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Patterns of inheritance with RAPD molecular markers reveal novel types of polymorphism in the honey bee

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Summary. The polymerase chain reaction (PCR) was used to generate random amplified polymorphic DNA (RAPD) from honey bee DNA samples in order to follow the patterns of inheritance of RAPD markers in a haplodiploid insect. The genomic DNA samples from two parental bees, a haploid drone and a diploid queen, were screened for polymorphism with 68 different ten-nucleotide primers of random sequence. Parents were scored for the presence or absence of individual bands. An average of 6.3 bands and 1.3 polymorphisms for presence/absence were observed per primer between the parents. Thirteen of these primers were used to determine the inheritance of RAPD marker alleles in the resulting progeny and in haploid drones from a daughter queen. Four types of polymorphisms were observed. Polymorphisms for band presence/absence as well as for band brightness were inherited as dominant markers, meeting Mendelian expectations in haploid and diploid progeny. Polymorphisms for fragment-length were also observed. These segregated in a near 1:1 ratio in drone progeny. The last type of polymorphism was manifested as a diploid-specific band. Mixing of amplification products after PCR showed that the diploid-specific band was the result of heteroduplex formation from the DNA of alternate alleles in heterozygotes. In two of the four cases of heteroduplex formation, the alternative alleles were manifested as small fragment-length polymorphisms, resulting in co-dominant markers. This is the first demonstration that a proportion of RAPD markers are not inherited in a dominant fashion.

Key words: Random amplified polymorphic DNA (RAPD) – *Apis mellifera* – Genetic markers

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Introduction

Much has been learned about honey bee (*Apis mellifera* L.) biology and behavior, yet little is known of its genome organization. Linkage tests with visible mutants of honey bee have demonstrated linkage between only three pairs of loci (Tucker 1986). The difficulty in finding linkage lies in the deficiency of suitable genetic markers. In order to have markers to construct a linkage map, we are investigating the inheritance of random amplified polymorphic DNA (RAPD) in the honey bee.

Other methods for finding variation at the level of DNA sequences have already been useful for honey bee genetics. Restriction fragment length polymorphisms (RFLPs) have provided genetic markers for population studies that document introgression between Africanized honey bees and European races in South America (Hall 1990; Sheppard et al. 1991). However, the RFLP technique is limited because individual insects contain insufficient quantities of DNA for extensive analysis. RFLP analysis using specific oligonucleotide repeats as probes can generate a "DNA fingerprint" and can be used to assess the relatedness of individual bees in colonies (Blanchetot 1991; Moritz et al. 1991). However, the banding patterns produced by these probes are complex due to the existence of many loci with related sequences. Genetic analysis of the banding pattern is further complicated because some of the loci are hypervariable. The complex patterns can be simplified only by cloning individual loci for use as probes (Wong et al. 1987). An alternative approach for obtaining genetic markers involves the polymerase chain reaction (PCR). Nanogram quantities of genomic DNA used as a template for PCR with ten-nucleotide primers of random sequence can generate RAPD markers in higher plants, humans, fungi and bacteria (Williams et al. 1990). RAPD markers have been

used as dominant markers for genome mapping in plants (Williams et al. 1990, 1992) and for the identification of plant disease-resistance genes (Martin et al. 1991; Michelmore et al. 1991). In mapping experiments RAPD markers have been shown to sample repetitive and single-copy sequences dispersed throughout the genome (Williams et al. 1992).

The honey bee is especially amenable for analysis with RAPD markers because it is haplodiploid. Honey bee females are diploid, but the male (drone) is haploid, the result of parthenogenetic development of an unfertilized egg (arrhenotoky). Moreover, due to an abortive meiosis, the sperm of a drone are all genetically identical (except for mutations) and hence all of a drone's progeny share this genome. Thus analysis of haploids can provide: (1) complete information for heterozygous loci in the parental queen that is not confounded by dominance effects and (2) clarification of the banding patterns in diploid siblings. This is a report on the frequency of polymorphism and some novel patterns of inheritance observed for RAPD markers in the honey bee.

Materials and methods

A virgin honey bee queen (referred to as the parental queen) was crossed to a single haploid male (the parental drone). DNA aliquots from these parents, from haploid male (drone) and diploid female (worker) progeny, and from one virgin queen daughter and her drone progeny were used individually as templates to generate RAPD markers. The frequency of polymorphism between the parents was observed and the mode of inheritance in haploid and diploid progeny was determined.

Honey bee samples and DNA extraction

A single virgin queen was raised (see Laidlaw 1979) and then instrumentally inseminated with the semen of a single, presumably unrelated, drone (Laidlaw 1977). Twenty haploid drone and 12 diploid worker progeny were subsequently collected. In addition, a daughter queen was raised and 94 of her drone progeny were also collected. All bees were quickly frozen on dry ice and stored at −70 °C prior to DNA extraction. Total genomic DNA was isolated from individual bees by a modification of the method of Saghai-Maroof et al. (1984). Bees were ground with plastic pestles in microcentrifuge tubes that contained a CTAB extraction buffer (1% hexadecyltrimethyl ammonium-bromide, 0.75 M NaCl, 50 mM Tris, pH 8, 10 mM EDTA and 100 µg/ml of Proteinase K). Samples were incubated at 60 °C for 2 h and extracted with phenol/chloroform and then with chloroform. The DNA was precipitated with two volumes of ethanol and the samples were centrifuged at 4000 g and 4 °C. After resuspension in 10 mM Tris, 1 mM EDTA, the DNA was then quantified with a fluorometer (Hoeffer) and diluted to 5 ng/µl in 10 mM Tris, pH 7.6, 0.3 mM EDTA.

Polymerase chain reaction and electrophoresis

Target DNA sequences were amplified by PCR in a Perkin Elmer Cetus (480) thermal cycler. A single ten-nucleotide oligomer of random sequence (Operon Technologies Inc., Alameda Calif.) containing at least 50% G-C served as a primer for each reaction. Reaction conditions were as reported by Williams et al. (1990) except that only 5 ng of honey bee DNA was used per reaction. Reactions were incubated for 45 cycles consisting of 1 min at 94 °C, 1 min at 35 °C, a 2 min temperature transition to 72 °C and 2 min at 72 °C.

Amplification products were resolved on either 1.9% agarose gels (0.9% Ultra-pure agarose, BRL, and 1% NuSieve agarose, Hoefer) or on 0.6% Ultra-pure agarose with 1% Synergel (Diversified Biotech, Newton Centre, Mass.). Gels were run in 0.75 × TBE buffer at 4 V/cm for 5 h 30 min and stained with ethidium bromide. In some cases amplification products were mixed in order to determine whether the observed diploid-specific bands were due to the reannealing of two homologous DNA strands that also contained a non-homologous region (heteroduplex formation). Samples were amplified separately as usual, then EDTA (5 mM) was added to the amplification products in order to inhibit residual *Taq* polymerase activity. The amplified DNA from two individual bees was mixed, heated to 94 °C and allowed to reanneal at room temperature.

Results and discussion

Frequency of polymorphism

Many RAPD markers were polymorphic for band presence/absence between the two original parents. The 68 different random decamers that were used for PCR with honey bee DNA generated 432 scoreable bands for an average of 6.3 bands per primer. Ninety of these bands were polymorphic for band presence/absence between the two parents, an average of 1.3 polymorphic loci per primer. However, other types of polymorphisms which could not be detected in the parents were revealed in the inheritance study. Altogether, polymorphisms of four types were found: band presence/absence, band brightness polymorphism, fragment-length polymorphism, and heteroduplex-band polymorphism. These types of markers occurred 20, six, eight and four times, respectively, for an average of about three polymorphisms per primer.

Inheritance of RAPD markers

Thirteen primers were used to follow the inheritance of polymorphic markers. Analysis included DNA from the two original parents, along with five drones derived from the parental queen and five of her worker progeny. The inheritance of some of the markers was studied in more detail with a larger sample of 20 drones and 12 workers. In addition, a daughter queen and 94 of her drones were analyzed with nine of the primers. All polymorphic markers for which the daughter queen was heterozygous segregated in ratios that were not significantly different from 1:1 (for all 24 loci; $P \geq 0.05$, chi-squared test).

Four types of RAPD marker polymorphisms were observed in the progeny (Table 1). The first two types, band presence/absence polymorphism and a novel type of polymorphism, band brightness, segregated as dominant markers. A 1:1 segregation ratio of these polymorphisms is expected in the haploid drones of a heterozygous queen because drones are equivalent to

Table 1. The observed inheritance of four types of polymorphic RAPD markers in haploid and diploid honey bee progeny. DNA samples from individual haploid males and diploid females were used in polymerase chain reactions with single ten-nucleotide primers to generate RAPD markers

Primer no.[a]	Type of polymorphism[b]	Scored as inherited in	
		Haploids	Diploids
OA1	+/−	+	+
	B	+	+
	B	+	+
OA4	+/−	+	+
	+/−	+	−
	B	+	+
	L	+	−
	L	+	−
OA9	L	+	−
	H	−	+
OA20	+/−	+	+
	+/−	+	−
	+/−	+	+
OA13	+/−	+	+
OA16	L	+	−
OB1	+/−	+	+
	+/−	+	−
	H	−	+
OB7	+/−	+	+
	+/−	+	+
	B	+	+
	L[c]	+	−
	H	−	+
OB8	+/−	+	+
	B	+	+
OB9	+/−	+	+
	+/−	+	+
OB11	+/−	+	+
	+/−	+	+
	L	+	−
	L	+	−
OC15	+/−	+	+
	+/−	+	+
	+/−	+	+
OD16	+/−	+	−
	B	+	+
	L[c]	+	−
	H	−	+

[a] Ten-nucleotide primers of arbitrary sequence were obtained from Operon Technologies

[b] Four types of polymorphisms were resolved in agarose gels: presence/absence of a band, +/−; band-brightness polymorphism, B; fragment-length polymorphism, L, and the occurrence of a heteroduplex band in diploid progeny, H. Each symbol in this column represents a different polymorphism seen

[c] These allelic fragments participated in heteroduplex formation. Individuals that were heterozygous for this fragment-length polymorphism showed the heteroduplex band listed for this primer

gametes of the queen. Segregation in the parental queen's drones showed that the queen was heterozygous for a presence/absence polymorphism when tested with primer OA-1 (Fig. 1, band c). All of the female progeny inherited the marker from the drone father and showed a band in this position, indicating dominance. The same allele is transmitted to all diploid progeny from the drone father because, barring mutation, all of the sperm are expected to be genetically identical.

Five primers generated polymorphisms for band brightness. All of the female progeny inherited a brightness polymorphism (generated with primer OA-1) from the drone father that appeared as a bright band. The parental queen, however, was homozygous for the presence of a dim band, as seen in her drone progeny (Fig. 1, band b). This marker also segregated in the drones of the heterozygous daughter queen. Analysis of 94 of the daughter queen's drone progeny showed a 1:1 segregation for this marker. Another brightness polymorphism was anaomalous because it segregated in a near 1:1 ratio in the drones of the daughter queen, even though she showed a dim band (Fig. 1, band a). For all of the other brightness polymorphisms that were observed, presence of a bright band was dominant. Band brightness polymorphism may be due to amplification of a fragment within a tandem repeat at a locus that is polymorphic for copy number. Alternatively, it may be caused by a difference in sequence homology of the primer binding site, resulting in differential amplification. The sequence homology hypothesis is supported by the observation that one of the presence/absence polymorphisms appeared as a brightness polymorphism in a subsequent reaction (data not shown).

In addition to these two types of markers, fragment-length polymorphisms were observed in amplification products from drone progeny of both of the queens. The near 1:1 segregation ratio of fragment-lengths in drones of the daughter queen, and the occurrence of only one of the two band sizes in each haploid drone both indicated that the fragment-length markers were allelic, or at least tightly linked in trans (Fig. 2). These fragment-length polymorphisms and three of the presence/absence polymorphisms could be seen clearly in haploid drones but were either obscured or absent in diploid workers or queens. Consequently over 25% of the segregating RAPD markers (11 of 38) were only informative in the haploid drones.

The lack in diploids of some bands that occur in haploids may be due to competition for primer binding sites. Competition between primer binding sites is thought to be an important factor controlling which RAPD fragments are amplified enough to be visible. Many of the primer binding sites must have some degree of mismatch because genomes of widely varying complexity produced the same average number of bands in

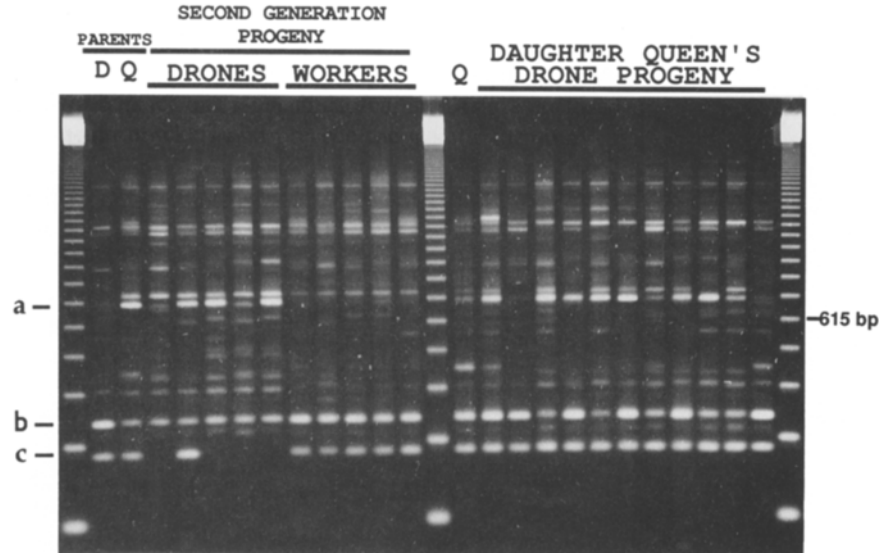

Fig. 1. RAPD markers from three generations of honey bees demonstrating the Mendelian inheritance of two types of polymorphisms. Each lane contained the PCR amplification products from a single bee's DNA with primer OA-1 resolved by electrophoresis in 1% Synergel and 0.5% agarose. The amplification products from the parental drone and queen (*D* and *Q*) are followed by the queen's progeny drones and their worker progeny. The daughter queen (*Q*) and her drone progeny follow the second marker lane. Segregation for the presence/absence of *band c* was seen in drones from the heterozygous parental queen. Two band-brightness polymorphisms were also visible (*bands b and a*). *Band b*, presence of a bright band was seen in all female progeny and the parental drone. Segregation for bright/dim is seen for *band b* in the drones of the daughter queen, showing dominant inheritance of bright. For the other brightness polymorphism, *band a*, the daughter queen had a dim band but her drone progeny segregated for dim/bright. This marker was unusual in that presence of a dim band was dominant

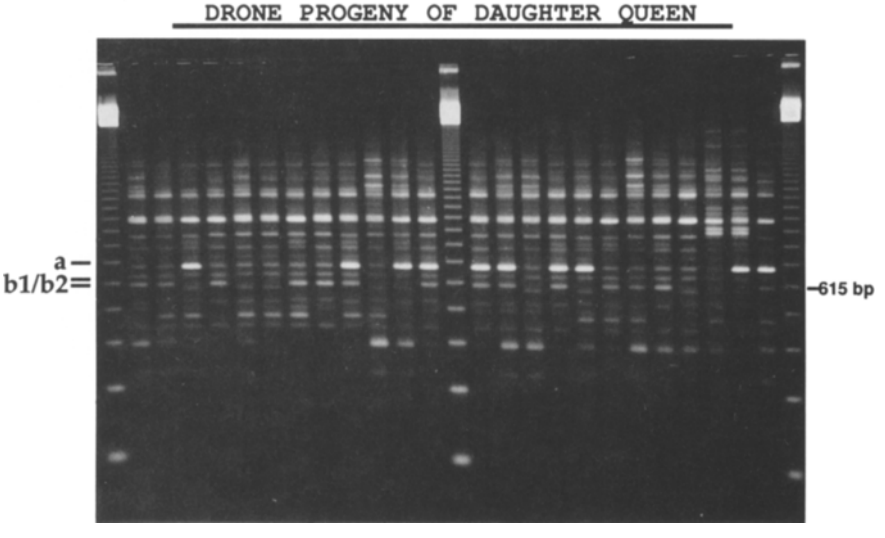

Fig. 2. Drones from the heterozygous daughter queen showed segregation for a fragment-length polymorphism. Each lane contained the PCR amplification products from a single drone's DNA with primer OA-4. Only one of the two fragments, *b1 or b2*, was present in each drone indicating allelism. In addition, the queen was heterozygous at a second locus for a brightness polymorphism (*band a*) which segregated 1:1 in the drones

RAPD reactions, even though the smaller genomes would be unlikely to have many priming sites with a perfect sequence match (Williams et al. 1992). The genome of a diploid female honey bee may contain more primer binding sites with sufficient sequence homology than the genome of a haploid male. The presence of a greater number of potential templates for amplification may explain the absence of some bands in diploids.

Heteroduplex bands

Four primers (OA-9, OB-1, OB-7 and OD-16) generated bands which were present in diploid workers and queens, but not in the haploid drones. One of these primers, OD-16, produced a band in the parental queen that was absent in her male progeny, but segregated in a 1:1 ratio in the worker progeny (Fig. 3, band a). The relative mo-

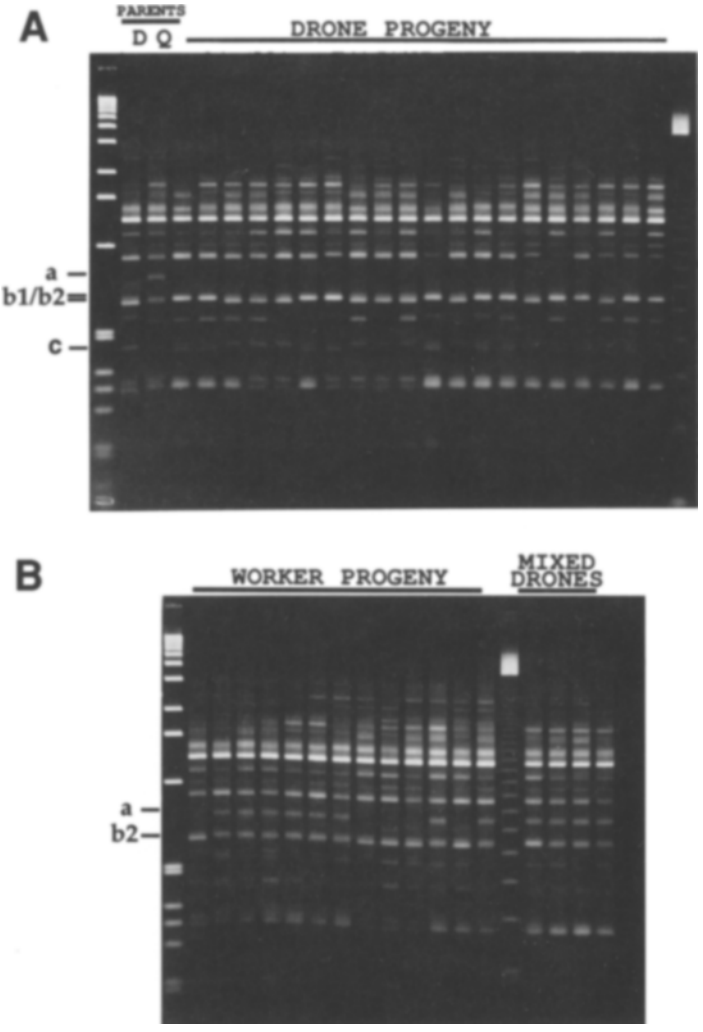

Fig. 3 A, B. Diploid-specific bands, generated by PCR with primer OD-16 and the DNA of single bees, were the result of heteroduplex formation. **A** *Band a* from the parental queen was not seen in any of her haploid drones. The drones segregated for a small fragment-length polymorphism (*bands b1 and b2*). The larger band, *b1*, co-segregated with *band c*. **B** *Band a* segregated in the queen's diploid worker progeny. The band could be regenerated by mixing amplified DNA from two drones which differed for the appearance of *bands b1 and b2*. The mixed amplification products were heated and allowed to reanneal before loading the gel (mixed drones). Thus *band a* represents a heteroduplex formed from alternative alleles from DNA of the heterozygote

bility of this diploid-specific band differed in the two types of gels used for electrophoresis. Thus we postulated that this was a heteroduplex band that did not migrate according to its true molecular weight. The heteroduplex band (band a) was formed when allele *b1* from the queen was present with allele *b2* from the parental drone in the worker progeny. When progeny were homozygous for allele *b2*, no heteroduplex band was formed. Bands b1 and b2 were slightly different in size and segregated in the progeny drones. The higher molecular weight band b1, co-segregated with a small band c, suggesting that a primer-recognition site was inside a larger template sequence. Mixing PCR products from the two types of drones, those with *b1* and those with *b2*, in the absence of polymerase activity regenerated the diploid-specific band seen in the parental queen and her worker progeny (Fig. 3). Thus the queen was heterozygous for two alleles generating fragments of similar mobility that can form a heteroduplex. One of these alleles was shared with the parental drone.

The parental queen did not show a heteroduplex band with primers OA-9, OB-1 or OB-7 but a band that was not present in either parent was seen in all of the female progeny. In these cases, the heteroduplex band could be regenerated by mixing the amplified DNA from the parental drone with amplified DNA from any of the parental queen's drones (or from the queen herself) but not by mixing the amplification products of the queen's drones with each other (data not shown). Thus the parental drone and queen had different alleles at this locus. One of these three heteroduplex bands was also associated with a fragment-length polymorphism, showing co-dominant inheritance, as found with primer OD-16. With the remaining two primers, the homoduplexes could not be identified and the alternative alleles could be distinguished only by mixing experiments.

The allelic nature of the heteroduplex-forming sequences was shown by analysis of DNA from 94 of the daughter queen's drones. Three of the four primers generated a heteroduplex band in the daughter queen but

not in any of her drones, indicating that these sequences were allelic (or tightly linked in trans) and the queen was heterozygous. If these sequences were not linked, then some drones would show heteroduplex bands. The generation of heteroduplexes from the same locus in diploid individuals suggests that small insertion/deletion or inversion events are common in these regions of the genome. These regions may represent repetitive sequences.

Conclusions

Segregations of RAPD markers fit the Mendelian expectations of a haplodiploid species for the four types of polymorphisms observed. Two of these types, band brightness polymorphism and heteroduplex-band polymorphism, had not been reported previously, whereas another type, fragment-length polymorphism, was observed only in haploid individuals in this study. The majority of RAPD markers were inherited in a dominant fashion as previously reported, such that heterozygotes could not be detected without segregation analysis. However, heterozygotes could be distinguished when heteroduplex bands were generated and approximately ten percent of the markers were heteroduplex polymorphisms. In addition, the availability of haploid drones in the honey bee reveals all heterozygous loci in the queen and will provide complete information for genome mapping.

RAPD markers will be useful for studies of honey bee genetics. Estimates of the amount of polymorphism demonstrated no lack of markers in honey bee genomes. The small amount of DNA required and the relative simplicity of RAPD markers make them ideal for studies involving insect population biology, genome mapping, and behavioral genetics.

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References

Blanchetot A (1991) Genetic relatedness in honeybees as established by DNA fingerprinting. J Hered 82:391–396

Hall, HG (1990) Parental analysis of introgressive hybridization between African and European honeybees using nuclear DNA RFLPs. Genetics 125:611–621

Laidlaw HH (1977) Instrumental insemination of honey bee queens. Dadant and Sons, Hamilton, Illinois

Laidlaw HH (1979) Contemporary queen rearing. Dadant and Sons, Hamilton, Illinois

Martin GB, Williams JGK, Tanksley SD (1991) Rapid identification of markers linked to a *Pseudomonas* resistance gene in tomato by using random primers and near-isogenic lines. Proc Natl Acad Sci USA 88:2336–2340

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88:1212–1216

Moritz RFA, Meusel MS, Haberl M (1991) Oligonucleotide DNA fingerprinting discriminates super- and half-sisters in honeybee colonies. Naturwissenschaften 78:422–424

Saghai-Maroof MA, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc Natl Acad Sci USA 81:8014–8018

Sheppard WS, Rinderer TE, Mazzoli JA, Stelzer JA, Shimanuki H (1991) Gene flow between African- and European-derived honey bee populations in Argentina. Nature 349:782–784

Tucker KW (1986) Visible mutants. In: Rinderer TE (ed) Bee genetics and breeding. Academic Press, New York London, pp 57–90

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18(22):6531–6535

Williams JGK, Rafalski JA, Tingey SV (1992) Genetic analysis using RAPD markers. Methods Enzymol (in press)

Wong Z, Wilson V, Patel I, Povey S, Jeffreys AJ (1987) Characterization of a panel of highly variable minisatellites cloned from human DNA. Ann Hum Genet 51:269–288