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PRIMER NOTE

Isolation and characterization of polymorphic microsatellite markers in *Zabrotes subfasciatus* Boheman (Coleoptera: Bruchidae)

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Abstract

Bruchid beetles of the genus *Zabrotes* (Coleoptera: Bruchidae) are important worldwide pests of legume seeds. Samples of *Zabrotes subfasciatus* and *Z. sylvestris* were collected from seeds of two *Phaseolus* species throughout Mexico to assess the role of host plant variation and plant domestication on the evolution of host use in this species. For this purpose six polymorphic microsatellite loci were isolated and characterized for the bruchid *Z. subfasciatus*. Cross-species amplification tests were performed on *Z. sylvestris* and revealed that three loci amplified successfully and were polymorphic in this closely related species.

Keywords: bean, Bruchidae, domestication, microsatellite, population structure, *Zabrotes*

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Bruchid beetles in the genus *Zabrotes* oviposit and feed on several legume species (Romero & Johnson 1999) and are considered a major pest of field crops and stored beans worldwide (Leroi *et al*. 1990). *Zabrotes subfasciatus* Boheman (Coleoptera: Bruchidae) specializes on lima bean (*Phaseolus lunatus*) and common black bean (*P. vulgaris*) but is also able to feed on seeds of other Fabaceae genera, such as *Vicia* (Sànchez 1992) and *Vigna* (Hills 1990). Originating from the New World (Credland & Dendy 1992), this species now has a cosmopolitan distribution mainly due to trading of the cultivated seeds which it attacks. Recently, a new species of the same genus has been described, Z. *sylvestris* (Romero & Johnson 1999), which is only distinguishable from *Z. subfasciatus* by examination of the genitalia. A study using allozyme markers investigated the degree of genetic similarity between *Z. subfasciatus* and *Z. sylvestris* (Gonzalez-Rodriguez *et al*. 2002). These authors found genetic evidence that these two bruchid species are sibling species as no gene flow was detected between them. Great genetic differentiation was detected among *Z. subfasciatus* populations but the low number of polymorphic allozyme loci (three out of six) may have led to bias estimates (Gonzalez-Rodriguez *et al*. 2002). The genetic differentiation among *Z. sylvestris* populations could not be evaluated as only one allozyme locus revealed polymorphism. Therefore, we isolated microsatellite loci from *Z. subfasciatus* and tested for cross-species amplification in *Z. sylvestris* to further investigate the degree of genetic variation among populations and to determine the role of host plant variation and plant domestication on the evolution of these species.

Total genomic DNA was extracted using a Puregene™ DNA isolation kit (Gentra Systems) from individuals of *Z. subfasciatus* collected in Tepoztlan, Tejupilco, Temascaltepec and Malinalco (Mexico State). Microsatellite-enriched libraries were built following Billotte *et al*. (1999): DNA was digested to completion with *Rsa*I (Eurogentec) and 500–1000-bp DNA fragments were selected after resolving on TAE 0.8% (w/v) agarose gel and isolated using an extraction kit (Promega). The enrichment step was as described in Kijas *et al*. (1994) and Edwards *et al*. (1996). The enriched microsatellite partial library was then constructed by ligating the PCR products into pGEM-T (Promega) according to the manufacturer's recommendations. Epicurian-coli XL1-Blue MRF' supercompetent cells (Stratagene) were used for the transformation of the cloned

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Table 1 Polymorphic microsatellite loci for *Zabrotes subfasciatus*

Locus	Motif	GenBank Accession no.	Primer sequences (5′–3′)	Size range (bp)	T_a (°C)	Tejupilco (n = 10)			Mazunte (n = 10)		
						n_a	H_E	H_O	n_a	H_E	H_O
Zs2	$(CA)_{11}C$	AY684292	F: CACAAGTGGTGTGAAAACGG R: AGTTCACTTAGCAGTTGCCC	252–278	66	4	0.61	0.20*	7	0.85	0.30*
Zs6	$(AC)_{10}$	AY684293	F: AGTCATCTTGCAGATAAGC R: ATCAGATCAGTTTCATCCG	248–270	49	6	0.84	0.50*	5	0.81	0.10*
Zs8	$(CA)_{19}C$	AY684294	F: GAAAACAGTTTCTCAATGGTG R: TGCAGAATGGCTACGATGG	214–332	59	5	0.82	0.40*	7	0.69	0.30*
Zs15	$(GT)_6TT(GT)_{14}$	AY684295	F: TCACCACGATTAGAATGTCC R: TAGCTCTTTGGCTACTTCGC	216–270	55	4	0.61	0.50	12	0.77	0.70*
Zs16	$(AC)_8$	AY684296	F: CAAACTTAGACAAATAGGCAG R: AGATGGTAAGCAAACTCAGCA	228–242	59	5	0.63	0.40*	2	0.19	0.00
Zs17	$(AC)_{11}A$	AY684297	F: ACAACCAATGCCCATTGGC R: GAACCTGTAGTAGAACGTCA	190–242	55	5	0.78	0.70	11	0.92	0.40*

T_a, annealing temperature; n_a, number of alleles detected; H_E, expected heterozygosity under Hardy–Weinberg equilibrium; H_O, observed heterozygosity; n, number of individuals tested.
*Significant deviation from H_E ($P < 0.05$).

DNA fragments. Following standard blue–white selection on X-Gal/IPTG/ampicillin plates, 184 white transformant clones were transferred on Hybond-N + nylon membranes (Amersham) and hybridized using the ^{32}P-labelled oligoprobes $(CT)_{15}$ and $(GT)_{15}$. After hybridization, the filters were washed twice with 4 × SSC (10 min, 57 °C) and then with 0.1 × SSC/10% (w/v) sodium dodecyl sulphate (10 min, 40 °C). Of these clones, 74 gave a strong positive signal of which 41 inserted DNA fragments were sequenced. Seventeen primer pairs were designed of which six gave satisfactory amplification patterns (i.e. PCR products of the predicted size and supernumerary bands of low intensity).

PCR amplifications were performed in a final volume of 5 μL which contained 1 μL of extracted DNA (2 ng/μL), 2.5 μL of HotStarTaq Master Mix (Qiagen), 0.25 μL of 10 μM reverse and forward primer (5′ IRD-700/800 modified) and 1 μL of double-distilled H_2O. PCRs were performed on a Biometra® T gradient thermocycler using the following cycling conditions: an initial denaturing step of 95 °C for 15 min followed by 40 cycles of 95 °C for 30 s, annealing temperature for 30 s and 72 °C for 40 s, and a final elongation at 72 °C for 10 min. PCR products were mixed with 2.5 μL of stop solution [95% (v/v) desionized formamide, 50 mM EDTA/10 mM NaOH/0.1% (w/v) bromophenol blue/0.1% (w/v) xylene cyanol green] and denatured at 94 °C for 2 min prior to electrophoresis on a denaturing 7.4 M urea–6.5% (w/v) polyacrylamide gel (Sequagel *XR*; National Diagnostics) on a Li-Cor DNA Analyser. Isolated bands were visualized and analysed using SAGA *IR*^{2} software (version 2.2.2).

The degree of polymorphism at the six loci was tested for *Z. subfasciatus* using females from two populations: Tejupilco (*P. vulgaris*) and Mazunte (*P. lunatus*). We calculated expected and observed heterozygosities, estimated heterozygote deficit using Hardy–Weinberg exact tests and checked for linkage disequilibrium (GENEPOP 3.3 package; Raymond & Rousset 1995).

All loci were polymorphic with two to 12 alleles per population and observed heterozygosities ranging from 0.00 to 0.70 (Table 1). A significant deficit of heterozygotes was observed for all loci (Zs2, Zs6, Zs8 and Zs16 in Tejupilco and Zs2, Zs6, Zs8, Zs15 and Zs17 in Mazunte), suggesting the presence of null alleles. No linkage disequilibrium was detected for either population.

Cross-species amplification tests on *Z. sylvestris*, using similar PCR conditions, on females from two populations (San José de los Laureles and Ahuehueyo on *P. vulgaris*) showed that three loci (Zs2, Zs6 and Zs16) out of six amplified consistently and were polymorphic (Table 2). Two to

Table 2 Polymorphic microsatellite loci for *Zabrotes sylvestris*

Locus	T_a (°C)	San José de los Laureles (n = 10)			Ahuehueyo (n = 10)		
		n_a	H_E	H_O	n_a	H_E	H_O
Zs2	66	2	0.19	0.00	2	0.27	0.10
Zs6	49	5	0.75	0.60	5	0.78	0.50*
Zs8	59	—	—	—	—	—	—
Zs15	55	—	—	—	—	—	—
Zs16	59	5	0.66	0.40	2	0.53	0.20
Zs17	55	—	—	—	—	—	—

Abbreviations as in Table 1.

five loci were detected and observed heterozygosities ranged from 0.00 to 0.60. One locus (Zs6) showed a heterozygote deficit suggesting the presence of null alleles. No linkage disequilibrium was detected.

Due to probable high null allele occurrence, these loci are not appropriate for studies of breeding systems. Nevertheless, these six novel polymorphic markers should enable us to estimate genetic diversity at these loci among and within populations to obtain a detailed picture of the population structure of *Z. subfasciatus* in relation to host plant variability and bean domestication status.

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