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Isolation and characterization of microsatellites in the harlequin ladybird, *Harmonia axyridis* (Coleoptera, Coccinellidae), and cross-species amplification within the family Coccinellidae

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Abstract

A total of 18 microsatellite DNA loci were isolated and characterized from the harlequin ladybird, *Harmonia axyridis* (Coleoptera: Coccinellidae). We optimized a multiplex panel consisting of two polymerase chain reactions, allowing the genotyping of all loci. The number of alleles and heterozygosity observed at each locus ranged from 1 to 12 and from 0 to 100%, respectively. After Bonferroni correction for multiple tests, none of the loci deviated significantly from Hardy–Weinberg equilibrium and there was no indication of significant linkage disequilibrium among pairs of loci. Successful cross-species amplification was obtained for only three of the seven tested species of Coccinellidae.

Keywords: Biological invasion, Insect, Invasive Species, Microsatellites, Nuclear marker

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The harlequin ladybird, *Harmonia axyridis*, is a coccinellid native to Asia, which has been used in numerous countries as a biocontrol agent for about a century (Koch 2003). This species has become invasive in northern America since the late 1980s and in Europe about 15 years later (Koch 2003; Coutanceau 2006). It has now spread worldwide (see DAISIE database at http://www.europe-aliens.org/ for European data) and is considered good model for the study of invasive species. It is expected to become one of the most widely distributed ladybirds in the world (Brown *et al*. 2008; Poutsma *et al*. 2008). We have developed here a large set of microsatellite markers to address various questions regarding the historical, demographical and ecological factors involved in the worldwide invasion of the harlequin ladybird.

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Harmonia axyridis genomic DNA was extracted using DNeasy Tissue Kit (QIAGEN) from ethanol-preserved larvae originating from the laboratory rearing of the biocontrol firm Biotop. A total of 2.5 μg of genomic DNA was digested using *RSA1* restriction enzyme (Promega). Fragments of digested DNA of 500–900 bp were isolated from an agarose gel, purified and ligated to *Mlu*I oligo adaptors (*RSA21* and phosphorylated *RSA25*; Edwards *et al*. 1996). Biotinylated oligo probes [$(TC)_{10}$, $(TG)_{10}$, $(ATCT)_6$, $(TGTA)_6$] were hybridized to the ligated DNA and selected using streptavidin magnetic particles (Promega). Polymerase chain reactions (PCR) were performed on the microsatellite-enriched solution using one of the oligo adaptors (*RSA21*) as primer. The PCR products were purified (QIAquick PCR Purification Kit, QIAGEN) and ligated into a plasmid vector (pGEM-T Easy Vector system, Promega), transformed

Table 1 Primer pairs used for the two PCR sets of the multiplex panel. For each of the 18 loci, the table includes the repeat motifs in the sequence used to design primers, sequence and fluorescent dye label used for each primer, number of individuals successfully genotyped, observed number of alleles, allelic size range, expected heterozygosity (Nei 1978), observed heterozygosity and *P* value (before Bonferroni correction) for the test of Hardy–Weinberg equilibrium (HWE). The loci HA-200 and HA-282 are not shown because they were found to be monomorphic in this population (details are available on request)

PCR set	Locus	Repeat motifs	GenBank Accession no.	Primers sequences 5′–3′	Fluorescent dye	*N*	Alleles	Size range (bp)	H_O	H_E	HWE
PCR 1	Ha-244	$(TG)_{10}TC(TG)_2$	FJ263403	F: TGACGGACGCACGAAGAT R: ACAGCTGACCATAGAGGATCG	FAM	26	8	81–96	0.81	0.86	0.43
	Ha-201	$(CA)_8$	FJ263399	F: CTTCGCCATCATCCACTAGG R: GTGCGGTCATTAATTCAGGC	FAM	26	6	306–319	0.73	0.77	0.33
	Ha-555	$(CA)_{10}$	FJ263408	F: GATGCGCCCTCTAGAAAAG R: CCCTATAACGCCAACAATG	VIC	26	6	75–85	0.62	0.72	0.8
	Ha-605	$(GA)_{16}$	FJ263410	F: TCCGACGCACAGATAACAGA R: GTTACGTTGACCCGTCGC	VIC	26	11	134–167	0.65	0.78	0.07
	Ha-281	$(TG)_7$	FJ263406	F: TTCGCACGTTCCATTGTTC R: GCCGTTTGCGGTATGTTC	NED	26	12	134–147	0.5	0.78	0.02
	Ha-627	$(GA)_{11}$	FJ263411	F: CGTAACTTTAACGATCACTCAGC R: GAACATTGTCTTCGCGTGG	NED	26	11	227–254	1	0.88	0.75
	Ha-565	$(GA)_{10}$	FJ263409	F: TCTGAACATTCGACCTACATAGT R: AATGCGTGATGAACGACC	NED	24	3	326–334	0.08	0.08	1
	Ha-234	$(CA)_8$	FJ263402	F: GCTAAAACCAACGTCAGG R: CTCGCGCGATTATTGGAC	PET	24	7	128–138	0.63	0.79	0.17
PCR 2	Ha-267	$(AC)_8$	FJ263405	F: AACCTGTAATTCGATTGTGGAAC R: CCGACCTGACCTTTCGTC	FAM	26	6	177–187	0.65	0.69	0.21
	Ha-005	$(GA)_5$	FJ263394	F: AGGGTGTGTATGTAGAACAGAGG R: AACCGCAATAACTCGATTGG	FAM	22	3	275–279	0.36	0.62	0.01
	Ha-253	$(CA)_7$	FJ263404	F: GATACATCGTCCTTTCAGTCCTC R: CCTGCAAACTCTTCCAGACC	VIC	26	6	182–188	0.65	0.66	0.48
	Ha-105	$(GA)_5$	FJ263396	F: CGCCTAACAAATAGGCATCAC R: AGGGTGGAGAATGGAATAACC	VIC	26	4	240–243	0.54	0.53	1
	Ha-194b	$(GCA)_4$	FJ263397	F: ACCAGATTGCTGCTTGGATT R: ACAAATTGGGCGTGAGAAAC	NED	26	2	80–83	0.35	0.34	1
	Ha-215	$(CA)_7$	FJ263400	F: CGAATCAATAACCCTAGGCG R: AGCGATCTCCTGTTCTACGG	NED	26	5	174–182	0.5	0.66	0.23
	Ha-223	$(TG)_6$	FJ263401	F: TCGTTTAACCGTGATAGGAGAG R: ACGAATTCCGAAAGATGAGG	NED	23	2	229–233	0.09	0.16	0.13
	Ha-094	$(TAGA)_5$	FJ263395	F: TTAGTCGGCGGGTCCATC R: GGGCCGATAAGTCAAACGAG	PET	24	6	350–359	0.5	0.67	0.05

into JM109 competent cells (Promega), and plated onto Luria-Bertani (LB) agar medium with ampicillin. Recombinant plasmids were identified by means of blue-white screening. Positive-transformed cells were grown on LB-agar, transferred onto Hybond-N + membranes (Amersham) and screened using digoxigenin-end-labelled $(TC)_{10}$, $(TG)_{10}$, $(ATCT)_6$, and $(TGTA)_6$ probes. Out of the 742 cell colonies detected as positive, 380 were sequenced with the standard primer *SP6*. Sequencing was performed by Macrogen Inc. using the BigDye Terminator chemistry and an ABI 3700 automatic sequencer. Finally, 284 readable sequences containing microsatellite motifs were obtained (84 contained no microsatellite motif and 16 were not readable). A set of 45 primer pairs was designed from nucleotide sequence regions flanking microsatellites using the online version of Primer3 (http://frodo.wi.mit.edu/primer3/input.htm). When possible, criteria used for designing primers were (i) 18–24 bp length (ii) G/C-3′ end, and (iii) annealing temperature around 60 °C. PCR primers were first tested in monoplex PCR, on individual DNA samples of adults originating from different populations and extracted using the DNeasy Tissue Kit (QIAGEN). Only the primers producing good quality PCR products for all samples were retained. These primers were then labelled with fluorescent dyes (Applied Biosystems) and integrated in a multiplex panel consisting of two multilocus PCR sets including eight and 10 loci, respectively (Table 1). Multiplex PCR were performed using the QIAGEN Multiplex kit and a thermocycler Mastercycler (Eppendorf). 2 µL of genomic DNA (~10 ng) were added

Locus/Species	*Harmonia quadripunctata*	*Harmonia conformis*	*Harmonia yedoensis*
Ha-244	81	81	83, 85, 88
Ha-201	—	307	308, 310, 312, 316
Ha-555	—	85, 89	77, 79, 81
Ha-605	—	122, 141	129, 137
Ha-281	—	135	136, 138, 141
Ha-627	—	—	228, 230, 232, 233, 234, 242
Ha-565	—	—	—
Ha-234	—	—	126, 128, 134, 136, 141
Ha-267	—	—	179, 181, 183
Ha-005	—	—	277, 279
Ha-200	—	—	110
Ha-253	—	—	178, 180, 181
Ha-105	235	—	239, 240
Ha-194b	—	—	80
Ha-215	—	—	174, 176
Ha-223	—	—	233
Ha-282	98	—	98
Ha-094	—	—	356

Table 2 Results of cross-amplification tests, for each locus and each species. The size of each amplified allele is given. Sample size is four diploid individuals per species. Amplification with DNA from all other tested species (i.e. *Adalia bipunctata*, *Coccinella undecimpunctata*, *Coccinella septempunctata* and *Hippodamia variegata*) did not give readable PCR product

to the 8 μL of mix consisting of 1X QIAGEN buffer, water and primers at final concentration of 0.2 μM, except for loci HA282 (0.04 μM), HA105, HA194b, HA223 (0.1 μM) and HA005, HA244, HA565 (0.3 μM). PCR conditions for both multilocus set were as followed: initial denaturation at 94 °C for 15 min; 25 cycles of denaturation (94 °C, 30 s), annealing (57 °C, 60 s) and elongation (72 °C, 2 min); final extension at 60 °C for 30 min. A total of 2 μL of diluted (1:10) PCR products was mixed with 0.25 μL of 500 LIZ Size Standard (Applied Biosystems) and 8.75 μL of formamide (Applied Biosystems). Products were then electrophoresed using an ABI PRISM 3130 sequencer (Applied Biosystems).

We have estimated the level of polymorphism of loci by genotyping 26 adult individuals of *H. axyridis* collected in Kazakhstan (Almaty, 43°14′22.2″N; 76°56′68.4″E). Genotypes were scored using Gene Marker version 1.5 (SoftGenetics). The software GenePop version 3.3 (Raymond & Rousset 1995) was used to estimate expected and observed heterozygosities and to test for genotypic linkage disequilibrium and deviation from Hardy–Weinberg equilibrium (HWE). The program Micro-Checker (Van Oosterhout *et al*. 2004) was used to detect null alleles and estimate their frequencies.

The number of alleles per locus ranged from 1 to 12. Two loci (HA200 and HA282) were found to be monomorphic but preliminary data from other populations shows that HA200 displays several alleles (unpublished). For other loci, expected heterozygosity values ranged from 0.08 to 1 (Table 1). We did not find any significant deviation from HWE after Bonferroni sequential correction for multiple comparisons. However, null alleles were detected by Micro-Checker at loci HA005 and HA281, with frequencies estimated at 0.18 and 0.17, respectively. Finally, there was no indication of significant linkage disequilibrium among pairs of loci.

The multiplex panel was also tested using the same PCR conditions on individual DNA extracts from seven coccinellid species (four individuals per species): *Adalia bipunctata*, *Coccinella undecimpunctata*, *Coccinella septempunctata brucki*, *Harmonia quadripunctata*, *Harmonia conformis*, *Harmonia yedoensis*, *Hippodamia variegata*. We obtained successful PCR amplification at three to 17 loci in the three most closely related species only: *H. yedoensis* and to a lesser extent, *H. conformis* and *H. quadripunctata* (Table 2).

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Development and characterization of nine polymorphic microsatellite markers in the Chilean kelp *Lessonia nigrescens*

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Abstract

A total of nine microsatellite loci were isolated and characterized in the Chilean kelp *Lessonia nigrescens* Bory. Using two different enriched libraries, we observed 1–14 alleles per locus in two samples of 21 kelp individuals each. The observed heterozygosities ranged from 0.05 to 0.80 and all loci are in Hardy–Weinberg equilibrium for one or both samples. Seventeen samples collected from different sites showed high allele diversity along the species distribution. The variation detected at these markers is currently being used for the study of populations of *Lessonia nigrescens* at different geographical scales.

Keywords: genetic variability, kelp, *Lessonia nigrescens*, microsatellite markers

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Lessonia nigrescens Bory is an intertidal kelp inhabiting the southeast Pacific coasts, from Cape Horn to southern Peru. It is a keystone species and a bioengineer of the intertidal communities. In order to establish kelp stocks, estimate some important demographic parameters and design effective conservation plans, the study of the distribution of the genetic diversity became a main concern. However, the progress of such research was severely limited by the poor quality and reduced statistical power of the random amplified polymorphic DNA markers used so far (Martínez *et al*. 2003; Faugeron *et al*. 2005), highlighting the need for highly polymorphic, codominant and reliable molecular markers. Microsatellite markers have been developed in only one kelp species, *Laminaria digitata* (Billot *et al*. 1998), and cross-amplification in different kelp species within the order Laminariales has been unsuccessful, in particular with *L. nigrescens* (Martínez *et al*. 2005). For all these reasons, species-specific microsatellites markers were developed.

We describe here the isolation and characterization of nine polymorphic microsatellites markers for *L. nigrescens* using two enriched libraries. Genomic DNA was extracted from a bulk of immature and healthy fronds of *L. nigrescens*

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