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PERMANENT GENETIC RESOURCES

New microsatellite loci isolated from the field cricket *Gryllus bimaculatus* characterized in two cricket species, *Gryllus bimaculatus* and *Gryllus campestris*

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Abstract

We have developed a new set of 27 polymorphic markers for each of two cricket species, *Gryllus bimaculatus* and *Gryllus campestris*. Initially, 14 published *G. bimaculatus* loci were tested in *G. campestris*; however, only five loci were polymorphic. Therefore, we isolated an additional 50 new microsatellite loci from *G. bimaculatus* and tested these in both species. In a minimum of 20 individuals, 27 of the new loci were polymorphic in *G. bimaculatus* and 25 in *G. campestris*.

Keywords: cricket, cross-species utility, Gryllidae, microsatellite, Orthoptera, primer

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Gryllus field crickets are widely used as model species for studies of sexual selection (e.g. Tregenza & Wedell 2002; Bretman *et al*. 2006). Polymorphic microsatellite loci for the field cricket *Gryllus bimaculatus* (De Geer) (Dawson *et al*. 2003) have been used successfully for studies involving parentage analysis (e.g. Bretman & Tregenza 2005). Their amplification, but not polymorphism, was tested in 10 other gryllid cricket species. The closest relative of *G. bimaculatus* is the field cricket *G. campestris* (Linnaeus), a species of interest not only to behavioural ecologists, but also to conservation biologists as it is endangered in the UK (UK Biodiversity Group 1999). We found that only five of 14 previously published *G. bimaculatus* loci were polymorphic in *G. campestris* (Table 1). Therefore, we isolated and characterized further *G. bimaculatus* microsatellite loci to identify a set of polymorphic loci for both species.

The development of the *G. bimaculatus* microsatellite-enriched genomic library is described in Dawson *et al*. (2003). Briefly, a single male *G. bimaculatus* was stored in 100% ethanol until DNA extraction using phenol:chloroform (Sambrook *et al*. 1989). The DNA was digested with *Mbo*I (ABgene) and enriched for $(CAGT)_n$ or $(GACT)_n$ sequences (Amersham Pharmacia Biotech). The method used was essentially that described by Armour *et al*. (1994) with two modifications. First, to prevent duplicate clones, the DNA fragments were not amplified by polymerase chain reaction (PCR) before the enrichment hybridization. Second, a different plasmid cloning vector was used that was supplied predigested and dephosphorylated. The enriched fragments were ligated into pUC18-*Bam*HI/BAP (Amersham Pharmacia Biotech) and transformed into XL1-Blue competent cells (Stratagene). For the current study, transformant colonies were screened with TTTC, GTAA, GATA, CTAA, AC and AG and their complement. From 1568 colonies picked, 100 positive clones were identified. Sixty-two positively hybridizing clones were sequenced in both directions using M13 primers (M13F-CACGACGTTG-TAAAACGAC, M13R-CAGGAAACAGCTATGACC) with BigDye terminators (Applied Biosystems) on an ABI3730 DNA Analyser (Accession nos AM398082–398140). Consensus sequences were created and checked for duplication using BLASTN 2.2.4 (Altschul *et al*. 1997). None of the new sequences duplicated any of those previously published (Dawson *et al*. 2003). However, the sequence of clone CR114C02 (Accession no. AM398092) duplicated that of clone CR115E02 (Accession no. AM398108) and primers were therefore only designed from one of these sequences (CR114C02, *Gbim28*). Three sequences did not

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Table 1 Five published *Gryllus bimaculatus* loci (Dawson *et al*. 2003) now characterized in *Gryllus campestris*

Locus	EMBL Accession no.	Repeat motif in *G. bimaculatus*	Primer sequence (5′–3′)†	T_a (°C)	$MgCl_2$ (mM)	N	*A*	Allele size range (bp)	H_O	H_E
Gbim03	AJ315355	$(CT)_{31}$ $(CA)_{17}$	(F) (6-FAM)-GCGAATCCCAGAGCAGTACCC	65	1.0	23	4	180–188	0.9	0.70*
			(R) AGACAGCACCGCTACACCCG						1	
Gbim04	AJ315356	$(GT)_{27}$	(F) (6-FAM)-CGACGTATGTAGGCCTGCGG	65	1.0	22	12	211–257	0.6	0.83
			(R) ATCCTACCAACACGGCACGG						4	
Gbim06	AJ315359	$(GT)_{25}$	(F) (PET)-GCGATGCGAATCTTGAACTGC	65	1.5	22	7	175–191	0.2	0.69*†
			(R) TTCCTCGCCTTGACGACTCC						3	
Gbim08	AJ315361	$(CA)_9$ and	(F) (NED)-ACGTCAATACCATCAAAGCCTTTCC	65	1.5	22	3	170–178	0.4	0.61*†
		$(GA)_3AGAC(GA)_2$	(R) TCACTTACAGGGCCAACGCC						1	
Gbim15	AJ315368	$(CA)_{16}$	(F) (VIC)-GACTGCGGGTACCCTTGTCG	65	1.0	21	9	167–197	0.9	0.79
			(R) ATCCGGAGCTTCAGCAAGGC						5	

T_a, annealing temperature; *N*, number of individuals genotyped; *A*, number of alleles observed; H_O, observed heterozygosity; H_E, expected heterozygosity.

*locus significantly deviates from Hardy–Weinberg equilibrium after sequential Bonferroni correction for multiple tests.

†heterozygote deficiency (H_E–H_O > 0.2).

The PCR profile used was 94 °C for 4 min (one cycle), followed by 94 °C for 30 s, T_a for 30 s, 72 °C for 30 s (35 cycles), and finally 72 °C for 10 min (one cycle).

contain a repeat region and for one clone, the flanking regions were too short for primer design (Accession no. AM398126).

Primers for 57 loci were designed using PRIMER 3 (Rozen & Skaletsky 2000). The new loci were characterized in *G. bimaculatus* and *G. campestris*. The previously published *G. bimaculatus* loci (Dawson *et al*. 2003) were characterized in *G. campestris* using the same approach. For primer testing, *G. bimaculatus* were taken from a large laboratory population derived from 40 mated females caught near Valencia, Spain (approximately 10 generations in the laboratory, with a population never smaller than 100). Females are highly polyandrous in the wild (Bretman & Tregenza 2005) and could have mated with up to 10 males prior to capture. *G. campestris* were from a wild population near Gijón, Spain. For primer testing, 30 individuals were selected at random from each of these populations, so are presumed to be unrelated. DNA was extracted using a salting-out method (see Bretman & Tregenza 2005). The 5′ end of the forward primer was labelled with 6-FAM fluorescent dye (Operon). PCR was performed on a PX2 Thermal Cycler (Thermo Electron). Each 10 μL PCR contained 0.1–10 ng of genomic DNA, 0.5 μM of each primer, 0.2 mM of each dNTP and 0.25 U of *Taq* DNA polymerase (Yorkshire Biosciences) in the manufacturer's buffer (final concentrations 100 mM Tris-Cl, 500 mM KCl, 1% Triton X-100), and $MgCl_2$ at four different concentration: 0.6 mM, 1.0 mM, 1.5 mM and 2.5 mM. Primers were initially tested for amplification using six individuals of each species and products were visualized on 1.5% agarose gels stained with ethidium bromide. Primer sets that amplified a strong specific product as analysed on agarose gel were used to amplify 20–30 individuals of each species under the same conditions. Genotypes were scored on an ABI 3130XL Genetic Analyser using Liz500 size standard (Applied Biosystems) and analysed with GENEMAPPER version 4.0 software (Applied Biosystems).

Fourteen of the 16 *G. bimaculatus* loci previously characterized in *G. bimaculatus* were found to amplify in *G. campestris* (Dawson *et al*. 2003) and were therefore tested in *G. campestris*. Only five were found to be polymorphic (Table 1).

Seven of the 57 new primer sets were abandoned due to poor amplification (some of these primer sequences included runs of single bases or were A/T-rich). Of the 50 new loci tested, 47 amplified in *G. bimaculatus* and 45 did in *G. campestris*. Of these, 20 loci produced nonspecific products in both species and therefore were not tested further. Twenty-seven loci were polymorphic in *G. bimaculatus* (displaying three to 12 alleles) and 25 loci were polymorphic in *G. campestris* (displaying three to 18 alleles). Primer sequences, numbers of alleles per locus and observed allele size range for each species tested are provided in Table 2.

Observed and expected heterozygosities for each locus were calculated using CERVUS version 3.0 (Kalinowski *et al*. 2007). All polymorphic loci were tested for deviation from Hardy–Weinberg equilibrium using GENEPOP version 3.4 (Raymond & Rousset 1995). After sequential Bonferroni correction, seven loci in *G. bimaculatus* and eight loci in *G. campestris* deviated significantly from Hardy–Weinberg equilibrium, possibly due to the presence of null alleles (Tables 1 and 2).

Linkage disequilibrium between loci was tested in two ways. Genotypes from the presumed unrelated individuals were analysed using GENEPOP version 3.4 (Raymond & Rousset 1995). After sequential Bonferroni correction, significant linkage disequilibrium was found in *G. bimaculatus* between *Gbim29* and *Ghim45*, *Gbim29* and *Ghim46*, and

Table 2 Twenty-seven new microsatellite loci isolated from *Gryllus bimaculatus* and characterized in two cricket species, *Gryllus bimaculatus* and *Gryllus campestris*

Locus	EMBL no.	Repeat motif in *Gryllus bimaculatus*	Primer sequence 5′–3′†	Species	T_a (°C)	$MgCl_2$ (mM)	N	A	Allele size range (bp)	H_O	H_E
Gbim20	AM398084	$(GA)_{23}$	(F) AGGCCACCCGTGAGTGAGAG	*Gbim*	65	1.0	20	9	189–255	0.90	0.89
			(R) TCAAAGAGGCCATCAGAGCATTAAG	*Gcam*	65	1.0	22	13	194–245	0.55	0.90*‡
Gbim21	AM398085	$(GA)_2TA(GA)_3$ GG$(GA)_2$GG$(GA)_5$	(F) GACCGCCACTAACCCACCAC	*Gbim*	65	0.6	27	10	246–387	0.63	0.81
			(R) GGAACGGGCAGCAGTTTGTC	*Gcam*	65	0.6	23	18	262–317	0.78	0.95
Gbim24	AM398088	$(GT)_{14}GC(GT)_3$	(F) CGGGACACCGCCTCAGTAAG	*Gbim*	65	1.0	23	8	153–182	0.83	0.78
			(R) CGGAGACTGACCCTCACAAACAG	*Gcam*	65	1.0	22	5	154–170	0.32	0.76*‡
Gbim26	AM398090	$(GT)_{26}$	(F) CGTTAAACTACACGTCAGCTTCTG	*Gbim*	58	1.0	23	7	157–191	0.70	0.85
			(R) GCTTTCCGTCTTCATTGTTTTC	*Gcam*	58	1.0	23	9	142–180	0.78	0.87
Gbim28	AM398092	$(CTTT)_{15}$	(F) GATCCCATGGGTACGCAAATATCG	*Gbim*	65	1.0	20	7	155–212	0.80	0.78
			(R) CCACGACGAGCGCATTGG	*Gcam*	65	1.0	20	7	114–160	0.42	0.77*‡
Gbim29	AM398093	$(CA)_3A(CA)_{16}$	(F) GATCCATTTCCGCCACTTCG	*Gbim*	65	1.0	22	7	270–299	0.50	0.65
			(R) AATGCAACGGCATCGTAGGG	*Gcam*	65	1.0	23	5	270–281	0.74	0.76
Gbim32	AM398096	$(GA)_{23}$	(F) ACCATCCGTTCGCTTTCTCG	*Gbim*	65	0.6	21	12	159–190	0.67	0.84
			(R) GAGCAGTAGACATAGTTCGAGGGTGTC	*Gcam*	—	—	—	—	—	—	—
Gbim33	AM398097	$(GATA)_{14}(GATT)_3$	(F) GCTTCAGAAGGCGAAGACACG	*Gbim*	65	1.0	20	7	265–347	0.50	0.80‡
			(R) TTGGTGGATTGTGACGATTATTGC	*Gcam*	65	1.0	23	11	203–276	0.83	0.87
Gbim34	AM398098	$(CA)_7$	(F) TTTCCTTCCTCTTCCTTGTCCTATCC	*Gbim*	65	0.6	22	5	190–204	0.55	0.61
			(R) ATCCAATGCCGACTTACAACAGC	*Gcam*	65	0.6	22	3	151–196	0.18	0.25
Gbim35	AM398099	$(CT)_{22}$	(F) ACTCGACAACACTTAACGGACTAATGC	*Gbim*	65	1.0	22	8	217–265	0.77	0.82
			(R) TGTGAACGGAAAGGCTTGACC	*Gcam*	65	1.0	23	3	215–219	0.13	0.20
Gbim38	AM398102	$(CA)_4TA(CA)_2$ TT$(CA)_{15}$	(F) GATCCTTAACAAACAGGACACGAAGC	*Gbim*	65	1.5	27	10	177–209	0.56	0.82‡
			(R) GGCACCAGTCAAGCCATCG	*Gcam*	65	1.5	23	6	183–193	0.52	0.80‡
Gbim40	AM398104	$(CA)_{18}$	(F) GATCTGTCCTATCATCACCTCTTGC	*Gbim*	61	0.6	20	12	125–183	0.60	0.90*‡
			(R) ACGGCAGGCGGAGTTTC	*Gcam*	61	0.6	25	9	142–166	0.80	0.65
Gbim41	AM398105	$(CA)_8$	(F) CATGGGCATCGCAAGC	*Gbim*	61	2.5	24	9	119–169	0.79	0.81*‡
			(R) AAATTACTTTAATCTGGAGAGAAAGTTGC	*Gcam*	61	2.5	22	7	117–147	0.46	0.68*‡
Gbim42	AM398106	$(CT)_7(CA)_5$	(F) TCCTTCACTTCATCCTTGCTTCG	*Gbim*	65	1.0	27	7	146–164	0.48	0.77
			(R) CTCCACCGCCGAGATACCAC	*Gcam*	65	1.0	22	8	144–177	0.59	0.79
Gbim45	AM398109	$(CA)_{18}$	(F) CGCGCAATCTTTCCTTCCTG	*Gbim*	65	1.0	23	6	105–135	0.52	0.52
			(R) TCCCGACCGGTATCCCAAG	*Gcam*	65	1.0	20	4	101–106	0.15	0.57*‡
Gbim46	AM398110	$(CT)_{23}$	(F) GTCGCTCTCTCTGGCAATTTCTG	*Gbim*	65	1.5	27	6	150–182	0.22	0.62*‡
			(R) GGGCCAAGGAGAGAAAGAGAGG	*Gcam*	65	1.5	20	7	130–188	0.30	0.44
Gbim48	AM398112	$(CT)_{25}(CA)_8$	(F) GATCTCTTCTTCCTCATTATTCTCC	*Gbim*	58	1.0	20	8	119–161	0.80	0.85
			(R) CCCGGTGGGTCTATCTATATG	*Gcam*	58	1.0	23	17	86–161	0.83	0.92
Gbim49	AM398113	$(GT)_{21}$	(F) TTGCCACATCTCCCGAGAAAG	*Gbim*	65	1.0	22	9	206–240	0.77	0.83
			(R) TTGGTCCGTGCGTGGTAATTC	*Gcam*	65	1.0	23	4	187–197	0.26	0.49‡
Gbim52	AM398116	$(CA)_{12}$	(F) ACACCAGGCGAATGTCGAAAC	*Gbim*	65	0.6	21	7	163–178	0.62	0.74
			(R) CCAGACGGGACTTGCTCAAAG	*Gcam*	65	0.6	23	4	161–168	0.78	0.69
Gbim53	AM398117	$(CT)_4TT(CT)_2TT(CA)_{12}$	(F) TCTTTCTTTCTTCACTCTTGACCACTCC	*Gbim*	65	1.0	20	12	120–186	0.65	0.90*‡
			(R) CGCCATGTGGGATGCTGTAG	*Gcam*	65	1.0	23	16	128–207	0.83	0.93

Table 2 *Continued*

Locus	EMBL no.	Repeat motif in *Gryllus bimaculatus*	Primer sequence 5′–3′†	Species	T_a (°C)	$MgCl_2$ (mM)	*N*	*A*	Allele size range (bp)	H_O	H_E
Gbim57	AM398121	$(CA)_{12}$	(F) TGCGAATGCCGGAGTAATACC	*Gbim*	65	0.6	20	6	157–181	0.55	0.69
			(R) CGGGAGGACAAGCTCTCACC	*Gcam*	65	0.6	22	6	163–178	0.64	0.73
Gbim58	AM398122	$(CA)_5CG(CA)_4CG(CA)_4CG(CA)_4CG(CA)_4CG(CA)_6CG(CA)_{15}$	(F) TCCTCATACATGAGACGTACTCCCTTC	*Gbim*	65	1.0	23	6	114–149	0.17	0.72*‡
			(R) TCTCGATTGGTCTCTAACAGGTAATGC	*Gcam*	65	1.0	22	3	95–99	0.36	0.49
Gbim59	AM398123	$(CA)_{17}$	(F) CCTCTCCCCTCATGCTCACG	*Gbim*	65	1.0	23	3	140–157	0.04	0.13
			(R) GGCGAGGAACGTCCTCCAG	*Gcam*	65	1.0	23	3	146–164	0.22	0.38
Gbim66	AM398130	$(CA)_{20}$	(F) AAGCTCATTTACCCTGCTGTTTGC	*Gbim*	65	1.0	30	12	310–437	0.67	0.88*‡
			(R) AACTCCAGGCAAGGGACACG	*Gcam*	65	1.0	23	7	303–320	0.70	0.60
Gbim71	AM398135	$(GT)_{17}$	(F) CACTGCCACGCAATATTTGGAC	*Gbim*	65	1.0	24	6	130–146	0.17	0.67*‡
			(R) GAGTGCCGAAAGCCGTTAGC	*Gcam*	65	1.0	23	4	132–139	0.52	0.64
Gbim72	AM398136	$(CA)_{16}$	(F) ACCAGGTGAATGTCGGAGCAG	*Gbim*	65	0.6	24	9	180–241	0.71	0.83
			(R) CAGTGTGGCACCACAGCAATC	*Gcam*	65	0.6	21	3	180–192	0.38	0.45
Gbim76	AM398140	$(GT)_{17}$	(F) ATCCGACGCCACACTACGG	*Gbim*	65	1.0	21	5	191–205	0.38	0.64‡
			(R) TTCCTCTTCCTTGTCATATCCTTACCC	*Gcam*	—	—	—	—	—	—	—

Species: G*bim*, *G. bimaculatus* and G*cam*, *G. campestris*.

†Forward primers were fluorescently labelled with 6-FAM.

T_a, annealing temperature; *N*, number of individuals genotyped; *A*, number of alleles observed; H_O, observed heterozygosity; H_E, expected heterozygosity.

*locus significantly deviates from Hardy–Weinberg equilibrium after sequential Bonferroni correction for multiple tests.

‡heterozygote deficiency (H_E–H_O > 0.2).

Loci *Gbim32* and *Gbim76* do not amplify in *G. campestris*.

The PCR profile used was 94 °C for 4 min (one cycle), followed by 94 °C for 30 s, T_a for 30 s, 72 °C for 30 s (35 cycles), and finally 72 °C for 10 min (one cycle).

Gbim40 and *Gbim72*. There was no evidence for linkage disequilibrium in *G. campestris*. For *G. bimaculatus* only, known parents and four to six of their offspring were genotyped. Linkage was then tested using CRI-MAP version 2.1 (Green *et al*. 1990). Evidence for linkage disequilibrium was found between two different pairs of loci: *Gbim52* and *Ghim72,* and *Gbim35* and *Ghim58*. We suggest these pairs of loci should not be used together in *G. bimaculatus*.

Twenty-seven of the newly isolated *G. bimaculatus* loci were polymorphic in *G. bimaculatus*. Combined with the 16 previously characterized *G. bimaculatus* loci (Dawson *et al*. 2003), these provide a powerful tool for studies in *G. bimaculatus*. Thirty *G. bimaculatus* loci were polymorphic in *G. campestris*, including 25 newly isolated loci and five loci from the previously published study. We will use these loci to examine natural and sexual selection in a wild population of *G. campestris*.

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References

Altschul SF, Madden TL, Schaffer AA *et al*. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, **25**, 3389–3402.

Armour JAL, Neumann R, Gobert S, Jeffreys AJ (1994) Isolation of human simple repeat loci by hybridization selection. *Human Molecular Genetics*, **3**, 599–605.

Bretman A, Rodríguez-Muñoz R, Tregenza T (2006) Male dominance determines female egg laying rate in crickets. *Biology Letters*, **2**, 409–411.

Bretman AJ, Tregenza T (2005) Measuring polyandry in wild populations: a case study using promiscuous crickets. *Molecular Ecology*, **14**, 2169–2179.

Dawson DA, Bretman AJ, Tregenza T (2003) Microsatellite loci for the field cricket *Gryllus bimaculatus* and their cross-utility in other species of Orthoptera. *Molecular Ecology Notes*, **3**, 191–195.

Green P, Falls K, Crooks S (1990) *Documentation for CRI-MAP*. Washington University in St Louis, St Louis, Missouri.

Kalinowski ST, Taper ML, Marshall TC (2007) Revising how the computer program CERVUS accommodates genotyping errors increases success in paternity assignment. *Molecular Ecology*, **16**, 1099–1106.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact test and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Rozen S, Skaletsky HJ (2000) PRIMER 3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds Krawetz S, Misener S), pp. 365–386. Humana Press, Totowa, New Jersey.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning. A Laboratory Manual, 2nd edn. Cold Spring Harbour Laboratory Press, New York, USA.

Tregenza T, Wedell N (2002) Polyandrous females avoid costs of inbreeding. *Nature*, **415**, 71–73.

UK Biodiversity Group (1999) *Tranche 2 Action Plans — Volume IV: Invertebrates*. DEFRA, UK Government, London, UK.