JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2005,** *53,* 8075−8081 **8075**

Organization and Potential Function of the *mrjp3* Locus in Four Honeybee Species

VIERA ALBERTOVÁ,[†] SONGKUN SU,[§] AXEL BROCKMANN,[#] JÜRGEN GADAU,[#] AND ŠTEFAN ALBERT*,[†]

Institut für Medizinische Strahlenkunde und Zellforschung (MSZ), Universität Würzburg, Versbacher Strasse 5, 97078 Würzburg, Germany; College of Animal Sciences, Zhejiang University, 268 Kaixuan Road, Hangzhou 310029, People's Republic of China; and Institut für Verhaltensphysiologie und Soziobiologie, Universität Würzburg-Biozentrum, Am Hubland, 97074 Würzburg, Germany

Royal jelly is a nutritious secretion produced by nurse honeybees to provision queens and growing larvae. Major proteins of royal jelly are mutually similar, and they all belong to the MRJP/yellow protein family (pfam03022). The *mrjp3* loci in four traditional honeybee species (*Apis mellifera*, *Apis cerana*, *Apis dorsata*, and *Apis florea*) were sequenced and found to share high sequence and structural similarities. PCR analyses confirmed the presence of an extensive repetitive region, which showed size and sequence polymorphisms in all species. The evolutionary history of *mrjp* genes and their repetitive regions was reconstructed from their nucleotide sequences. The analyses proved that the repeat region appeared early in the evolution of the *mrjp* gene family and that the extreme elongation of the repeat is *mrjp3* specific. In the MRJPs was documented a correlation between nitrogen content and repeat length. Therefore, it is argued that the repeat occurred due to a selection for an increase in nitrogen storage for a more efficient nutrition of queens and larvae.

KEYWORDS: Royal jelly; honeybee; nutrition; nitrogen storage; protein; MRJP; evolution; genomic sequence; computer program

INTRODUCTION

Royal jelly (RJ) is a milky secretion that nurse honeybees provide as larval food, and it plays a central role in the reproductive caste determination. Queens are produced by feeding chosen larvae a surplus of royal jelly throughout their larval development. RJ is a complex mixture of water, fats, sugars, low molecular mass compounds, and proteins produced by nurse bees. Proteins make up ∼50% of the RJ dry weight, and ∼90% of the total protein content are major royal jelly proteins (MRJPs) (*1*, *2*). These MRJPs are closely related to each other and share sequence homologies with *yellow* proteins of *Drosophila* (*3*). MRJPs, *yellow* proteins of *Drosophila* and other insects, together with several bacterial proteins constitute a protein family termed MRJP (pfam03022). Hereafter, we will refer to the MRJP subfamily or MRJP/yellow protein family if we mean the closely related MRJP proteins only or the whole protein family, respectively. The majority of spots of two-dimensional fractionated royal jelly (*4*, *5*) or hypopharyngeal glands extract (*6*) were identified as MRJP proteins.

In the *Drosophila* genome 14 genes (termed *yellow-b* to *yellow-k*) were found, which encode *yellow*-like proteins (*7*, *8*). The original *yellow* protein has a function in cuticle melanization and male courtship behavior (*9*, *10*). *yellow-f* and *yellow-f2* were identified as dopachrome conversion enzymes (*11*); *yellow-g* and *yellow-g2* were shown to be essential for follicle function in eggshell development (*12*).

Among all MRJP proteins, MRJP3 holds a distinct position due to its antiallergic and anti-inflammatory activities (*13*, *14*). It is also the only major protein of RJ showing size polymorphism among individual honeybees (*4*, *15*). This size polymorphism is caused by a variable number of recurring 15-mer units found within the coding part of different *mrjp3* alleles (*15*). Due to the relatively large basic repetitive unit, PCR-amplified polymorphic alleles can easily be distinguished by agarose gel electrophoresis. *mrjp3* can thus also be used as a marker to genotype individual honeybees.

Very little is known about RJ proteins in other honeybee species. Only recently have three MRJP proteins and one MRJP1 cDNA of *Apis cerana* been partially characterized and shown to share high homologies with MRJPs of *Apis mellifera* (*16*). We set out to characterize the *mrjp3* loci of four *Apis* species, to determine the similarities/differences among honeybee species and the evolutionary implications of these differences in the MRJP proteins and their repeats. For that purpose we sequenced the *mrjp3* loci of four honeybee species (*A. cerana*, *A. dorsata*, *A. florea*, and *A. mellifera*) using specifically developed primers. We found that in all four species the *mrjp3* repetitive region is

* Corresponding author (telephone +49 931 201 45838; fax +49 931 201 45887; e-mail Stefan.Albert@mail.uni-wuerzburg.de).
[†] Institut für Medizinische Strahlenkunde und Zellforschung (MSZ).
[§] Zhejiang University.
[#] Universität Würzburg-Biozentrum.

10.1021/jf051417x CCC: $30.25
Published on Web 09/07/2005

polymorphic but the number of alleles and allele size differences varied considerably between species. We discuss the role of the repetitive region in nutrition and implications of our findings for the evolution of the MRJPs.

MATERIALS AND METHODS

Biological Samples. *A. dorsata*, *A. cerana*, and *A. florea* workers were collected from multiple feral colonies at the campus of the Indian Institute of Science, Bangalore, and at the campus of the University of Agricultural Sciences, GKVK, Bangalore. For studies of the *mrjp3* polymorphism in *A. florea*, additional workers were collected from six different locations of Guangxii province, in southern China. European honeybees, *Apis mellifera carnica*, were collected in Germany from the apiary of the University of Würzburg and from a private apiary in Göttingen. Individual honeybees were first anesthetized on ice, then stored and transported in 100% ethanol.

Experimental Procedures. Honeybee genomic DNA was isolated according to a standard phenol/chloroform extraction (*17*) or the QIAmp DNA extraction kit (QIAGEN).

Long-distance Polymerase Chain Reactions (PCR) were done in 50 μL volumes as follows: after an initial 2 min denaturation step and the subsequent addition of a DNA polymerase mix (Taq:DeepVent = 5:1; AmpliTaq, Perkin-Elmer and New England Biolabs), 35 cycles of 30 s at 94 °C, 30 s at 52 and 72 °C (2 min/1000 bp) were run. The reactions were completed by a final elongation step at 72 °C for 10 minutes. PCR products were separated by agarose gel electrophoresis using appropriate agarose concentrations (0.8−2%) and directly sequenced or cloned into the multiple cloning site of the PCR 2.1 TA vector (Invitrogen).

PCR amplifications of the repetitive region with Taq polymerase (Perkin-Elmer) were performed as described previously (*15*).

DNA sequencing was performed by the cycle sequencing method using the Prism Ready Reaction Dyedeoxy Terminator kit (Perkin-Elmer) on an ABI 373A sequencing device.

Sequences were assembled and analyzed using the Wisconsin GCG program package (*18*). Further analyses were done using the honeybee genome server (http://www.hgsc.bcm.tmc.edu/projects/honeybee/).

Assembled sequences were deposited in GenBank under following accession numbers: *am-mrjp3*, AY663104; *ac-mrjp3*, AY663105; *ad-mrjp3*, AY663106; *af-mrjp3*, AY663107.

Phylogenetic Analyses. DNA sequences were unambiguously aligned using ClustalX and translated into protein sequences using MEGA v 2.1 (*19*). Only the coding sequences were used for phylogenetic analysis in PAUP*4.0b10 (*20*). Additionally, all repeat sequences were excluded, and gaps were treated as missing characters. We performed phylogenetic analyses following the principles of maximum parsimony, maximum likelihood, and distance analysis. Bootstrap analyses were done with 1000 replications under the parsimony and distance settings.

Nitrogen Content Calculations with the Program NITCONT. The computer program NITCONT was written in FORTRAN-77 to calculate variations in the average N content along the sequences of proteins (the program is available on request from the corresponding author). We used a sliding window approach similar to that used by Kyte and Doolittle (*21*) to calculate the hydropathic character of proteins. Briefly, the program continuously determines the average N content (either as percentage molecular mass/amino acid or as nitrogen atoms/amino acid) within a protein segment of a given length as it advances through the sequence. All calculations in this study were performed using a window size of 23 amino acids. The calculated results from the NITCONT program were plotted using the program KALEIDAGRAPH (Synergy Software, Reading, PA).

RESULTS

Sequencing of the *mrjp3* Loci of Four *Apis* Species. Our previous data showed that PCR primers designed for two *mrjp* genes of *A. mellifera* produced specific fragments with genomic DNA of *A. dorsata*. Moreover, the sequences of the amplified fragments of *A. dorsata* were similar to these of *A. mellifera* (*22*). Therefore, we employed a PCR-based approach for the isolation and characterization of the *mrjp3* loci in *A. mellifera*, *A. dorsata*, *A cerana*, and *A. florea*. High-accuracy PCR reactions were used for amplification of overlapping fragments, which were sequenced and used for assembly of the loci. Primers derived from the known cDNA sequence of *A. mellifera* MRJP3 are listed in the Supporting Information. Several primer combinations did not yield any PCR fragments with non-*mellifera* DNAs. In one case (primer combination P57-P84), a specific fragment was amplified from *A. dorsata* and *A. cerana* that did not assemble with the corresponding *mrjp3* gene. Interestingly, the amplified fragments from both species are highly homologous to each other (95% identity), and their closest homologue is *mrjp6* of *A. mellifera* (*23*). We conclude that the P57-P84-amplified DNA fragments represent *mrjp6* orthologues of *A. dorsata* and *A. cerana* (GenBank accession numbers AY082889 and AY732221, respectively). Developing new primers derived from the previously obtained sequences of *mrjp3* genes, we could assemble complete DNA sequences of *mrjp3* for all four species. As primers derived from the noncoding regions of the MRJP3 cDNA did not work with non-*mellifera* DNAs, the assembled loci of *A. florea* and *A. dorsata* *mrjp3* (termed *af-mrjp3* and *ad-mrjp3*, respectively) are truncated at their termini. Nevertheless, the obtained sequences cover the gene portions coding for complete mature MRJP3 proteins.

Comparison of *mrjp3* Genes of Four Honeybees. Introns of *am-mrjp3* were identified by comparing the genomic sequence with the corresponding cDNA. Introns in non-*mellifera* species were determined by comparison with *am-mrjp3* considering the GT-AG rule for the intron borders. The accuracy of intron positions determined in this way was confirmed by sequencing of MRJP3 cDNA from *A. cerana* (*24*).

Overall, the intron−exon organizations of honeybee *mrjp3*s were identical (**Figure 1** and **Table 1**). All *mrjp3* genes contain five introns located at the same positions, have the same translational phase, and are of similar sizes. A notable exception is the significantly shorter size (175 versus 409−420 bp) of the fourth intron in *A. mellifera* (**Figure 1**; **Table 1**). We found a segment duplication in the fourth intron of *A. cerana* and *A. dorsata* (larger size of the fourth intron) as compared to that of *A. mellifera* (smallest fourth intron). Significant size differences were also observed in the size of the sixth and largest exon, which contains recurring 15-mer sequences. The size differences between species are caused exclusively by a different number of repetitive units. It should be noted that the size of the sixth exon may vary also among individuals of the same species (see below).

The sequence of the *mrjp1* locus of *A. mellifera* published recently (*25*) shows the same organization (see **Table 1**).

A genomic contig containing the *mrjp3* locus was found in the recent release of *A. mellifera* genome assembly (Amel_3.0) in the linkage Group 11.23 (GenBank accession number NW_059510).

Comparison of MRJP3 Proteins and Their Repetitive Regions. The proteins encoded by the honeybee *mrjp3* genes are extremely similar, as expected for orthologues of closely related species (**Figure 2**). A protein with an N-terminal sequence identical to that of acMRJP3 was recently identified by peptide sequencing of fractionated proteins of *A. cerana* RJ (*16*), which strongly supports the notion of the functional homology of the *mrjp3* genes presented here. However, MRJP3 proteins can differ by as much as 115 amino acids in length (504 versus 619 amino acids; **Figure 2** and see below).

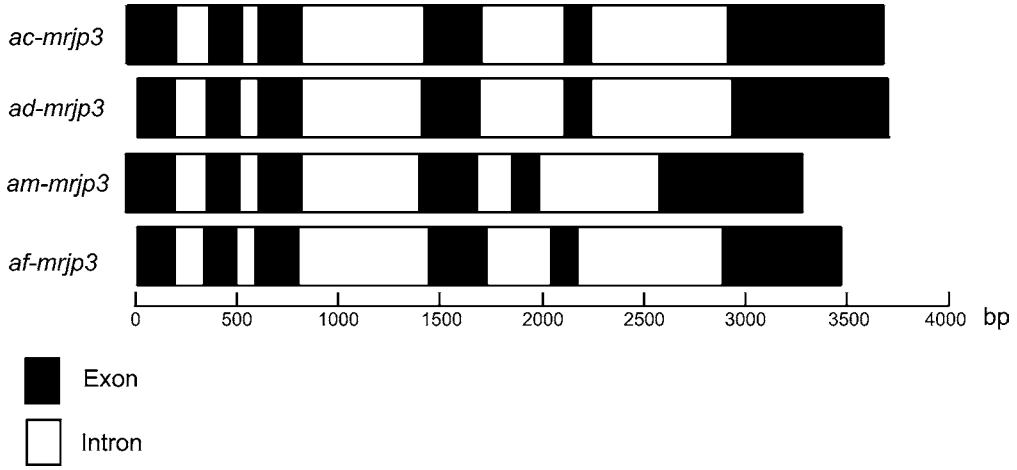

Figure 1. Schematic representation of the *mrjp3* gene organization in four honeybees. Black and white boxes represent exons and introns, respectively. The scale indicates the size in base pairs. The 5′-terminal extensions of *am-mrjp3* and *ac-mrjp3* were obtained by sequencing the respective cDNAs. These sequences are missing in the two other species because no cDNAs were available for *A. dorsata* (*ad-mrjp3*) and *A. florea* (*af-mrjp3*).

Table 1. Comparison of Introns Found in *mrjp3* of Four Honeybee Species and in *mrjp1* of *A. mellifera*

	intron length (bp) [translational phase]				
intron	*am-mrjp3*	*ad-mrjp3*	*ac-mrjp3*	*af-mrjp3*	***am-mrjp1***[a]
1	152 [+1]	153 [+1]	155 [+1]	146 [+1]	113 [+1]
2	89 [0]	98 [0]	85 [0]	88 [0]	82 [0]
3	580 [0]	592 [0]	606 [0]	641 [0]	653 [0]
4	175 [–1]	420 [–1]	409 [–1]	317 [–1]	322 [–1]
5	590 [0]	739 [0]	672 [0]	711 [0]	570 [0]

[a] Data from Malecova et al. (*25*).

In each species, at least three different alleles of the *mrjp3* repetitive region were characterized by DNA sequencing. Overall, the repetitive regions of all species have very similar makeups. Namely, the core repetitive units have the same size [15 bp encoding 5 amino acids (aa)] and the same consensus sequence. Repetitive regions of each of the species contain some unique characteristics not found in other honeybees. For example, the repetitive region of *A. dorsata* always contains a single recognition sequence for *Bst*EII restriction enzyme. In *A. cerana* the repeat contains a truncated glycine−asparagine−asparagine (GNN) repeat unit at the boundary between the asparagine-starting and arginine/lysine-starting segments. For *A. mellifera* and *A. florea* an incomplete repeat is found close to the end of the repeat sequence. Finally, we found several species-specific perturbations of the otherwise well-conserved amino acid sequences (see the Supporting Information, Figure S1). Taken together, although of common origin, the MRJP3 proteins of each species show signs of independent evolution particularly visible in the rapidly evolving repeats.

Studies of the Polymorphic *mrjp3* Repetitive Region of Four *Apis* Species. To get a consistent and species-independent amplification, we synthesized another primer (termed P23) shifted 23 bases in the 5′ direction from the primer P77. This region is conserved among *mrjp3* of all four honeybee species (Supporting Information, Figure S2).

To document the inter- and intracolonial polymorphisms in different species, genomic DNAs were isolated from individual bees of different colonies and subjected to PCR using primers P23 and P76. **Figure 3** documents that by using this primer combination, we could distinguish polymorphic fragments in all four *Apis* species tested. The allele sizes differ substantially among species (**Figure 3**). Length polymorphism seems to be less pronounced among *A. florea* workers. In a colony from India, all amplified fragments had the same DNA sequence with 24 repetitions. Therefore, we additionally genotyped six *A. florea* workers from different locations of the Guangxi province of China. Of these, only one worker yielded two fragments of different size (**Figure 3**). Both alleles of this bee were cloned and sequenced. Sequence comparison revealed that the larger fragment contains two repetitive units more than the smaller fragment (26 vs 24 units). Interestingly, when we compared the sequence of the smaller fragment from China with the Indian allele, we found nine base substitutions (7xG-A plus 1xT-C transitions and 1xT-A transversion) despite an identical overall size (**Figure 3**). All nucleotide changes occurred in the central part of the repetitive regions (Supporting Information, Figure S3, bold characters).

Possible Role of the Repetitive Region in Nitrogen Storage. It is generally accepted that nitrogen, which is an essential component of biogenic polymers (proteins, nucleic acids), is a bottleneck component of the eukaryotic diet. Because animals are not able to fixate atmospheric nitrogen, they all depend on exogenous nitrogen sources. However, low molecular weight nitrogen-rich compounds such as urea, uric acid, or ammonium are toxic at higher concentrations and therefore not suitable for storage or transport of nitrogen. Amino acids are suitable for nitrogen delivery but have on average a lower nitrogen content than the previously mentioned molecules. However, some amino acids have more nitrogen than others, for example, asparagine and glutamine, which are used as shuttles to deliver the nitrogen for biosynthetic reactions by transamidation. Due to the osmotic pressure of concentrated free amino acids (a situation similar to that of mono- and polysaccharides), the polymers (peptides, proteins) are supposed to be the principal nitrogen suppliers for growing organisms.

Haydak (*26*) calculated that between 4 and 6 mg of nitrogen is required to rear one bee larva. If the nurses (producing RJ to feed the brood) are deprived of protein food, they continue to nourish the brood for ∼1 week. During this period, the nitrogen content of their tissues decreases due to the use of their own tissue to rear larvae (reviewed in ref *27*). In summary, RJ is the principal source of nitrogen for all bee larvae and the queen, and the MRJPs of RJ are the major nitrogen storage (hereafter N storage) component.

Because MRJPs are dominant components of total RJ protein [82−90%, (*2*)] we hypothesized previously that some MRJPs

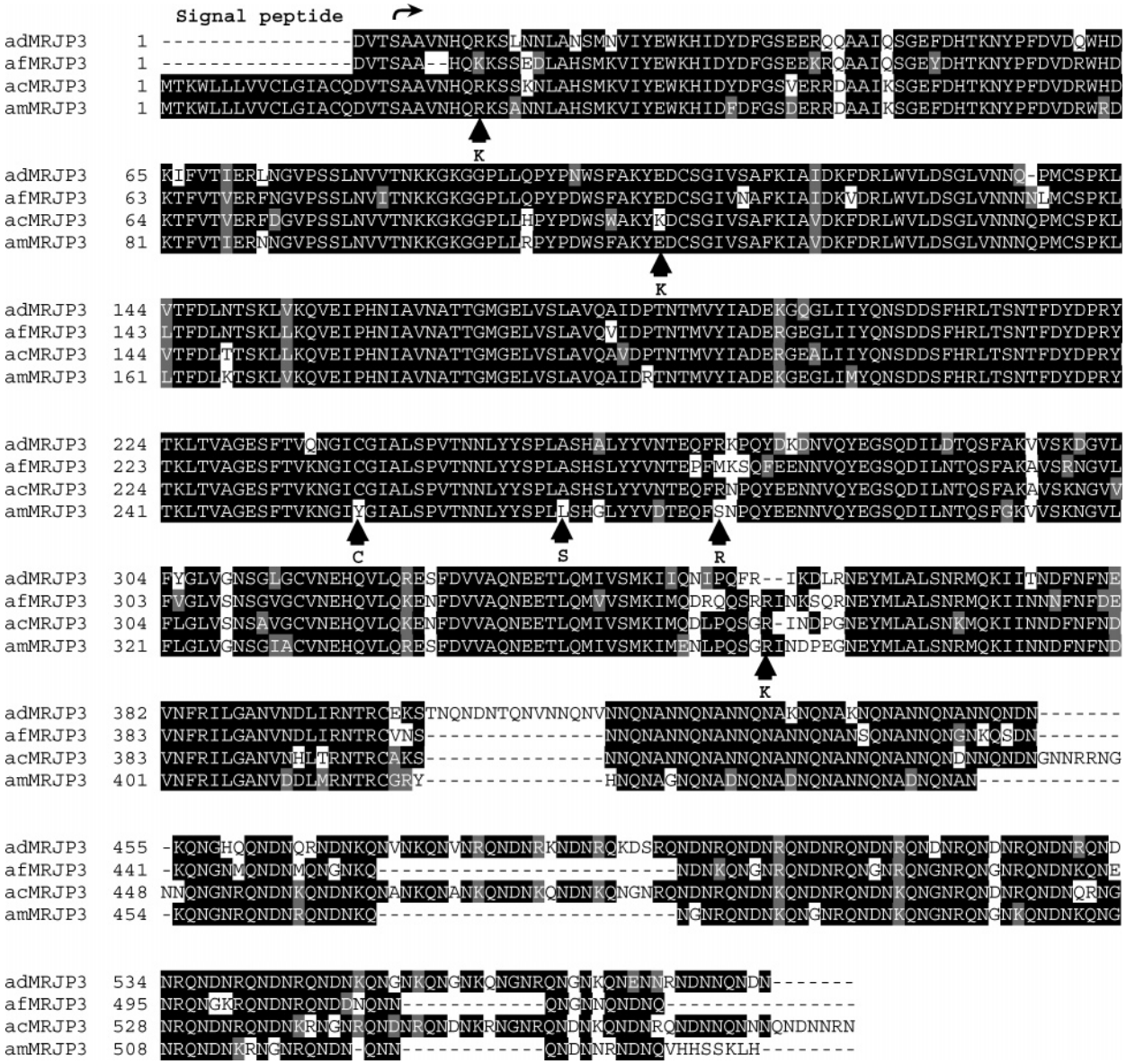

Figure 2. Multiple alignment of MRJP3 protein sequences of four honeybee species. Identical amino acid residues are shaded black, and substitutions with amino acids of similar physical properties are shaded gray. A horizontal arrow indicates the amino termini of the mature protein sequence as determined by Edman sequencing of the MRJP3 of *A. mellifera* (*2*) and *A. cerana* (*16*). Vertical arrows point to the amino acids that were found to be polymorphic in *A. mellifera*. The letters below the arrows indicate the amino acid (in single-letter code) found in alternative alleles.

might have gained a novel N-storage function during their evolution (*15*, *28*). To test this hypothesis, we first attempted to understand the dependence of N content on the nutritional function of a protein. The program NITCONT was written and used to measure both the local average "concentration" of nitrogen and the total average N content expressed as the number of nitrogen atoms per amino acid (N/aa). We used several randomly chosen protein sequences and calculated their N contents (see Supporting Information, Table 1). To have a "benchmark" we needed to calculate a grand average value. We applied the program on all open reading frames found in the *Haemophilus influenzae* Rd and *D. melanogaster* genome sequences. The average N contents of the average "*Haemophilus* and *Drosophila* proteins" were nearly identical. Therefore, we used the *Haemophilus* grand average N content (1.357 N/aa) as a "baseline". We further tested several proteins that have been described as having a storage or nutrition function (Supporting Information, Table 1). Briefly, all tested plant seed storage proteins and egg yolk vitellogenins, including bee vitellogenin, were above the grand average. Of note, bee vitellogenin binds to hypopharyngeal glands of nurse bees and possibly serves as a precursor of royal jelly (*29*). The analyses with NITCONT supported our hypothesis of a correlation between increased N content and the nutritional function of a protein. Therefore, we analyzed the N content of the different members of the MRJP family. These analyses showed that all members of the MRJP family in the honeybee, except MRJP1and MRJP8 (that completely lack the N-rich repeat region, discussed later), have greater N contents than the grand average (**Figure 4**) and that the N content correlates with the length of the repetitive region. The inspection of local N-content profiles reveals a significant peak at the C termini of most MRJP proteins (**Figure 4**). This peak is particularly extended in MRJP3 due to the presence of the longest N-rich repeat at the C terminus. A similar peak can also be observed in the repeat region of MRJP5, although these two repeat regions do not share sequence similarity and are of different origin (*28*).

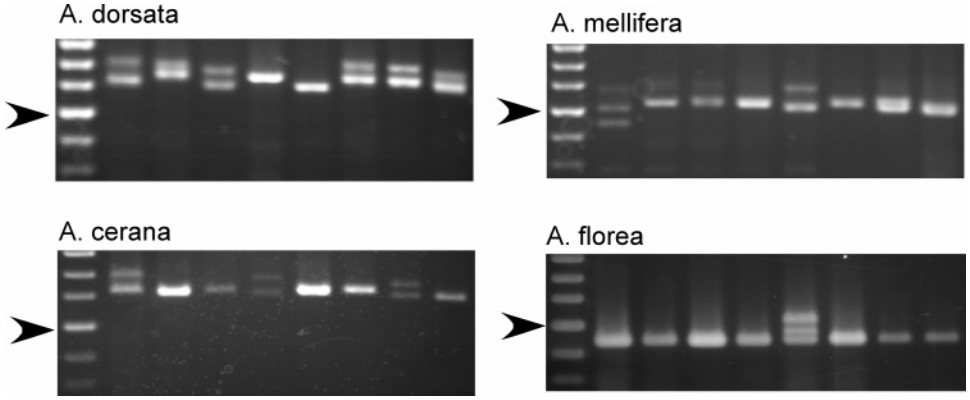

Figure 3. Variability and intraspecific length polymorphism of the MRJP3 repeat by PCR. MRJP3 repeats of individual bees (from two colonies, four animals each) were PCR-amplified and size-separated by electrophoresis in 1.6% agarose gel. The arrows to the left show the position of the 500 bp marker band. High polymorphism is seen in all species except *A. florea*, where only one sample showed a different banding profile. The longest repeats were found in *A. dorsata* and *A. cerana* (600–700 bp), and the shortest repeat was found in *A. florea* (~450 bp).

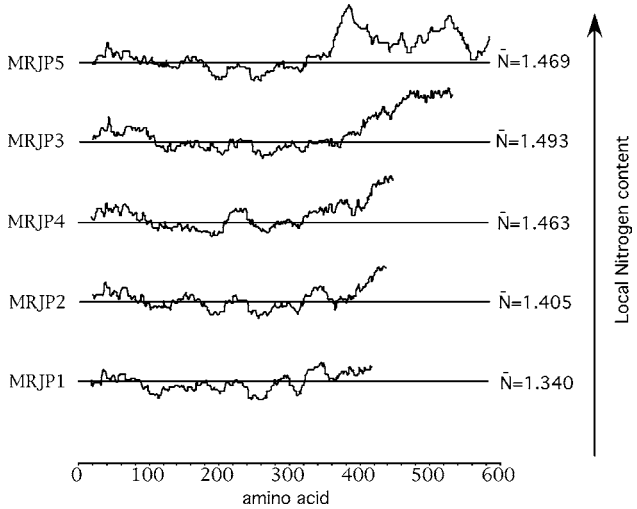

Figure 4. Nitrogen content profiles of the five abundant MRJP proteins. The average values of N contents (*y*-axes) were calculated along the protein sequence (*x*-axes) with the program NITCONT. Profiles are similar throughout the MRJP sequences except for the N-rich regions, where these values increase dramatically. Repeats are located in the N-rich regions, that is, at the C termini of MRJP2,3,4. A reference line on each plot shows the baseline of *Haemophilus* total protein (N = 1.357) as calculated in the text. Calculated N contents for a given protein are shown to the right.

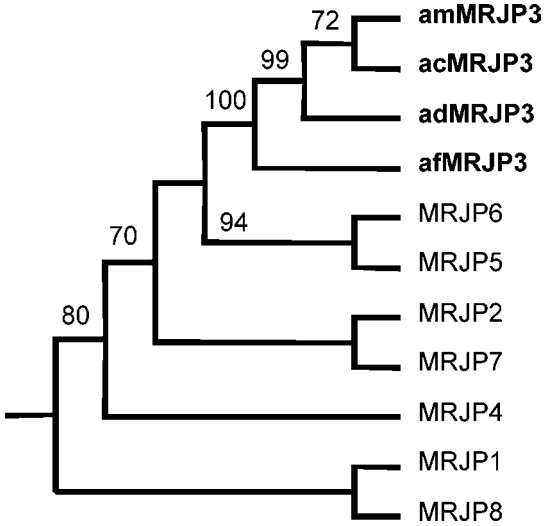

Figure 5. Bootstrap consensus tree (1000 repeats, parsimony) of *mrjp* genes based on analysis of 1120 bp of genomic sequences of *mrjps* loci. Bootstrap values >50 are shown above branches. Tree topology was identical for all analyses independent from the method used (parsimony, maximum likelihood, neighbor joining). The tree was rooted using the *yellow* gene of *Drosophila*.

Phylogenetic Analyses. A total of 1120 bp of genomic sequences were used for the phylogenetic analyses. Of these, 445 were constant and 357 parsimony-informative. The Ti/Tv ratio is 1.46, and the mean AT content for all *mrjp3* genes is 61%. All MRJPs form a monophyletic group with a bootstrap value of 100 (**Figure 5**). Within this group MRJP1 and MRJP8 are basal and most likely represent the ancestral MRJP proteins. The remaining MRJP proteins form a monophyletic group. Therefore, we can conclude that the N repeat has evolved only once in the common ancestor molecule of MRJP2−7. All MRJP3 molecules form a terminal clade in our tree, demonstrating a single origin of this MRJP variant with the long N repeat (**Figure 5**, bold).

DISCUSSION

Our results demonstrated that the genes encoding the MRJP3 protein, a major component of the honeybee royal jelly, are highly similar among four honeybee species. All *mrjp3*s contain a similar repetitive region with a 15 bp long core unit that shows considerable inter- and intraspecific polymorphisms. The *mrjp3* loci of the four honeybee species share high sequence similarity and have the same intron−exon structure. The gene structure of *mrjp3* is very similar to that of *mrjp1* (*25*) and other *mrjp* genes (unpublished results). The phylogenetic analyses also demonstrated that the honeybee *mrjp* genes constitute a distinct cluster within the MRJP/yellow protein family (*23*) and, furthermore, that *mrjp3* genes of the four investigated honeybee species also form a significantly distinct cluster (bootstrap value of 100%) separated from all other *mrjp* genes (**Figure 5**).

This indicates that the genes coding for major proteins of RJ evolved by duplications of a pre-existing genomic segment, rather than by retrotransposition of mRNAs, and that the ancestor of current honeybee species already had multiple *mrjp* genes. Further support for gene duplication comes from the current release of the honeybee genome Amel_3.0 (http://www.hgs.bcm.edu/projects/honeybee), where several *mrjp* genes occur in tandem.

On the basis of the similarity of the *mrjp3* genes it can be presumed that the *A. mellifera* genome, which is just being

completed, will also accelerate the genetic analyses of other honeybee species.

Evolution of *mrjp* Genes in Honeybees Inferred from the Repetitive Regions. The sequences of *mrjp3* genes and inferred proteins document that a *mrjp3* gene containing a polymorphic repeat must have existed in the ancestor of modern honeybees. Six of the eight MRJPs contain shorter and often degenerated repeats near the C termini (Figure S4), supporting a common single origin of the 15-mer repetitive region.

The presence of multiple alleles differing only in repeat numbers in the populations of all analyzed honeybee species suggests that the repetitive region of at least *mrjp3* continuously undergoes expansions and/or deletions. We found multiple nucleotide substitutions (often resulting in amino acid exchange) in the repetitive regions of the same taxon (see *A. florea* from India and China, Figure S3, as an example). Truncated units and numerous substitutions were also found at different positions of repeats of *A. mellifera*, *A. cerana*, and *A. florea* (Figure S1). Apparently, the repetitive regions represent a rapidly evolving part of the MRJP3 proteins. The species-specific differences in allele length of the *mrjp3* homologues might reflect differences in nutritional requirements of the different species (size, growth rates, etc.).

MRJPs as a major component of RJ are clearly a central component of honeybee nutrition. However, they do not share any homology with established nutritive insect proteins, such as vitellogenins. Rather, they seem to share a common origin with the proteins found in insect cuticle, brains, and saliva. We have hypothesized that the evolution of feeding by sterile nurse siblings was accompanied by gene multiplication of former saliva components, which gained a novel nutritive function (*23*). On the basis of the N-content analyses, it is tempting to speculate that the presence of the repeat increasing the N-storage capacity or the protein is the consequence of the selection for an increase in nutritive efficiency of these proteins.

Controversially, MRJP1, the most abundant RJ protein, does not contain any repeats. However, MRJP1 is not RJ-specific, it is expressed also in the honeybee brain (*30*). Recent identification of jelleines, antimicrobial peptides found in RJ as tryptic digests of the C terminus of MRJP1 (*31*), suggest that one of the physiological functions of MRJP1 might be to serve as a precursor of jelleines, protecting the RJ against bacterial infections. This function is incompatible with the presence of the repeat found in other MRJPs, because it disrupts the region coding for jelleine peptides (Figure S4).

But is nitrogen storage the only function of the MRJP repeats? Several authors suggested signaling and/or regulatory functions for MRJP/*yellow* proteins (*8*, *10*). In fact, nutritive and signaling functions are not necessarily mutually exclusive. Repetitive regions of many proteins often form extended surface-exposed areas that commonly participate in protein−protein interactions (*32*). Notably, despite many amino acid substitutions and deletions, the translational reading frame of *mrjp3* repeat never shifts; no frameshift or precocious STOP codon could be detected among 20 sequenced alleles of four *Apis* species. This indicates that despite an increased mutation rate of the repetitive region, there is a selection pressure that eliminates frameshift or termination mutations.

MRJP3 has recently been shown to possess several valuable pharmacological activities such as inhibition of IL-4 production by spleen cells, and it functions as an anti-inflammatory agent (*13*, *33*). It would be interesting to map the sites of these activities on the MRJP3 polypeptide. The repetitive region, although present in shorter and degenerated forms in some other MRJPs, is a distinct feature of MRJP3. Therefore, we think that it would be interesting to see in the future whether the repetitive region of MRJP3 has any functional role concerning its demonstrated pharmacological effects.

ABBREVIATIONS USED

MRJP, major royal jelly protein; RJ, royal jelly; N content, nitrogen content; aa, amino acid; PCR, Polymerase Chain Reaction; bp, base pairs.

ACKNOWLEDGMENT

We are very grateful to Raghavendra Gadagkar for inviting A.B. to Bangalore and generously letting him use his facilities. Ponnanna helped in collecting the honeybees. We thank Gavi Gowda for offering to collect *A. dorsata* workers on the GKVK Campus.

Supporting Information Available: Supplemental Figures S1−S4, supplemental Table 1, and list of primers. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Klaudiny, J.; Hanes, J.; Kulifajova, J.; Albert, S.; Simuth, J. Molecular cloning of two cDNAs from the head of the nurse honey bee (*Apis mellifera* L.) for coding related proteins of royal jelly. *J. Apic. Res.* **1994**, *33*, 105−111.

(2) Schmitzova, J.; Klaudiny, J.; Albert, S.; Schroeder, W.; Schreckengost, W.; Hanes, J.; Judova, J.; Simuth, J. A family of major royal jelly proteins of the honeybee *Apis mellifera* L. *Cell. Mol. Life Sci.* **1998**, *54*, 1020−1030.

(3) Nash, W. G. Patterns of pigmentation color states regulated by the y locus in *Drosophila melanogaster*. *Dev. Biol.* **1976**, *48*, 336−343.

(4) Sano, O.; Kunikata, T.; Kohno, K.; Iwaki, K.; Ikeda, M.; Kurimoto, M. Characterization of royal jelly proteins in both Africanized and European honeybees (*Apis mellifera*) by two-dimensional gel electrophoresis. *J. Agric. Food Chem.* **2004**, *52*, 15−20.

(5) Scarselli, R.; Donadio, E.; Giuffrida, M. G.; Fortunato, D.; Conti, A.; Balestreri, E.; Felicioli, R.; Pinzauti, M.; Sabatini, A. G.; Felicioli, A. Towards royal jelly proteome. *Proteomics* **2005**, *5*, 769−776.

(6) Santos, K. S.; dos Santos, L. D.; Mendes, M. A.; de Souza, B. M.; Malaspina, O.; Palma, M. S. Profiling the proteome complement of the secretion from hypopharyngeal gland of Africanized nurse-honeybees (*Apis mellifera* L.). *Insect Biochem. Mol. Biol.* **2005**, *35*, 85−91.

(7) Drapeau, M. D., The family of yellow-related *Drosophila melanogaster* proteins. *Biochem. Biophys. Res. Commun.* **2001**, *281*, 611−613.

(8) Maleszka, R.; Kucharski, R. Analysis of *Drosophila* yellow-B cDNA reveals a new family of proteins related to the royal jelly proteins in the honeybee and to an orphan protein in an unusual bacterium *Deinococcus radiodurans*. *Biochem. Biophys. Res. Commun.* **2000**, 270, 773−776.

(9) Chia, W.; Howes, G.; Martin, M.; Meng, Y. B.; Moses, K.; Tsubota, S. Molecular analysis of the yellow locus of *Drosophila*. *EMBO J.* **1986**, *5*, 3597−3605.

(10) Drapeau, M. D. A novel hypothesis on the biochemical role of the *Drosophila* yellow protein. *Biochem. Biophys. Res. Commun.* **2003**, *311*, 1−3.

(11) Han, Q.; Fang, J.; Ding, H.; Johnson, J. K.; Christensen, B. M.; Li, J. Identification of *Drosophila melanogaster* yellow-f and yellow-f2 proteins as dopachrome-conversion enzymes. *Biochem. J.* **2002**, *368*, 333−340.

(12) Claycomb, J. M.; Benasutti, M.; Bosco, G.; Fenger, D. D.; Orr-Weaver, T. L. Gene amplification as a developmental strategy: isolation of two developmental amplicons in *Drosophila*. *Dev. Cell* **2004**, *6*, 145−155.

(13) Kohno, K.; Okamoto, I.; Sano, O.; Arai, N.; Iwaki, K.; Ikeda, M.; Kurimoto, M. Royal jelly inhibits the production of proinflammatory cytokines by activated macrophages. *Biosci., Biotechnol., Biochem.* **2004**, *68*, 138−145.

(14) Taniguchi, Y.; Kohno, K.; Inoue, S.; Koya-Miyata, S.; Okamoto, I.; Arai, N.; Iwaki, K.; Ikeda, M.; Kurimoto, M. Oral administration of royal jelly inhibits the development of atopic dermatitis-like skin lesions in NC/Nga mice. *Int. Immunopharmacol*. **2003**, *3*, 1313−1324.

(15) Albert, S.; Klaudiny, J.; Simuth, J. Molecular characterization of MRJP3, highly polymorphic protein of honeybee (*Apis mellifera*) royal jelly. *Insect Biochem. Mol. Biol.* **1999**, *29*, 427−434.

(16) Srisuparbh, D.; Klinbunga, S.; Wongsiri, S.; Sittipraneed, S., Isolation and characterization of major royal jelly cDNAs and proteins of the honey bee (*Apis cerana*). *J. Biochem. Mol. Biol*. **2003**, *36*, 572−579.

(17) Fondrk, M. K.; Page, R. E.; Hunt, G. J. Paternity analysis of worker honey bee using random amplified polymophic DNA. *Naturwissenschaften* **1993**, *80*, 226−231.

(18) Devereux, J.; Haeberli, P.; Smithies, O. A comprehensive set of sequence analysis programs for VAX. *Nucleic Acids Res.* **1984**, *12*, 387−395.

(19) Kumar, S.; Tamura, K.; Nei, M. MEGA: molecular evolutionary genetics analysis software for microcomputers. *Comput. Appl. Biosci*. **1994**, *10*, 189−191.

(20) Swofford, D. L.; Waddell, P. J.; Huelsenbeck, J. P.; Foster, P. G.; Lewis, P. O.; Rogers, J. S. Bias in phylogenetic estimation and its relevance to the choice between parsimony and likelihood methods. *Syst. Biol.* **2001**, *50*, 525−539.

(21) Kyte, J.; Doolittle, R. F. A simple method for displaying the hydrophatic character of a protein. *J. Mol. Biol.* **1982**, *157*, 105−132.

(22) Albert, S.; Schmitz, J. Characterization of major royal jelly protein-like DNA sequences in *Apis dorsata*. *J. Apic. Res.* **2002**, *41*, 75−82.

(23) Albert, S.; Klaudiny, J. The MRJP/YELLOW protein family of *Apis mellifera*: identification of new members in the EST library. *J. Insect Physiol.* **2004**, *50*, 51−59.

(24) Su, S. K.; Albert, S.; Chen, S.; Zhong, B. Molecular cloning and analysis of four cDNAs from the heads of *Apis cerana cerana* nurse honeybees coding for major royal jelly proteins. *Apidologie* **2005**, *36*, 389−401.

(25) Malecova, B.; Ramser, J.; O'Brien, J. K.; Janitz, M.; Judova, J.; Lehrach, H.; Simuth, J. Honeybee (*Apis mellifera* L.) mrjp gene family: computational analysis of putative promoters and genomic structure of mrjp1, the gene coding for the most abundant protein of larval food. *Gene* **2003**, *303*, 165−175.

(26) Haydak, M. H. Honey bee nutrition. *Annu. Rev. Entomol.* **1970**, *15*, 143−156.

(27) Crailsheim, K. The protein balance of the honey bee worker. *Apidologie* **1990**, *21*, 417−429.

(28) Albert, S.; Bhattacharya, D.; Klaudiny, J.; Schmitzova, J.; Simuth, J. The family of major royal jelly proteins and its evolution. *J. Mol. Evol.* **1999**, *49*, 290−297.

(29) Amdam, G. V.; Norberg, K.; Hagen, A.; Omholt, S. W. Social exploitation of vitellogenin. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 1799−1802.

(30) Kucharski, R.; Maleszka, R. Evaluation of differential gene expression during behavioral development in the honeybee using microarrays and northern blots. *Genome Biol.* **2002**, *3*, RESEARCH0007.

(31) Fontana, R.; Mendes, M. A.; de Souza, B. M.; Konno, K.; Cesar, L. M.; Malaspina, O.; Palma, M. S. Jelleines: a family of antimicrobial peptides from the royal jelly of honeybees (*Apis mellifera*). *Peptides* **2004**, *25*, 919−928.

(32) Andrade, M. A.; Perez-Iratxeta, C.; Ponting, C. P. Protein repeats: structures, functions, and evolution. *J. Struct. Biol.* **2001**, *134*, 117−131.

(33) Okamoto, I.; Taniguchi, Y.; Kunikata, T.; Kohno, K.; Iwaki, K.; Ikeda, M.; Kurimoto, M. Major royal jelly protein 3 modulates immune responses in vitro and in vivo. *Life Sci.* **2003**, *73*, 2029−2045.

Received for review June 14, 2005. Revised manuscript received August 1, 2005. Accepted August 3, 2005. This project was supported in part by the National Natural Science Foundation of China (No. 30200206) and the Zhejiang Provincial Natural Science Foundation of China (No. 302113).

JF051417X