: TACTCGTGCTGCCTGTTGAC R: GGGTGCGAGACATGCTAACT	$(TG)_2(CG)_2$ $(TG)_5$	58	227–231	3	Monomorphic	0.429/0.343	1.000/0.513	AY820825
B198	F: GCCTACCCGGGCACA R: ACTGCGTGAGTTCCGAGAAG	$(CA)_9(CG)_3$	60	227–231	3	0.655/0.506	Monomorphic	Monomorphic	AY820823

PCR primers were designed using the computer software, PRIMER 3 (Rozen & Skaletsky 2000). The optimal PCR conditions were found for the designed primers by running PCRs over a range of $MgCl_2$ concentrations and annealing temperatures, in all three species of *Bacillus*. Each PCR consisted of a l0-μL mixture containing 4 ng of DNA, 1 mM $MgCl_2$, 10 μM of each primer, 200 μM each dNTP, 10 mM PCR buffer 10× (Invitrogen) and 0.25 U of *Taq* polymerase (Invitrogen). Amplifications were performed in a GeneAmp PCR System 2700 (Applied Biosystems) as follows: initial denaturation at 94 °C for 3 min, 40 cycles at 94 °C annealing temperature for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 7 min. In all, 110 positive clones were screened for the presence of microsatellite loci and 19 clones contained useful simple sequence repeats, but only five of them reliably amplified a single locus (see Table 1).

To characterize each locus, we genotyped 27 amphigonic individuals of *B. rossius rossius* collected in Montiano, Toscana, 29 individuals of amphigonic *B. rossius redtenbacheri* collected in Torino di Sangro Marina, Abruzzo, and 20 individuals of *B. whitei* collected at Canicattini Bagni, Sicily. Genotyping of the individuals was performed in a Beckman CEQ8000 sequencer using 5′-labelled (Proligo) forward primers. Only two loci were polymorphic in *B. rossius rossius* (locus *B101* and *B152*), each having two alleles, the rest being monomorphic. In *B. rossius redtenbacheri*, all loci were polymorphic with two or three alleles per locus, except locus *B152*, which was monomorphic. In *B. whitei*, loci *B101* and *B152* were polymorphic with the presence of fixed heterozygotes, the rest being monomorphic. Observed (H_O) and expected heterozygosities (H_E) were calculated using GENEPOP version 1.2 (Raymond & Rousset 1995). Probability to fit Hardy–Weinberg equilibrium (HWE) was calculated for each polymorphic locus and test for linkage disequilibrium (LD) between the polymorphic loci within each species were calculated using FSTAT vesion 2.9.3.2 (Goudet 2002). No deviation from HWE was observed for the polymorphic loci in *B. rossius rossius* and *B. rossius redtenbacheri*, but a significant deviation from HWE ($P < 0.05$) was found in *B. whitei* due to the fixed heterozygoseties. No significant LD was found. We found always one or two peaks in all our amplifications, and have tested them for the presence of null alleles according to Brookefield (1996). We found two loci with significant presence of null alleles, loci *B154* and *B67* in *B. rossius redtenbacheri*, with 0.06 and 0.16 estimated frequencies, respectively. Locus *B101* allele 241 was found only in *B. whitei*, which could be an allele originating from the parental *B. grandii grandii*. We cannot exclude the presence of null alleles in *B. whitei* because it has not been possible to test if the primers amplify in the parental species *B. grandii grandii*. The five loci investigated showed low allelic variation, when compared to the variation found in other insect species (Vargo 2000), but different allelic patterns of the microsatellite markers among the species considered could make them useful in future studies of hybridization and population dynamics.

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