Research article

Seven polymorphic microsatellite loci in honeybees (*Apis mellifera*)

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Summary

Seven microsatellite markers identified by probing clones made from a honeybee population from Victoria, Australia with a GA oligonucleotide are polymorphic in collections of honeybees from Iberia. Five of the microsatellites are GA repeats (three perfect repeats, two imperfect repeats, and one interrupted perfect repeat); of two others found in $(GA)_n$-containing clones, one is a perfect AT repeat and one a mononucleotide T repeat. A further perfect GA repeat is monomorphic in the Iberian bees. Primer sequences are given for each of these microsatellites, and for seven others either not amplifying or giving multiple bands under the conditions used, or which were not tested. In all, eighteen microsatellites were found in twelve clones. One clone contained a mini-satellite repeat, not assayed for polymorphism.

Introduction

Populations of Hymenoptera, both eusocial and non-eusocial, are generally believed to have relatively low levels of polymorphism at allozyme loci (Graur, 1985; Pamilo and Crozier, 1981; Pamilo et al., 1978; Shoemaker et al., 1992), although the generality of this tendency for non-eusocial forms needs further investigation (Sheppard and Heydon, 1986). In populations where allozymic variation is relatively low, such as most honey bee populations, population genetic and sociobiological analyses are hindered. Much attention is therefore now being given to DNA markers in the search for polymorphisms adequate for such studies. DNA markers can be divided into substitution differences, such as restriction site variation (Nei

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Table 3. Microsatellite primer sequences not assayed for variability using the Iberian bees

Microsatellite	Primer name	Primer sequences 5′ to 3′	Expected length (bp)
B7	B7-f B7-r	AGTATGAATTACCGATTAACC CCTTTGGATAATTACTTTCC	170
ED-R	ED2-f ED2-r	GCGTTATTATTATTTATCGAG GAGTTTGAATTGGTAACCG	130
FE1*	FE1-f FE1-r	CATCGCTCGCGATTCTATAG ATGTCGTCTCGATGGCTACC	180
FE2*	FE2-f FE2-r	CTTGGTCGACCGATATTCG CCCGCCTTCGTTCCCGG	120
FM-F	FM1-f FM1-r	CCCCTTCCTCGTCCAACAGC CAATAATCATTCACAATAAAC	270
GH-F	GM1-f GM1-r	TGCTTTGCATCGCGAA AAATTTCAGAAAGCAATAC	220
GJ	GJ-f GJ-r	GACAATGGACACCTCAAAAGC CGCAGACTTTCTCCCGAAAG	130
HC*	HC-f HC-r	TTTTCCATTCGCGGGACG ACGTTTCACCGCAAATATGC	110

* Primers not tested.

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Table 2. Microsatellite primer sequences and corresponding allele frequencies observed in Iberian honeybees

Micro-satellite	Allele lengths (bp) Numbers seen						Primer sequences (5′ to 3′)-name	
CD	289	290	291	296	297	304	CAGAAATATTTCCATATAC	-CD1
	2	1	1	1	1	5	CTTTATCCACGCTTTGAGC	-CD2
	305	306	307	309	310	311		
	4	7	2	4	4	2		
	312	313	314	315	317	321		
	2	2	2	1	2	1		
						n = 44		
DJ1	149	150	151	152			TTCGAGATTCTTCGATGGGGC	-DJ1-f
	10	29	7	8		*n = 54*	AAGATTATTTCTTATCATTAAGC	-DJ1-r
DJ2	270						GAAAGACGATTGCCAAAAGC	-DJ2-f
	48					*n = 48*	CGATATTTAAAAATCGATTGAAC	-GJ2-r
ED1	174	175	176	177	179	181	CAACAGCCGTGAACGCTATC	-ED1-f
	1	2	1	2	4	8	TCATCGTGTACCAATAACG	-ED1-r
	182	183	184	185	186	187		
	1	2	6	3	3	2		
	188	190	203	204	206			
	1	1	1	1	2	*n = 44*		
FM2	291	292	293	294	295	296	ATTCCCGGTATCATCTCTTG	-FM2-f
	4	14	1	9	1	1	AATTCGTGGTTAAATTCAAAG	-FM2-r
	297	298	300					
	1	1	4		*n = 40*			
GH2	125	127	128				CGGAATATAGTCAAGAAAAT	-GH2-f
	27	11	2		*n = 40*		GATGTATATTTTTTTCTCTTG	-GH2-r
IC1	209	211	212	213	215		GGTTTGATGCTCGTAAGGA	-IC1-f
	3	33	1	1	6	*n = 44*	GGCACCTCTTGCCATCTG	-IC1-r
IC2	197	198					GGATCGATGCGCCTACA	-IC2-f
	22	18			*n = 40*		CTTCCCTTTTCCACGTAG	-IC2-r
IM	203	204	206	207	209	211	ACGCAAATGACAAGTATTAG	-IM1
	3	1	2	7	1	24	GAGTGTATTTCGAAATCGATG	-IM2
	212	213	214	220	227	228		
	1	1	3	15	1	1		
						n = 60		

Table 1. Microsatellite motifs and primer pairs. The third column shows the outcome of PCR amplification; "–" indicates that recorded primers were not synthesised

Micro-satellite	Repeat motif	Amplification	Primers and annealing temp (°C)
B7	perfect GA repeat $(GA)_{12}$	no priming	B7-f B7-r 68 °C
CD	imperfect interrupted GA repeat $(GA)_7AA(GA)_8GTGGGGAGGG$ $(AG)_3GAAGGAAGAAAGACA$ $GAGGAGGGAGAGAGG(GA)_8$	18 alleles 289–321 bp	CD-f CD-r 54 °C
CK	perfect CT repeat interrupted with tetranucleotide TATC repeat $(CT)_{14}(ATCT)_4(CT)_4$	no priming	CK-f CK-r 56 °C
DJ-F	imperfect poly-T mononucleotide repeat $(T)_7(T)_8A(T)_7(T)_7AC(T)_4C(T)_3$	4 alleles 149–152 bp	DJ1-f DJ1-r 58 °C
DJ-R	perfect GA repeat $(GA)_{10}$	monomorphic 270 bp	DJ2-f DJ2-r 58 °C
ED-F	perfect CT repeat $(CT)_8NNNN(CT)_{10}$	17 alleles 174–206 bp	ED1-f ED1-r 62 °C
ED-R	perfect poly-A mononucleotide repeat $(A)_{48}$	no priming	ED2-f ED2-r 56 °C
FE1	interrupted CT repeat $(CT)_5TTT(CT)_{13}$	–	FE1-f FE1-r 60 °C
FE2	perfect GA repeat $(GA)_{23}$	–	FE2-f FE2-r 58 °C
FM-F	perfect GA repeat followed by a perfect TA repeat $(GA)_{23}(N)_{130}(AT)_8$	multiple bands	FM1-f FM1-r 60 °C
FM-R	2 × perfect AT repeats $(AT)_{10}(N)_{149}(AT)_9$	12 alleles 291–303 bp	FM2-f FM2-r 56 °C
GH-F	minisatellite-like repeat ATAAGTACCAGCTAAATTT TTTTTTTTTTTTT	no priming	GH1-f GH1-r 60 °C
GH-R	perfect GA repeat $(GA)_{13}$	3 alleles 125–128 bp	GH2-f GH2-r 54 °C
GJ	perfect GA repeat $(GA)_{18}$	multiple bands	GJ-f GJ-r 60 °C
HC	perfect GA repeat $(GA)_{15}$	–	HC-f HC-r 56 °C
IC-F	interrupted perfect GA repeat $(AG)_{13}(N)_{39}(GA)_{13}$	5 alleles 209–215	IC1-f IC1-r 58 °C
IC-R	imperfect CT repeat $(CT)_9GTAT(CT)_9$	2 alleles 197 and 198 bp	IC2-f IC2-r 54 °C
IM	perfect GA repeat $(GA)_{15}$	12 alleles 203–228 bp	IM-f IM-r 56 °C

volumes with 0.5 µl of template DNA solution, 164 µmol dNTP, 0.8 µmol of one pri-
mer (cold), 0.11 µmol of the other primer endlabelled with γ^{33}P, 200 ng BSA and 0.4
units of Taq Polymerase (Promega) in 1× buffer (Promega). Thermal cycling was
standard for all primers (with specific annealing temperatures as shown in Table 1):
3 min initial denaturation at 94 °C, then 30 cycles of 94 °C for 45 sec, 45 sec at the
primer-specific annealing temperature and 72 °C for 45 sec, the last cycle being
followed by a 10 min extension step at 72 °C. The PCR products were electro-
phoresed on 5 % polyacrylamide denaturing gels together with sequencing ladders
made using pBR322 as template, for sizing the products.

Results

Twelve clones, among approximately 2,000 showing inserts, were identified using
the (GA)$_{10}$ probe and sequencing identified eighteen microsatellite sequences and
one putative minisatellite sequence (GenBank accession numbers U73917–
U73934). In some instances microsatellites with different repeat motifs occurred
close together in the same clone. The occurrence of more than one microsatellite
sequence in several clones accords with the observation that microsatellite sequen-
ces are not evenly distributed in the apid genome (Estoup et al., 1993).
    Primer pairs were devised to sixteen potential microsatellite markers (Tables 1,
2); of these eight were assayed for polymorphism at Baton Rouge and several were
found to be variable in the Iberian bees, with numbers of alleles varying from two
to 18 (Table 2). Table 3 lists primer pairs (and predicted repeat sequence lengths)
which have not been assayed for variability but which may be useful to other
researchers.
    Clone GH contained an apparent minisatellite sequence; the highly repeated
sequence immediately adjacent to the minisatellite sequence proved refractory
to sequencing, perhaps indicating hairpin loop formation (between GT and AC
repeating regions).

Discussion

Our assays of primer sets using the Iberian bees show that seven of these reveal
polymorphism, and of these five determine highly polymorphic loci with more than
five alleles each. Those primers which show little or no variability in our test bees
may well reveal variation in other areas; possibly also in non-apid bees.
    The rapid hybridization method described here significantly reduced the time
between cloning and sequencing. Selecting large inserts (600 to 800 bp) for cloning
detected some microsatellite sequences different from the (GA/CT) probe motif,
probably reflecting concentration of dinucleotide microsatellites through their joint
occurrence in non-coding regions. With automated methods for sequencing which
can reliably generate more than 500 base pairs of sequence from purified plasmid
DNA, cloned inserts greater than 500 bp can be sequenced. A marked improve-
ment in the efficient isolation and characterization of microsatellite loci is possible
using these methods together.

Microsatellite markers, being highly characterised and easily-analysed genetic markers, present the prospect of not only tracing specific chromosome segments in populations but a more powerful means of locating loci important in sociality than anything available before. The demonstration that some genotypes within honeybee colonies escape worker policing (Oldroyd et al., 1994 a) illustrates the potential afforded by such highly variable loci. Tanatalizing glimpses of a true genetics of sociality (Ross, 1992; Ross and Shoemaker, 1993) are likely to give way to a clear vision of the genetical evolution of social behavior within a few decades. For this reason it is important to develop further microsatellite markers and to determine their linkage relationships.

In this paper we present primer sequences for 18 honeybee microsatellite markers along with data on their variation in bees from a transect of 30 collections across the Iberian peninsula as part of a larger study.

Materials and methods

DNA was extracted from thoraces of three honeybees from a single colony from Wyperfeld National Park (Victoria, Australia), using an established protocol (Oldroyd et al., 1995), modified for the volumes involved. Approximately 1 µg of DNA was digested to completion with Sau3A and Hae*III*, electrophoresed in a 2 % NuSieve (FMC) agarose gel and fragments between 600–800 bp were extracted from the gel using the Prep-A-Gene DNA purification system (BioRad). Phagemid vector pTZ 18R (Pharmacia) was digested with Bam*HI* and Hinc*II*, cleaned with Prep-A-Gene and a filter unit (ultrafree-MC 30,000, Millipore), ligated to the bee DNA and transformed in *Escherichia coli* XL1 Blue (Stratagene) cells.

Colony lifts were taken with nylon membranes (Hybond N+, Amersham) to screen approximately 2,000 white colonies. Prehybridization was carried out for 10 min at 65 °C in 60 ml of Church and Gilbert buffer (Church and Gilbert, 1984) and then 50 pmoles of $(GA)_{10}$ end-labelled with 60 µCi of γ^{33}ATP (activity 1300 to 2000 Ci/mMol) and 25 units of T4 PNK was added to the hybridization solution. After a 20 min hybridization at 65 °C, the membranes were washed in $6 \times$ SSC for 10 min at 65 °C and then in $2 \times$ SSC at 55 °C for another 10 min. The final wash was repeated twice. After overnight exposure of the membranes to Kodak X-AR film, positive colonies were picked, restreaked on selective agar plates, and rescreened to confirm the initial positive signals. Plasmid DNA was prepared from the positive clones using a standard miniprep protocol (Sambrook et al., 1989) and the inserts were sequenced by cycle sequencing using the fmol™ DNA sequencing kit (Promega) and pUC/M13 forward and reverse primers end-labelled with γ^{33}ATP.

Polymerase chain reaction (PCR) primers flanking microsatellite sequences were determined and sent to Baton Rouge where the primers were made and used to assay the Iberian bees.

Template DNA for PCR reactions was extracted using a 10 % Chelex solution (Biorad). Degastered bees were ground in aliquots of 400 µl of solution, vortexed for 10 minutes, incubated at 56 °C for 30 minutes, vortexed for 10 seconds, boiled for 5 minutes, vortexed for 10 seconds, then spun for 3 minutes at 12000 rpm. Template DNA was then stored at -20 °C until used. PCR was carried out in 10 µl

and Li, 1979) and RAPDs (Welsh and McClelland, 1990; Williams et al., 1990) and length variations, involving either minisatellites ["DNA-fingerprinting" (Jeffreys et al., 1985)] or microsatellites (Weber and Wong, 1993).

Minisatellites are tandem repeats of length 15 bp or more, generally located in intergenic spaces. Microsatellites have a very short repeat length of 1–6 bp, which can occur in any area of the genome (although microsatellites within protein coding regions are expected to generally be trinucleotide repeats because otherwise the DNA reading frame is disrupted). Microsatellites show a high mutation rate thought to involve replication slippage due to mispairing of the DNA (Li and Graur, 1991); a well-known insect example within a coding region is that in the *Drosophila per* gene (Rosato et al., 1994). Minisatellites are typically studied using appropriate probes and restriction enzyme digests of total DNA, whereas microsatellite variation is revealed as the size of products amplified using the polymerase chain reaction and primers matching DNA sequences flanking the variable region.

Most current information on population structure and relatedness in social insects is based on allozyme data (Crozier and Pamilo, 1996), but DNA markers are being increasingly used following their first use in the study by (Davis et al., 1990) using restriction site variation in the ribosomal RNA genes of *Polistes* wasps. Restriction site variation has also been used to demonstrate genetic polyethism in the dwarf honeybee *Apis florea* (Oldroyd et al., 1994b). RAPDs have been used in the mapping of the honeybee genome (Hunt and Page, 1992; Hunt and Page, 1994; Hunt and Page, 1995), paternity analysis in honeybee colonies (Fondrk et al., 1993), and in a study of a fire-ant hybrid zone (Shoemaker et al., 1994).

DNA fingerprinting has been used to determine relatedness within colonies of honeybees (Blanchetot, 1991; Moritz, 1993; Moritz et al., 1991), halictid bees (Mueller et al., 1994), *Camponotus* ants (Heinze et al., 1994), and solitary bees (Blanchetot, 1992) and wasps (Pfennig and Reeve, 1993).

Although there are methods for estimating relatedness from multilocus DNA fingerprinting (Reeve et al., 1992), such analyses are complicated by the difficulty of assigning bands to alleles. Similar problems exist with the analysis of RAPD data. Single-locus DNA fingerprinting is achievable using sufficiently specific probes (e.g., Moritz, 1993; Moritz et al., 1991), and is usually then amenable to precise genetic analysis.

Because of the more technically difficult molecular biological procedures required for the development of microsatellite markers (Glenn, 1995), these markers have only relatively recently been developed for social insects where, however, they are proving extremely valuable sources of prolific genetic variation. Microsatellite markers have been reported for honeybees (Estoup et al., 1994; Estoup et al., 1993; Estoup et al., 1995b; Hall, 1991; Oldroyd et al., 1994a), bumblebees (Estoup et al., 1995a; Estoup et al., 1993), vespid wasps (Choudhary et al., 1993; Hughes and Queller, 1993; Thorén et al., 1995), and myrmicine (Evans, 1993; Hamaguchi et al., 1993) and formicine ants (Gertsch et al., 1995). Queller et al. (Queller et al., 1993) give an introduction to the use of microsatellites in social insect studies. Indicative of the power of the method is the ability to type males from their sperm in a female's spermatheca (Evans, 1993; Peters et al., 1995). Also encouraging is the fact that some microsatellites developed for *Apis* reveal variation in *Bombus*, and *vice versa*, although the proportion of such portable markers is small (Estoup et al., 1993).

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