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PRIMER NOTE

Microsatellite loci for the field cricket, *Gryllus bimaculatus* and their cross-utility in other species of Orthoptera

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Abstract

We have isolated 16 microsatellite loci in the field cricket, *Gryllus bimaculatus*. Nine loci were found to be polymorphic in *G. bimaculatus* and the number of alleles varied from seven to 14. All 16 loci were tested for amplification in nine other species. In the five species tested belonging to the same subfamily (Gryllinae), a minimum of nine loci amplified. These loci will be used to determine paternity as part of a study to investigate the genetic benefits of polyandry.

Keywords: cross-species amplification, field cricket, Gryllidae, *Gryllus bimaculatus*, microsatellite, Orthoptera

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The field or two-spot cricket, *Gryllus bimaculatus*, is an example of a species in which polyandry increases female reproductive success without obvious nongenetic benefits (Tregenza & Wedell 1998, 2002). In order to further investigate the genetic benefits of polyandry, we have isolated and characterized nine polymorphic microsatellite loci in this species.

DNA was extracted using a phenol : chloroform extraction method (Sambrook *et al.* 1989). For the production of the enriched microsatellite library, genomic DNA from a single male *G. bimaculatus* individual was digested with *Mbo*I (ABgene, Epsom, Surrey, UK) and enriched for $(CA.GT)_n$ or $(GA.CT)_n$ sequences (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). The method used was essentially that described by Armour *et al*. (1994) with two modifications. To prevent duplicate clones, the DNA fragments were not PCR-amplified (polymerase chain reaction) before the enrichment hybridization. Secondly, a different plasmid cloning vector was used which was supplied pre-digested and dephosphorylated. The enriched fragments were ligated into pUC18-*Bam*HI/BAP (Amersham Pharmacia Biotech) and transformed into XL1-Blue competent cells (Stratagene, La Jolly, CA, USA). Six hundred transformant clones were screened by hybridization to the sequences $(CA.GT)_n$ or $(GA.CT)_n$ radiolabelled with [α^{32}P]-dCTP, which identified 71 positives. Twenty-eight of the strongest positives were sequenced using BIG DYE Terminators on an ABI 377 Sequencer (Applied Biosystems, Foster City, CA, USA). Seventeen sequences were found to possess motifs with at least nine dinucleotide repeats and suitable for designing primers. These 17 sequences were confirmed unique using GENEJOCKEY Sequence Processor software (Biosoft, Cambridge, UK) and submitted to the EMBL database (Accession numbers: AJ315353–AJ315369). After submission to the EMBL database, the 17 sequences were double-checked unique using BLASTN 2.2.4 software (Altschul *et al.* 1997). All sequences were confirmed to be unique. Loci *Gbim10* and *Gbim12* (EMBL Accession numbers AJ315363 and AJ315365) are unique loci but exhibit 76% sequence homology between their respective flanking regions. The high number of base differences between the flanking sequence of *Gbim10* when compared with *Gbim12* suggests that these sequences are two different, unique loci and not different alleles of the same locus. The repeat region of *Gbim12* possesses additional (CG) motif repeats and fewer (CA) repeats. A different primer set was developed for each locus and different Orthoptera species were found to amplify with each primer set (Table 2), also suggesting these are two separate loci.

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Table 1 Characterization of nine polymorphic microsatellites in the field cricket, *Gryllus bimaculatus*

Locus	EMBL no. and id	Repeat motif*	Primer sequence 5′–3′ (and 5′ primer label)	T_a (°C)	$MgCl_2$ (mM)	Scored on	N	A	Expected allele size (bp)*	Observed allele size range (bp)	H_O	H_E
Gbim04	AJ315356 CR1E11	$(GT)_{27}$	(F) **(6-FAM)-**CGACGTATGTAGGCCTGCGG (R) ATCCTACCAACACGGCACGG	65	1.5	ABI	15	11	241	205–239	0.80	0.89
Gbim06	AJ315359 CR2A05	$(GT)_{25}$	(F) GCGATGCGAATCTTGAACTGC (R) TTCCTCGCCTTGACGACTCC	65	1.5	SS	15	7	204	160–220	0.80	0.83
Gbim07	AJ315360 CR2A12	$(CG)_3(CA)_{10}$	(F) TCTTCCTTATCCTATTCGCATCCC (R) CGGTGCCGACTTACACGTTG	65	1.0	SS	19	9	189	170–220	1.00	0.88
Gbim08	AJ315361 CR2B12	$(CA)_9$ and $(GA)_3AGAC(GA)_2$	(F) ACGTCAATACCATCAAAGCCTTTCC (R) TCACTTACAGGGCCAACGCC	65	1.5	SS	20	9	176	170–210	0.85	0.86
Gbim09	AJ315362 CR2E08	$(CA)_9CC(CA)_4CT(CA)_2$	(F) GATCTTTCCTTCTTCTTCCTTGTC (R) TCTCACCTCATATCCTGGGC	60	1.0	SS	19	11	165	150–210	0.58	0.88
Gbim11	AJ315364 CR3A05	$(CA)_{14}$	(F) **(6-FAM)-**CCCTTCCATATCCTACCCTCACC (R) CCAGATATCTTCCTCCTGCGG	65	1.5	ABI	18	7	141	130–162	0.67	0.74
Gbim13	AJ315366 CR3E12	$(GT)_{21}TT(GT)_2$	(F) TCCGGCACCAACTCTTAAGTCAG (R) GATCAACACCACGGGAACGTC	65	1.0	SS	16	9	246	200–310	0.88	0.86
Gbim14	AJ315367 CR3G03	$(CA)_7TA(CA)_{11}CTA(CA)_2AG(CA)_2$	(F) **(HEX)-**GATCATTATTCCTCTTCCTTGTTC (R) ATCCTTCGCCGAGCTAAC	57	1.0	ABI	15	14	206	188–226	0.93	0.92
Gbim15	AJ315368 CR4E02	$(CA)_{16}$	(F) GACTGCGGGTACCCTTGTCG (R) ATCCGGAGCTTCAGCAAGGC	65	1.0	SS	20	8	179	150–220	0.95	0.87

PCR profile used: 94 °C 2 min, then 94 °C 1 min, T_a °C 30 s, 72 °C 30 s for 5 cycles, then 94 °C 30 s, T_a °C 30 s, 72 °C 30 s for 30 cycles, then 72 °C for 3 min.
*Of sequenced clone.
T_a, annealing temperature; SS, genotypes scored on 6% polyacrylamide gels stained with silver; ABI, genotypes scored on an ABI 377 Sequencer; N, number of individuals tested; A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity.

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Seventeen primer pairs were designed with the assistance of PRIMER, version 3 (Rozen & Skaletsky 2000) and tested for amplification and polymorphism using 15–20 individuals. The *G. bimaculatus* individuals used to test for polymorphism were randomly selected from a laboratory population cultured in Leeds, which was originally established from individuals collected from the outskirts of Pretoria, South Africa. Each 10-μL PCR contained 0.1–10 ng of genomic DNA, 1 μM of each primer, 0.2 mM of each dNTP and 0.25 units *Taq* DNA polymerase (Thermoprime-Plus, ABgene) in the manufacturer's buffer [final concentrations 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl pH 9.0, 0.01% (w/v) Tween], including 1.0 or 1.5 mM $MgCl_2$ (see Table 1). For two loci, PCR amplification was improved by the addition of 0.5% bovine serum albumin, fraction V (BSA; Sigma, St Louis, MO, USA) and 1% dimethyl sulphoxide (DMSO; Sigma), and by reducing the amount of template DNA to 1.0 ng or 0.1 ng. PCR amplification was performed in a Hybaid Touchdown thermal cycler (Thermo Hybaid, Ashford, Middlesex, UK). The PCR profile adopted is provided in Table 1. PCR amplification was confirmed on 2% agarose gels stained with ethidium bromide. Only one of the 17 primer sets, did not amplify a product (clone CR1F04, EMBL Accession number AJ315357).

Ten loci were characterized for polymorphism. Seven loci were analysed using 6% polyacrylamide gels stained with silver (Bassam *et al*. 1991; Promega, Southampton, UK) and three loci using an ABI 377 Sequencer. For the loci characterized on the ABI 377 Sequencer, the 5′ end of the forward primer was labelled with a fluorescent phosphoramidite and gels analysed using GENESCAN software (version 3.1) and GENOTYPER DNA fragment analysis software (version 2.5). One locus was monomorphic (*Gbim12*, Table 2). Nine loci were polymorphic and these displayed between seven and 14 alleles in 15–20 *G. bimaculatus* individuals (Table 1). Primer sequences, expected PCR product sizes, allele size range and numbers of alleles for the nine polymorphic loci are given in Table 1. The observed and expected heterozygosity for each locus was calculated using CERVUS v2.0 (Marshall *et al*. 1998, 1999; Table 1). Only the locus (*Gbim09*) had a significant difference between observed and expected heterozygosity ($\chi^2 = 16.1$, $P < 0.001$). This could be due to the presence of a null allele.

Three loci, *Gbim04*, *Gbim11* and *Gbim14*, have been successfully used to assign paternity using an ABI 377 Sequencer (Bretman *et al*., unpublished data).

The utility of all 16 *G. bimaculatus* loci in other species was assessed using three high quality DNA samples from each of nine other Orthopteran species (Table 2). The loci tested included: the nine characterized polymorphic loci, the monomorphic locus and six loci which amplified well and were polymorphic in *G. bimaculatus* but which were not characterized fully. EMBL Accession numbers and primer sequences for these loci are provided in Table 2. The same PCR constituents were used as for *G. bimaculatus* but a different $MgCl_2$ concentration and PCR profile was adopted (Table 2).

Loci *Gbim04* and *Gbim06* jointly amplified in the highest number of other Orthopteran species (eight species). When tested in five species from the same subfamily, Gryllinae [genera: *Gryllus* (two species), *Teleogryllus*, *Acheta* and *Gryllodes*] between 9 and 14 loci amplified (56–88%). This high level of cross-amplification suggests these loci will be useful for genotyping in most of the 100–800 species belonging to the subfamily Gryllinae (species numbers dependent on the taxonomy used, Otte & Naskrecki 1997 or Hewitt 1979). Other studies have shown cross-species amplification of Orthopteran loci. Hockham *et al*. (1999) found 33–100% amplification of six bushcricket loci across 11 species of the same subfamily and King *et al*. (1998) found 50–100% amplification of four tree weta loci across four species in the same genus.

Four loci amplified in all five species of the subfamily Gryllinae but no other species. Within *Oecanthus nigricornis*, belonging to the same family as *G. bimaculatus* (Gryllidae) but a different subfamily (Oecanthinae), fewer loci (five) amplified. In species from three non-Gryllidae families a similar number of loci amplified (two to five loci; Table 2). More species need testing but this suggests that the genetic distance among species from different Gryllidae subfamilies is similar to the distance between species from different Orthopteran families. This may be due to the traditional classification, based upon phenotypic characteristics, not accurately reflecting phylogenetic relationships.

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Table 2 Cross-amplification of 16 *Gryllus bimaculatus* microsatellite loci in nine other species belonging to the order Orthoptera

						Family	G	G	G	G	G	G	A	T	P			
						Subfamily	Gr	Gr	Gr	Gr	Gr	Gr	O	na	na	H	No. of spp. amplifying per locus	
Locus	EMBL no. and id.	Repeat motif*	Primer sequence (5′–3′)	PCR profile	$MgCl_2$ (mM)	Species†	GB	GC	GT	TO	AD	GS	ON	HF	CN	CS	Other Gryllidae spp. (/6)	Non-Gryllidae spp. (/3)	Total other Orthoptera spp. (/9)
Gbim01	AJ315353 CR1B06	$(CA)_{24}$	(F) ATTGGGCCAAGCATAGCACG (R) AATGAGATATCCAGCGCGGC	65.td	2.0		+	+	+	+	+	–	+	+	+	–	5	2	7
Gbim02	AJ315354 CR1D02	$(CA)_{10}$	(F) GATCCTTCCTTCCATTTTCCTATC (R) CCGACTGGTCTCCCGACT	60.td	0.6		+	+	+	+	+	+	–	+	–	–	5	1	6
Gbim03	AJ315355 CR1E04	$(CT)_{31}(CA)_{17}$	(F) GCGAATCCCAGAGCAGTACCC (R) AGACAGCACCGCTACACCCG	65.td	1.0		+	+	+	–	–	+	–	–	–	–	3	0	3
Gbim04	AJ315356 CR1E11	$(GT)_{27}$	(F) CGACGTATGTAGGCCTGCGG (R) ATCCTACCAACACGGCACGG	65.td	1.5		+, P	+	+	+	+	–	+	+	+	+	5	3	8
Gbim05	AJ315358 CR1G07	$(GT)_{12}(GTAA)_7$	(F) CCGGCATGTGCGGTAGAGTAG (R) TATCAAACGGGCCACGGAAC	65.td	0.6		+	+	+	+	+	+	–	–	–	–	5	0	5
Gbim06	AJ315359 CR2A05	$(GT)_{25}$	(F) GCGATGCGAATCTTGAACTGC (R) TTCCTCGCCTTGACGACTCC	65.td	1.5		+, P	+	+	+	+	+	+	–	+	+	6	2	8
Gbim07	AJ315360 CR2A12	$(CG)_3(CA)_{10}$	(F) TCTTCCTTATCCTATTCGCATCCC (R) CGGTGCCGACTTACACGTTG	65.td	1.0		+, P	+	+	+	+	+	–	–	–	–	5	0	5
Gbim08	AJ315361 CR2B12	$(CA)_9$ and $(GA)_3$ $AGAC(GA)_2$	(F) ACGTCAATACCATCAAAGCCTTTCC (R) TCACTTACAGGGCCAACGCC	65.td	1.5		+, P	+	+	+	+	–	+	+	–	–	5	1	6
Gbim09	AJ315362 CR2E08	$(CA)_9CC(CA)_4CT$ $(CA)_2$	(F) GATCTTTCCTTCTTCTTCCTTGTC (R) TCTCACCTCATATCCTGGGC	60.td	0.6		+, P	–	–	–	–	–	–	–	–	–	–	0	0	0
Gbim10‡	AJ315363 CR2G11	$(CA)_{21}$	(F) ACACCAGGCGAATGCTGGAG (R) CCATCCCAGACAGAACGAGC	60.td	1.0		+	+	+	+	+	+	–	–	–	–	5	0	5
Gbim11	AJ315364 CR3A05	$(CA)_{14}$	(F) CCCTTCCATATCCTACCCTCACC (R) CCAGATATCTTCCTCCTGCGG	65.td	1.5		+, P	+	+	+	+	+	–	–	–	–	5	0	5
Gbim12‡	AJ315365 CR3C07	$(CG)_4(CA)_{12}$	(F) ATCATCACCAGCGAATGCCC (R) ACCCATGTCAGCTTGCGGAG	65.td	0.6		+, M	–	–	–	–	–	–	–	–	–	–	0	0	0
Gbim13	AJ315366 CR3E12	$(GT)_{21}TT(GT)_2$	(F) TCCGGCACCAACTCTTAAGTCAG (R) GATCAACACCACGGGAACGTC	65.td	1.0		+, P	+	+	+	–	–	–	–	–	–	3	0	3
Gbim14	AJ315367 CR3G03	$(CA)_7TA(CA)_{11}CTA$ $(CA)_2AG(CA)_2$	(F) GATCATTATTCCTCTTCCTTGTTC (R) ATCCTTCGCCGAGCTAAC	60.td	1.0		+, P	+	+	+	–	+	–	–	–	–	4	0	4

Table 2 *Continued*

						Family	G	G	G	G	G	G	A	T	P			
						Subfamily	Gr	Gr	Gr	Gr	Gr	Gr	O	na	na	H	No. of spp. amplifying per locus	
Locus	EMBL no. and id.	Repeat motif*	Primer sequence (5′–3′)	PCR profile	$MgCl_2$ (mM)	Species†	GB	GC	GT	TO	AD	GS	ON	HF	CN	CS	Other Gryllidae spp. (/6)	Non-Gryllidae spp. (/3)	Total other Orthoptera spp. (/9)
Gbim15	AJ315368 CR4E02	$(CA)_{16}$	(F) GACTGCGGGTACCCTTGTCG (R) ATCCGGAGCTTCAGCAAGGC	65.td	1.0	+, P	+	+	+	–	+	+	–	+	–	5	1	6
Gbim16	AJ315369 CR6B11	$(CGG)_4$ and $(GAGCG)_5$	(F) GACGCGCCCGGAATCAGTAG (R) CCAGCCGCGAAGCACGAG	65.td	3.0	+	+	+	+	+	–	–	+	–	–	4	1	5
			Total number of loci amplifying (/16)			16	14	14	13	10	9	5	5	4	2			

PCR profiles used:
65.td = 94 °C for 2 min for one cycle, then 94 °C for 30 s, X°C for 30 s, 72 °C for 30 s for two cycles (where *X* = 65 °C then 63, 61, 59, 57) then 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s for 30 cycles, and finally 72 °C for 3 min.
60.td = 94 °C for 2 min for one cycle, then 94 °C for 30 s, Y°C for 30 s, 72 °C for 30 s for two cycles (where *Y* = 60 °C then 58, 56, 54, 52) then 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s for 30 cycles, and finally 72 °C for 3 min.
*Of sequenced clone.
†Three unrelated individuals of each species were tested and species are ordered as most closely related to *Gryllus bimaculatus* (based on Gwynne 1995; Huang *et al*. 2000).
‡The sequences of Gbim10 and Gbim12 (AJ315363 and AJ315365) exhibit 76% sequence homology in their respective flanking regions (see text).
na, not applicable.
G, Gryllidae family; A, Anostostpmatidae family; T, Tettigoniidae family; P, Prophalangopsidae family; Gr, Gryllinae Subfamily; O, Oecanthinae subfamily; H, Haglidae subfamily.

GB, *Gryllus bimaculatus*, field cricket (origin of loci); GC, *Gryllus campestris*, field cricket (*G. campestris* is the sister species of *G. bimaculatus*); GT, *Gryllus texensis*, field cricket; TO, *Teleogryllus oceanicus*, field cricket; AD, *Acheta domestica*, black house cricket; GS, *Gryllodes sigillatus*, tropical house cricket; ON, *Oecanthus nigricornis*, black horned tree cricket; HF, *Hemiandrus furcifer*, ground weta; CN, *Conocephalus nigropleurum*, black sided meadow katydid; CS, *Cyphoderris strepitans*, sage bush cricket.

+, amplification; –, no amplification; P, polymorphic (see Table 1); M, monomorphic.

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