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MRJP microsatellite markers in Africanized *Apis mellifera* colonies selected on the basis of royal jelly production

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ABSTRACT. It is important to select the best honeybees that produce royal jelly to identify important molecular markers, such as major royal jelly proteins (MRJPs), and hence contribute to the development of new breeding strategies to improve the production of this substance. Therefore, this study focused on evaluating the genetic variability of *mrjp3*, *mrjp5*, and *mrjp8* and associated allele maintenance during the process of selective reproduction in Africanized *Apis mellifera* individuals, which were chosen based on royal jelly production. The three loci analyzed were polymorphic, and produced a total of 16 alleles, with 4 new alleles, which were identified at *mrjp5*. The effective number of alleles at *mrjp3* was 3.81. The observed average heterozygosity was 0.4905, indicating a high degree of genetic variability at these loci. The elevated F_{IS} values for *mrjp3*, *mrjp5*, and *mrjp8* (0.4188, 0.1077, and 0.2847, respectively) indicate an excess of homozygotes. The selection of Africanized *A. mellifera* queens for royal jelly production has maintained the *mrjp3 C*, *D*, and *E* alleles; although, the *C* allele occurred at a low frequency. The heterozygosity and F_{IS} values show that the genetic variability of the queens is decreasing at the analyzed loci, generating an

excess of homozygotes. However, the large numbers of drones that fertilize the queens make it difficult to develop homozygotes at *mrjp3*. Mating through instrumental insemination using the drones of known genotypes is required to increase the efficiency of Africanized *A. mellifera*-breeding programs, and to improve the quality and efficiency of commercial royal jelly apiaries.

Key words: Major royal jelly proteins; *mrjp3*; Genetic improvement; Polymorphism; Honeybee queen selection

INTRODUCTION

Royal jelly is a creamy secretion that is synthesized by the mandibular and hypopharyngeal glands of honeybees, located on the head of nurse worker honeybees and secreted between the 5th and 15th days of worker lifespan (Haydak, 1970; Knecht and Kaatz, 1990; Schmitzová et al., 1998). In general, royal jelly has a milky texture, is highly acidic and nitrogenated, and has a characteristic odor and taste (Chen and Chen, 1995; Melliou and Chinou, 2005; Liu et al., 2008). It has a density of approximately 1.1 g/cm^3 (Lercker et al., 1992) and is partially soluble in water. The viscosity of royal jelly varies with the age of honeybees and water content. Royal jelly is widely known for its complex composition, including various minerals, proteins, amino acids, steroids, phenols, carbohydrates, vitamins, lipids, and other, as yet, unidentified substances (Garcia-Amoedo and Almeida-Muradian, 2007).

Royal jelly is a natural source of essential amino acids, lipids, vitamins, acetylcholine, and other nutrients to the honeybees (Schmitzová et al., 1998), and plays an important role in their reproduction and development. Every female larva of less than 3 days of age may develop into a worker or queen honeybee, depending on the food provided by the nurse honeybees. After 3 days, only the larvae selected by the worker honeybees to become queens receive royal jelly exclusively as a source of food (Winston, 1992; Drapeau et al., 2006).

Among the many products obtained from honeybees, most studies have focused on royal jelly, because of its role as a food source for honeybees and its therapeutic properties. In general, the therapeutic effects of royal jelly have been associated with antibiotic, antifungal, and anti-inflammatory activity, as well as its ability to enhance immune resistance, tissue regeneration, the reduction of plasma cholesterol and triglyceride levels, and antihypertensive activity (Yatsunami and Echigo, 1985, Oka et al., 2001, Matsui et al., 2002; Koshio and Almeida-Muradian, 2003; Münsted and Georgi, 2003, Kohno et al., 2004).

Royal jelly contains several proteins that have molecular weights ranging between 47 and 80 kDa (Hanes and Simúth, 1992). These proteins belong to the group of major royal jelly proteins (MRJPs), which together account for 82-90% of the total protein content of the jelly. The MRJPs contain relatively large amounts of amino acids, supporting the hypothesis that MRJPs play important roles in the nutrition of honeybees (Schmitzová et al., 1998). A large number of studies performed to identify, characterize, and sequence the MRJPs of *Apis mellifera* (Klaudiny et al., 1994; Albert et al., 1996; 1999a,b; Schmitzová et al., 1998; Albert and Klaudiny, 2004; Drapeau et al., 2006). Nine proteins from the MRJP family have been identified, of which 5 (MRJP1, MRJP2, MRJP3, MRJP4, and MRJP5) represent about 82% of the total protein content of royal jelly. The genes encoding these proteins are located on chromosome 11 (Drapeau et al., 2006). Sequencing studies have revealed that *mrjp3* and *mrjp5* contain regions of hypervariable numbers of tandem repeats (VNTRs) of different sizes, sequences, and locations that create high genetic variability and,

therefore, may be used for genotyping individuals (Schmitzová et al., 1998; Albert et al., 1999b).

Using microsatellite analysis, Albert et al. (1999b) detected high variability at the *mrjp3* locus, because of the presence of VNTR regions located in the *mrjp3* 3rd region. This high genetic variability revealed the value of this gene as a marker for a variety of studies on *A. mellifera* (Beye et al., 1998).

Although the *A. mellifera* MRJP family has been extensively studied, knowledge remains limited about the possible uses of these molecular markers in population genetic studies or as selective markers associated with royal jelly production. Thus, this study evaluated the genetic variability of the *mrjp3*, *mrjp5*, and *mrjp8* loci in Africanized *A. mellifera* producers of royal jelly to identify the molecular markers that are associated with its production. The obtained results are anticipated to provide baseline information on which to develop new strategies to increase the production of royal jelly in commercial apiaries.

MATERIAL AND METHODS

Collection of biological samples

The Central Apiary of the Iguatemi Experimental Farm, State University of Maringa, Brazil, provided the Africanized *A. mellifera* nurse worker honeybees used in this study. In 2006, the 15 queens that produced the highest amounts of royal jelly were selected. From these selected matrices, daughter queens were produced and continue to be maintained in the Central Apiary. The nurse honeybees were collected from seven colonies matrices, placed in Langstroth-type hives, and destined for royal jelly production. The queens from these boxes were naturally mated and evaluated for the production of royal jelly. All queens showed high royal jelly production. Twenty nurse honeybees were collected from each colony, which were selected by their color and age, and extracted from the combs. These honeybees were immediately sacrificed, stored in closed flasks, properly identified, and frozen at -20°C for laboratory analyses.

Extraction and quantification of genomic DNA

Genomic DNA was extracted from 20 nurse workers from each colony analyzed, totaling 140 individuals. The total DNA extraction method described by Bardakci and Skibinski (1994) was adapted for *A. mellifera*. The thoraxes were macerated in 400 µL lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0, 100 mM NaCl, 1% SDS) and 10 µL proteinase K (5 µg/µL). The samples were maintained at 55°C for 4 h. DNA was purified with a solution of phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). From the recovered volume, DNA was precipitated using 3 M sodium acetate and cold absolute ethanol at a ratio of 0.25:2.5 and incubated overnight at -20°C. The precipitated DNA was separated by centrifugation at 10,300 *g* for 10 min, resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and treated with 3 µL RNase (10 µg/mL).

DNA integrity and concentration were verified on 0.8% agarose gels with 1X TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0). The amount of DNA in each sample was estimated by comparison with known and graded concentrations of standard DNA (λ phage). The agarose gels were stained with 0.5 µg/mL ethidium bromide solution. The DNA bands were then visualized with UV light, and the images were captured with an L-PIX HE (Loccus Biotecnologia, Brazil), molecular imaging system.

Polymerase chain reaction (PCR) amplification

PCR was performed using synthesized specific primers to amplify the repetitive regions of *mrjp3* (Albert et al., 1999b) and *mrjp5* (Albert et al., 1999a), and to amplify the *mrjp8* locus (Klaudiny et al., 1994): *mrjp3* (*forward:* ATG TAA TTT TGA AGA ATG AAC TTG; reverse: TGT AGA TGA CTT AAT GAG AAA CAC); *mrjp5* (forward: AGA CTC TTC AAA CGG TCG TTG C; reverse: CTG TAA TTT CAT ACT TAA AGC CAT); and *mrjp8* (forward: TTG CGA AGT GAA TGG ATC; reverse: TTA TTT TTG GCA ACC ACT TCG).

In a reaction volume of 20 µL, we used 1X Tris-KCl buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 0.5 µM of each primer, 0.1 mM of each dNTP, and 1 U Platinum *Taq* DNA Polymerase (Invitrogen). Optimizing the PCRs, we adopted specific concentrations of $MgCl_2$ and DNA template according to the primer that was used (Table 1). Thus, for amplification with the *mrjp3*, *mrjp5*, and *mrjp8* primers, the $MgCl_2$ concentrations adopted were 1.7, 1.5, and 3.0 M/L, respectively. The concentration of the DNA template for the *mrjp3* primer was 10 ng/µL, and was 20 ng/µL for the *mrjp5* and *mrjp8* primers.

Table 1. PCR conditions for the amplification of MRJP loci from Africanized *Apis mellifera* honeybees.

Parameter	*mrjp3*		*mrjp5*		*mrjp8*	
$MgCl_2$ (M/L)	1.7		1.5		3.0	
DNA (ng/µL)	10.0		20.0		20.0	
PCR conditions	T (°C)	Time	T (°C)	Time	T (°C)	Time
Denaturation	94	30 s	94	30 s	94	50 s
Annealing	54	30 s	54	30 s	50	50 s
Extension	72	1 min	72	1 min	72	100 s

PCRs were performed in a Techne TC-512 thermocycler (Keison, UK), and the conditions for the amplification with the *mrjp3* and *mrjp5* primers were based on Albert and Schmitz (2002). The process started with an initial denaturation step of 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 54°C, and 1 min at 72°C. The reaction was completed with a final extension step of 10 min at 72°C. For DNA amplification using the *mrjp8* primer, the PCR conditions were based on Albert and Klaudiny (2004). In brief, an initial denaturation step of 5 min at 94°C was used, followed by 35 cycles of 50 s at 94°C, 50 s at 50°C, and 100 s at 72°C. The reaction was completed with a final extension step of 10 min at 72°C.

The amplification products were separated on 2% agarose gels prepared with 0.5X TBE buffer (44.5 mM Tris, 44.5 mM borate, and 1 mM EDTA). Preparing the agarose gel, we used 50% common agarose and 50% MetaPhor agarose (Cambrex). The separation was performed at 60 V for 5 h. The gels were stained and visualized as described in the section for the extraction and quantification of genomic DNA, using a100-bp DNA ladder (Invitrogen) to determine the sizes of the amplified alleles.

Data analysis

The molecular data analyses involved the interpretation of the amplified fragments of genomic DNA. F statistics (F_{IS}, F_{IT}, and F_{ST}), Nei (1973) H statistic, and the effective number of alleles were estimated using the POPGENE 1.31 software (Yeh et al., 1999).

RESULTS

The *mrjp3*, *mrjp5*, and *mrjp8* loci encoded 16 polymorphic alleles (Table 2). The effective number of alleles was 3.81 for *mrjp3*, 3.13 for *mrjp5*, and 2.56 for *mrjp8*, with an average value of 3.17 alleles. Figure 1 presents the amplified fragments of the 3 loci analyzed and their respective alleles.

Table 2. MRJP loci from Africanized *Apis mellifera* producers of royal jelly, showing the size of each allele in bp and the allelic frequencies (F) obtained from the seven colonies analyzed.

Allele	mrjp3		*mrjp5*		*mrjp8*	
	bp	F	bp	F	bp	F
A	410*	-	570*	-	360*	0.0143
B	460*	-	590*	-	370*	0.1786
C	480*	0.0321	610*	-	390*	0.5321
D	510*	0.3357	620*	0.2643	420*	0.2750
E	530*	0.3107	680*	0.4393	-	-
F	580*	0.1786	720*	0.2357	-	-
G	610*	0.1429	-	-	-	-
H	-	-	650	0.0036		-
I	-	-	750	0.0250	-	-
J	-	-	780	0.0143	-	-
K	-	-	800	0.0179	-	-

*Described by Baitala et al. (2010).

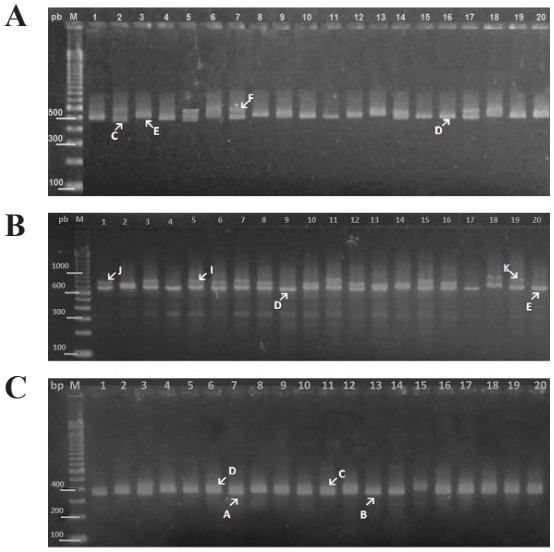

Figure 1. Electrophoresis profile of MRJP loci of the Africanized *Apis mellifera* honeybees. **A.** Amplified fragments of the *mrjp3* locus. **B.** Amplified fragments of the *mrjp5* locus. **C.** Amplified fragments of the *mrjp8* locus. M = DNA molecular weight marker (DNA Ladder, Invitrogen). The arrows and letters indicate the alleles identified for each locus.

The values obtained by the χ^2 test are presented in Table 3. All seven of the colonies analyzed showed deviations from the Hardy-Weinberg equilibrium (HWE) at the *mrjp3* locus. Five of the colonies showed deviations at the *mrjp5* locus, while three of the colonies showed deviations at the *mrjp8* locus.

Table 3. Chi-squared (χ^2) test to verify the Hardy-Weinberg equilibrium of the MRJP loci in seven colonies of Africanized *Apis mellifera*.

Colony	*mrjp3*	*mrjp5*	*mrjp8*
1	χ^2 = 22.41 d.f. = 1 P = 0.000002	χ^2 = 42.88 d.f. = 10 P = 0.000005	χ^2 = 1.91 d.f. = 3 P = 0.590386
2	χ^2 = 25.92 d.f. = 6 P = 0.000230	χ^2 = 5.30 d.f. = 1 P = 0.021285	χ^2 = 8.28 d.f. = 3 P = 0.040488
3	χ^2 =15.30 d.f. = 6 P = 0.01801	χ^2 = 3.73 d.f. = 3 P = 0.291503	χ^2 = 2.95 d.f. = 6 P = 0.814983
4	χ^2 = 12.31 d.f. = 3 P = 0.006387	χ^2 = 18.99 d.f. = 10 P = 0.040307	χ^2 = 2.32 d.f. = 3 P = 0.507901
5	χ^2 = 13.78 d.f. = 3 P = 0.003207	χ^2 = 3.10 d.f. = 6 P = 0.795217	χ^2 = 0.43 d.f. = 1 P = 0.509893
6	χ^2 = 12.00 d.f. = 3 P = 0.007364	χ^2 = 1.85 d.f. = 10 P = 0.997351	χ^2 = 3.27 d.f. = 1 P = 0.070525
7	χ^2 = 5.20 d.f. = 3 P = 0.157724	χ^2 = 11.65 d.f. = 3 P = 0.0088686	χ^2 = 3.52 d.f. = 1 P = 0.060373

d.f. = degrees of freedom; P = probability value. P > 0.05 is in Hardy-Weinberg equilibrium. P < 0.05 is not in Hardy-Weinberg equilibrium.

The average heterozygosity value observed for the three loci analyzed was 0.4905, indicating a high degree of genetic diversity for the samples analyzed (Table 4). The F_{IS} values presented in Table 5 indicate that the colonies analyzed had an excess of homozygotes.

Table 4. Observed (H_O) and expected heterozygosity (H_E) values of MRJP loci from the analyzed colonies of Africanized *Apis mellifera*.

Locus	H_O	H_E
mrjp3	0.4286	0.7285
mrjp5	0.6071	0.6848
mrjp8	0.4357	0.6113
Mean	0.4905	0.6749
Standard deviation	0.1011	0.0593

Table 5. Fixation indices (F_{IS}) for the MRJP loci of the Africanized *Apis mellifera* royal jelly producers.

Allele/locus	*mrjp3*	*mrjp5*	*mrjp8*
A	-	-	-0.0145
B	-	-	0.3183
C	0.4260	-	0.1250
D	0.3274	0.1918	0.4805
E	0.6165	0.1155	-
F	0.5617	0.0484	-
G	0.0667	-	-
H	-	-0.0036	-
I	-	-0.0256	-
J	-	-0.0145	-
K	-	-0.0182	-
Total	0.4188	0.1077	0.2847

Table 6 shows the genotypes of the queens and the probable drones that produced the worker honeybees for this study. The drones made a significant contribution to the genetic variability in the analyzed MRJP loci, as the queens were fertilized by a minimum of two and a maximum of five drones.

Table 6. Genotypes of seven Africanized *Apis mellifera* queens and of the probable drones that participated in the fertilization.

	mrjp3		*mrjp5*		*mrjp8*	
Colony	Queen	Drone	Queen	Drone	Queen	Drone
1	*DE*	*D;E*	*DE*	*D;E;K;I;J*	*CD*	*A;C;D*
2	*DE*	*E;F;G*	*EF*	*E;F*	*BC*	*B;C;D*
3	*DE*	*D;E;F;G*	*DE*	*D;E;F*	*BC*	*A;B;C;D*
4	*CE*	*C;E;G*	*DE*	*D;E;F;I;J*	*BC*	*B;C*
5	*EF*	*D;E;F*	*DE*	*D;E;F;I*	*BC*	*B;C*
6	*DE*	*C;D;E;F*	*DE*	*D;E;F;H;J*	*CD*	*C;D*
7	*FG*	*E;F;G*	*EF*	*D;E;F*	*CD*	*C;D*

DISCUSSION

Queen selection for royal jelly production has been kept in apiary at Universidade Estadual de Maringá, Brazil, since 2003 hence Baitala et al. (2010) developed an initial study for selection of Africanized *A. mellifera* queens' royal jelly producers. After the period of 3 years, high degree of genetic variability was detected for MRJP loci analyzed, but it has decreased with the selection time. Moreover, alleles *mrjp3 C*, *D* and *E*, initially proposed as important for the production of royal jelly, continue to occur in hive matrices.

Initially in 2010 were identified seven alleles of *mrjp3*, with sizes of 410 bp (*A*), 460 bp (*B*), 480 bp (*C*), 510 bp (*D*), 530 bp (*E*), 580 bp (*F*), and 610 bp (*G*). The *mrjp5* locus had 6 alleles, with sizes of 570 bp (*A*), 590 bp (*B*), 610 bp (*C*), 620 bp (*D*), 680 bp (*E*), and 720 bp (*F*). In the present study, we detected different allelic compositions of *mrjp3* and *mrjp5* compared to those observed by Baitala et al. (2010). For instance, the *A* and *B* alleles of *mrjp3* that were previously detected by Baitala et al. (2010) were not found in the queens that were analyzed in the current study (Table 2). At the *mrjp5* locus, alleles *D*, *E*, and *F* were detected in this study, together with four new alleles, called *H* (650 bp), *I* (750 bp), *J* (780 bp), and *K* (800 bp). Therefore, it will be necessary to sequence these alleles and compare the results with sequences to determine whether they have been previously described, or whether they are new alleles. The current study found the same alleles in *mrjp8* as those described by Baitala et al. (2010) (Table 2).

The *D* and *E* alleles at the *mrjp3* locus had the highest frequencies (0.3357 and 0.3107, respectively) (Table 2). This observation indicates that these alleles are being maintained in the queen selection for the production of royal jelly. Only the *C* allele, which also has the potential as a marker for royal jelly production, was rare (0.0321). The effective number of alleles for *mrjp3* (3.81) shows that the alleles that are being selected for at this locus (*C*, *D*, and *E*) are probably those that make the greatest contributions to royal jelly production.

HWE was tested, as shown in Table 3. The results indicate that the *mrjp3* locus is under selection. HWE was not observed at *mrjp3* in any of the seven colonies analyzed. The *mrjp5* and *mrjp8* loci are probably not under selection, as HWE was observed in colonies 3, 5, and 6 at *mrjp5* and in colonies 2, 6, and 7 at *mrjp8* (Table 3). Based on these results, *mrjp5* and *mrjp8* are not

under strong selection, whereas *mrjp3* is. Future studies should continue to examine the *mrjp3* and *mrjp5* genotypes in relation to royal jelly production. The development of studies at the genetic or proteomic level is also required to identify how these proteins contribute to royal jelly production.

The estimated heterozygosity values indicate that there is a high degree of polymorphism at the three loci analyzed (Table 4), whereas the F_{IS} values show that the three loci have an excess of homozygotes (Table 5). Only the *H*, *I*, *J*, and *K* alleles of *mrjp5* and the *A* allele of the *mrjp8* locus showed no deficiency of heterozygotes. Baitala et al. (2010) obtained values of average heterozygosity of 0.5827 to *mrjp3*, *mrjp5* and *mrjp8*, higher than the value obtained in the present study that was 0.4905. In the present study, the average heterozygosity was 0.4905. Therefore, selection for MRJP alleles and for royal jelly production seems to be leading to homozygosity at these loci, particularly at *mrjp3*, which had the lowest heterozygosity value, 0.4286 (Table 4).

De la Rúa et al. (2001) also evaluated the heterozygosity values of honeybees under selection for the production of royal jelly. In this previous study, the average heterozygosity observed for eight microsatellite loci of *A. mellifera* from the Canary Islands ranged from 0.312 to 0.432. The relatively higher heterozygosity values observed in the current study might be explained by the number of drones that mated with the selected queens (Table 6). During natural mating, *A. mellifera* females copulate with up to 17 drones, enhancing the genetic variability. In the seven queens analyzed here, genes from two to five drones per queen were identified (Table 6). Even under natural mating conditions, the genetic characteristics of the queens are shifting towards homozygosity, as indicated by the elevated values of F_{IS}. *mrjp3* had the highest F_{IS} values, demonstrating that the selective process is maintaining the alleles of interest for royal jelly production.

Reproductive selection alters the genetic characteristics of a given population, which is influenced by the process of gene transfer from generation to generation (Falconer, 1987). Moreover, it is beneficial for a population to maintain a certain degree of genetic variability, because polymorphisms within a population result in a greater ability to respond to selective pressures, especially in animal breeding programs for consumption purposes (Page and Kerr, 1991). In recent years, it has become necessary to use instrumental insemination, each female is fertilized with the semen from a single drone. This method is important because natural mating promotes increased genetic variability, introducing new alleles that are not of interest for directed genetic improvement. The high heterozygosity values detected in the current study are indicative of this artificial interference, and correspond to the alleles introduced by the drones (Table 6).

The maintenance of Africanized *A. mellifera* matrices, which are the best producers of royal jelly, will facilitate the establishment of a genetic improvement program, which would enable queens to be supplied to commercial apiaries and would improve the value of this commercial product. The selection of Africanized *A. mellifera* queens has maintained the *C*, *D*, and *E* alleles at the *mrjp3* locus, with the *D* and *E* alleles showing the highest frequencies in the queens analyzed in this study. In conclusion, instrumental insemination with a single drone is necessary to obtain homozygous queens for the *mrjp3* alleles of interest, and to establish a breeding program of Africanized *A. mellifera* that maximizes royal jelly production.

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