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Royal Jelly Proteome Comparison between *A. mellifera ligustica* and *A. cerana cerana*

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In this study, we compared royal jelly (RJ) produced by *Apis mellifera ligustica* and *Apis cerana cerana* in production, protein profiles, and abundances using proteomic approaches. The RJ proteome was displayed using two-dimensional gel electrophoresis (2DGE), and proteins were identified using MALDI-TOF MS and LC-Chip/ESI-QTOF MS. Differences in the RJ proteome between the two bee species were validated using western-blot analysis. RJ production by *A. cerana cerana* (3.21 ± 0.43 g) is significantly lower than that of *A. mellifera ligustica* (80.5 ± 7.83 g). The 2DGE based MS approach identified 52 and 60 proteins in the RJ of *A. mellifera ligustica* and *A. cerana cerana*, respectively. The majority of the identified proteins were major royal jelly proteins (MRJPs). Peroxiredoxin 2540, glutathione *S*-transferase S1, and MRJP5 were detected only in the RJ of *A. mellifera ligustica*, and MRJP1 was the most abundant MRJP. In contrast, MRJP7 was found only in the RJ of *A. cerana cerana.* But, similar to *A. mellifera ligustica*, MRJP1 was found most abundantly in this case too. In this study, glucose oxidase was identified for the first time in the *A. cerana cerana* RJ. Comparing the protein levels of MRJP1, 2, 3, 4, and 5 between the two species, they were significantly higher in the RJ of *A. mellifera ligustica* than in *A. cerana cerana*. This observation was supported by Western blot analysis using anti-MRJP1, 2, 3 antibodies. The result suggested that *A. mellifera ligustica* needs more nutrition to nurse the developing larvae and queens as compared to that of *A. cerana cerana*. This study improved our understanding of protein composition of RJ from Western and Eastern honeybees. RJ produced by *A. mellifera ligustica* exceeds the RJ from *A. cerana cerana* both in terms of production and health purposes.

Keywords: royal jelly • *A. mellifera ligustica* • proteome • *A. cerana cerana* • protein

Introduction

Due to increasing health-consciousness, the number of royal jelly (RJ) consumers has tremendously increased around the world, which created a high demand and made RJ one of the popular foods for promotion of health. RJ is a proteinaceous secretion of the hypopharyngeal glands and mandibular gland of nurse honeybees. It plays a central role in honey bee queen feeding and development.[1] European honeybees (*Apis mellifera ligustica*) are the most known RJ producer in the world. Several chemicals such as jelleines, defensin, royalisin, apisin, and 10-hydroxy-2-decenoic acid[2−5] have been documented in RJ and are known to have medical and health promotional effects.[6,7] Protein accounts for up to 50% dry weights in RJ,[7] and the major royal jelly proteins (MRJPs) family alone accounts for about 90% of the total proteins.[8−10] Their hypothetical sizes range from 49 to 87 kDa with the assumption of different sized RJ from different honeybee species having different protein contents, which may lead to special health-supporting features. In our previous study, we found that there is no difference in the protein composition between the RJ from high RJ producing bees (*A. m. ligustica*) (a line selected from Italian bees to increase RJ production in China) and RJ from native Italian bees (*A. m. ligustica*). But, the difference in RJ protein content of high RJ producing bees and that of Carnica bees (*A. m. carnica*) was significantly higher.[11] On the other hand, heterogeneity of the MRJPs between the Africanized and European honeybees (*Apis mellifera*) has been reported.[12] Likewise, differences in the chemical composition and water-soluble proteins between RJ of *A. mellifera* and *A. cerana* has been reported.[13] Detailing their differences through their biological effects, it has been documented that *A. cerana* queens cannot be reared successfully with the RJ produced from *A. mellifera* and *vice versa*.[13,14] In terms of protein form, RJ produced by *A. cerana cerana* contains less protein forms of MRJP2, 3 than that of European bees.[15] However, this study has limitations in that it detected only 23 protein spots using 2D gel in which only 14 proteins were identified as MRJPs. Hence, studies focusing on the differences in the RJ protein content between the two honeybee species (*A. mellifera* and *A. cerana*) are limited, and we felt that the available information is not complete.

Currently, *A. cerana cerana* is a dominant honeybee species and a major honey producer in China. Because of their innate

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resistance to bee mites, diseases, and wasps, over 2 million colonies of *A. cerana cerana* are commercially maintained in the mountain areas of China. Unfortunately, their RJ production is much lower than that of *A. mellifera*. The low RJ production by *A. cerana cerana* becomes a concern for the RJ industry, as high production is in great need.[16] Before the RJ industry turns away from *A. cerana cerana* for higher RJ producing bee species, one needs to address the issue of whether the low RJ production by *A. cerena cerena* is justified by its possibly higher quality for medical or health properties. Therefore, in addition to its information gap bridging effect, the aim of this study is to compare the RJ between *A. cerena cerena* and *A. mellifera ligustica*, hoping to make judgment about each species based on the protein differences. This could have scientific implication in terms of its knowledge contribution as well as practical suggestions to both RJ consumers and RJ producing beekeepers.

Materials and Methods

RJ Production and Sampling. RJ was collected from 10 colonies of Italian honeybees (*A. mellifera ligustica*) and 10 of Chinese honeybees (*A. cerana cerana*). The bee colonies were kept in the apiary of Bee Research Institute, Chinese Academy of Agricultural Science, and organized into similar sized populations and received similar management. Each colony was provided with 90 queen cell cups into which the larvae were grafted. RJ harvesting was done into sterile bottles from the queen cell cups from which the larvae had been grafted before 72 h and then weighed by an electronic scale. The RJ outputs were presented as mean ± standard error (SE) (n = 10). Generally, 10 batches of RJ samples from each bee species were collected to compare the RJ production capacity between two bee species. Different batches of RJ samples from each bee species were combined together, homogenized, subdivided into different aliquots, and extracted immediately for further analysis.

Protein Extraction and Two-Dimensional Gel Electrophoresis. The total proteins were extracted following our previous protocol.[11,17] RJ (1 mg RJ/10 μL PBS) was mixed in phosphate-buffered saline (PBS: 32.5 mM K_2HPO_4, 2.6 mM KH_2PO_4, 400 mM NaCl, pH 7.6). The mixture was homogenized for 5 min on ice and sonicated for 2 min, then centrifuged at 12 000× g for 10 min at 4 °C and recentrifuged at 15 000× g. The supernatant was transferred to a tube for use. The pellets were mixed in the PBS (1 mg RJ/2 μL PBS) and centrifuged at 15 000× g for 10 min at 4 °C. The supernatant was collected and pooled with the first collection as PBS-soluble RJ protein extract. The pellets containing PBS-insoluble proteins, were mixed in lysis buffer (LB) {8 M urea, 2 M thiourea, 4% CHAPS (3-[(3-Cholanidopropyl)dimethylammonio]-1-propanesulfonate), 20 mM Tris-base, 30 mM dithiothreitol (DTT), 2% Biolyte pH 3−10} at the rate of 1 mg RJ/10 μL buffer, then the mixture was sonicated for 2 min and centrifuged at 15 000× g for 10 min at 4 °C. The supernatant was collected and mixed with PBS-soluble proteins extract. Trichloroacetic acid (TCA) was added to the collected supernatants constituting 10% of the final volume of the supernatant, and then the mixture was kept on ice for 10 min to allow protein precipitation and desalting. Subsequently, the mixture was centrifuged twice at 15 000× g for 10 min at 4 °C. The supernatant was discarded and the pellets were redissolved in LB (1 mg RJ/4 μL LB), then the mixture was homogenized for 5 min on ice and sonicated for 2 min and subsequently adjusted to pH 7.0 with 2 M NaOH. The protein concentration was determined according to the Bradford method.[17]

2DGE was performed using Immobiline polyacrylamide system as previously described.[11,18] The isoelectric focusing (IEF) was carried out on immobilized linear pH gradient, from pH 3 to 10, of 17 cm length using PROTEAN IEF Cell (Bio-Rad, Hercules, CA). Analytical IPG strips were rehydrated, at 18 °C, with 200 μg of protein in LB and then mixed with rehydration buffer (8 M urea, 2% CHAPS, 0.001% Bromophenol Blue, 45 mM DTT, 0.2% Biolyte, pH3−10). The strips were then focused according to the following program: active rehydration for 14 h at 50 V; 250 V for 30 min 4 times; 1000 V for 60 min; 9000 V for 5 h; 9000 V until the total 60 000 Vh was reached.

After focusing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the IPG strips were first equilibrated in equilibration buffer 1 (6 M urea, 0.375 M Tris-HCl pH 8.8, 20% glycerol, 2% SDS, 2% DTT) for 15 min and then continued in equilibration buffer 2 (6 M urea, 0.375 M Tris-HCl pH 8.8, 20% glycerol, 2% SDS, 2.5% iodoacetoamide) for another 15 min. The gel strips were then transferred to SDS-PAGE gel, 12% separating gel (1.00 mm) for protein separation in the second dimension. The SDS-PAGE was performed on PROTEAN xi Cell (Bio-Rad, Hercules, CA) at 25 mA/gel for 6.5 h. Each sample was repeated 3 times to ensure protein pattern reproducibility.

Gel Staining, Imaging, and Statistics. The SDS-PAGE gels were visualized by Coomassie Brilliant Blue G-250 (CBB G250). The analytical gels were scanned with transparent model at 300 dpi (dot per inch) resolution, and image analysis was performed with PDQuest V 8.0 (Bio-Rad Hercules, CA) following the user's manual. The authenticity and outline of each spot were validated by visual inspection and editing.

To accurately compare the gels, the intensity of each protein spot was normalized to the total abundance of all valid spots. After normalization and background subtraction, matched sets were created for all samples. Quantitative tables with all normalized optical spot densities were generated and analysis of variance (ANOVA) to detect statistical differences between the measurements of the same spot in all replicate samples were conducted. ANOVA (Version 6.12, SAS Institute, Cary, NC) and Duncan parametric tests were performed at an error probability of $p < 0.05$ to check the significance of the normalized volume in the total density of the identified proteins in all gels.

Protein Identification. Proteins with higher abundances were identified by matrix assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) and lower abundant proteins by liquid chromatography-chip/electrospray ionization-qudropole time-of-flight mass spectrometry (LC-Chip/ESI-QTOF MS). First, the proteins were digested in-gel with bovine trypsin (Trypsin, Roche) according to our previous methods.[11,18] Then, the peptides were collected and vacuum-dried. Before obtaining the mass spectra of the peptide mixture, the digested peptides were desalted and cleaned with ZipTip C18 pipet tips (Millipore Corp., Bedford, MA) according to the manufacturer's instructions. All analyses were performed using Bruker Daltonics Autoflex (Bruker Daltonics, Billerica, MA) operated in the delayed extraction of 190 ns and reflector mode with an accelerating voltage of 20 KV. The peptides mixture were analyzed using saturated solution of α-cyano-4-hydroxy-cinnamic acid (CHCA, Bruker Daltonics, Billerica, MA) in 50% acetonitrile/0.1% trifluoroacetic acid. External calibrations were performed with peptide calibration standard (Bruker Daltonics

Billerica, MA), and internal calibrations with trypsin autoproteolytic fragments. Finally, the masses of proteolytic peptide fragments were obtained by peptide mass fingerprinting. To interpret the mass spectra of protein digests, the generated peaks lists of the tryptic peptide masses were searched using Mascot (http://www.matrixscience.com). Search parameters were: trypsin cleavage; allow up to one missed cleavage; no restriction on protein mass; peptide mass tolerance 100 ppm; fixed modification: carbamidomethyl (C) and variable modification: oxidation (M). Proteins were considered valid only when the statistical significance values were above the threshold ($p < 0.05$) and contained at least 2 identified peptides.

Proteins with lower abundances were identified by LC-Chip/ESI-QTOF-MS (QTOF G6530, Agilent Technologies Inc., Santa Clara, CA), equipped with capillary pump G1382A, nano pump G2225A, auto sampler G1377D and the Chip Cube G4240A. The LC-Chip used (Agilent Technologies Inc., Santa Clara, CA) was constituted of Zorbax 300SB-C18 enrichment column (40 nL, 5 μm) and a Zorbax 300SB-C18 analytical column (75 μm*43 mm, 5 μm). The loading flow rate was 4 μL/min and the loading mobile phase was water with 0.1% formic acid. Elution from the analytical column was performed by binary solvent mixture composed of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acids (solvent B). The following gradient program was used: from 3 to 8% B in 1 minutes, from 8 to 40% B in 5 minutes, from 40 to 85% B in 1 minutes and 85% B for 1 minutes. The Chip flow rate was 300 nL/minutes. The mass spectrometric conditions were performed as follows: positive ion mode; Vcap: 1900 V; drying gas flow rate: 5 L/minutes; drying gas temperature: 350 C; fragmentor voltage: 175 V; skimmer voltage: 65 V; reference masses: m/z 149.02332 and 1221.02332. The digested samples were diluted in 20 μL water with 0.1% formic acid; all the samples were centrifugated for 5 minutes at 10 500× g and about 15 μL of upper solution was analyzed from which 8 μL of sample were injected. Tandem mass spectra were retrieved and stored in combined mgf file using the MassHunter software. MS/MS data were searched using the Mascot search engine online. Search parameters: Carboxymethyl (C) and Oxidation (M) were selected as variable modifications and no fixed modification was selected. The other parameters used were: Taxonomy: all entries; Enzyme: trypsin; Missed cleavages: 1; Peptide tolerance: ±1.2 Da, MS/MS tolerance: ±0.6 Da. Protein identifications were accepted if they could be established at greater than 95% probability and contained at least 2 identified peptides.

Western Blot. Following the identifications of the proteins, MRJP1, MRJP2, and MRJP3, which have shown differentially abundance in the two RJ samples, were selected for further verification of the results. MRJP1, MRJP2, and MRJP3 were each subjected to 3 replication runs, and 4 μg of protein sample were loaded on each lane separated by stacking (4%) and separating (12%) SDS-PAGE gels. Gels were run at 120 V for about 1.5 h using Mini-Protein II Gel electrophoresis System (Bio-Rad Laboratories Ltd.). Resolved proteins were transferred to Nitrocellulose transfer membrane (0.2 μm pore size) (invitrogen) using an invitrogen iBlot Gel Transfer System. Nonspecific binding was blocked with 5% (w/v) nonfat milk powder in Tris buffered saline containing 0.1% (v/v) Tween-20 (TBST) at room temperature for 1 h. The membranes were then incubated with primary rabbit polyclonal anti-MRJP1, MRJP2, and MRJP3 antibodies (developed by our laboratory) at dilution of 1:5000 in 2% milk powder in TBST at 4 °C overnight. Following three washes, the membranes were further incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (Pierce Biotechnology, Inc., Rochford, IL) (1:10 000 in 2% milk powder in TBST), rolling for 1.5 h at room temperature. At the end of this process, the NC membranes were washed for 2 h rolling at room temperature. Immunoreactive protein bands were then visualized by enhanced chemiluminescence detection (ECL, Pierce Biotechnology, Inc., Rochford, IL) regents and quantified by densitometry using Quantity-one image analysis system (Bio-Rad Laboratories Ltd.). The volume of each band was normalized as percentage values by dividing each value by the total sum of all of the detected bands.

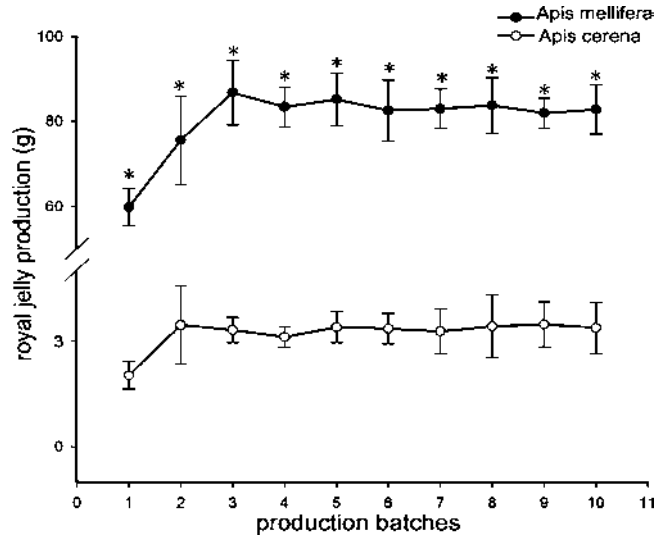

Figure 1. Royal jelly output represented as mean ± SE ($n = 10$). The upper line indicate *A. mellifera ligustica* and the lower line is *A. cerana cerana*. Asterisks indicate the statistic significant differences between royal jelly yield of the two bee species at different batches ($p < 0.05$).

Results

RJ Production Comparison. As *A. cerana cerana* did not accept plastic queen cell cups, the queen cell cups made of bee wax were used and the readily available plastic queen cell cups were provided to *A. mellifera ligustica* and accepted. Comparing the RJ production, there is a great difference between the two species. According to the recorded result in this study, one *A. mellifera ligustica* colony produced 80.5 ± 7.83 g of RJ each time, which is a much higher amount as compared with the amount produced by *A. cerana cerana*, which is only 3.21 ± 0.43 g, and this is statistically very significant (Figure 1), suggesting the impracticality of RJ mass production from *A. cerana cerana*.

Identified RJ Proteins. In the course of identifying RJ proteins, about 85 and 89 protein spots were detected in RJ from *A. mellifera ligustica* and RJ from *A. cerana cerana*, respectively. From these spots, about 52 and 60 reproducible proteins were successfully identified in the two bee species correspondingly, with a better number of identified protein in RJ from *A. cerana cerana*. It is also recognized that most of the identified proteins in both species belong to MRJPs (Figures 2 and 3). However, there are protein spots that remained unidentified in both cases, and this might be attributed to their low abundance to produce enough spectra, or it might be because of the confidence interval of the database search that was not higher than 95% to yield clear results.

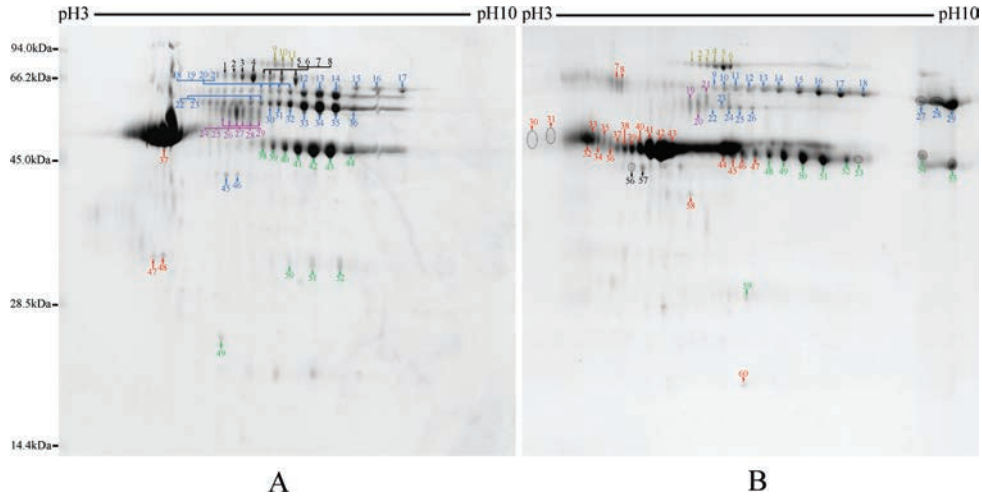

Figure 2. Representative 2DGE protein profiling royal jelly from (A) *A. mellifera ligustica* and (B) *A. cerana cerana*, respectively. Two-hundred micrograms of protein was loaded per gel and stained with CBB-G250. The number labels indicate positively identified proteins. Red, green, blue, pink, and yellow represent MRJP1−4 and glucose oxidase, respectively. Black represents proteins only present in royal jelly samples of *A. mellifera ligustica* and *A. cerana cerana.*

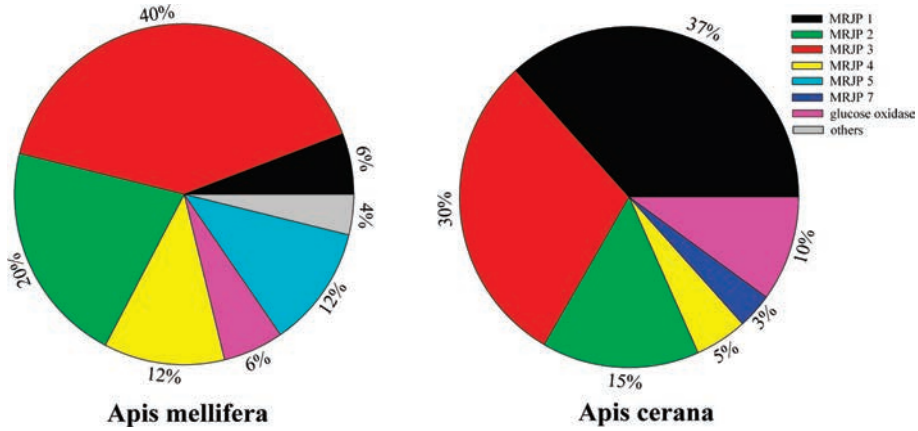

Figure 3. Percentage proportion of the identified protein from the royal jelly samples of *A. mellifera ligustica* ($n = 52$) and *A. cerana cerana* ($n = 60$) based on the total values from Tables 1 and 2, respectively.

Out of the 52 proteins identified in *A. mellifera ligustica* RJ, 3 protein spots were classified as MRJP1, 11 protein spots as MRJP2, 21 protein spots as MRJP3, 6 protein spots as MRJP4, 6 protein spots as MRJP5, 3 protein spots as glucose oxidase (GOD), 1 protein spot as peroxiredoxin 2540 (PRX2540), and 1 protein spot as glutathione S transferase S1 (GST S1). Comparing the relative molecular mass (M_r) and isoelectric point (p*I*) of the MRJPs, there are variations between the observed experimental and theoretical values (Figure 2, Table 1). These variations can be explained as naturally occurring protein isoforms that result from *in vivo* PTMs such as phosphorylation, glycosylation, or acetylation. One major MRJP1 was detected with theoretical M_r 48.86 kDa, p*I* 5.10 (Figure 2A spot 37, Table 1), while 2 forms of MRJP1 may be the degradation products of MRJP1 showing the same M_r and p*I* as the major MRJP1 (Figure 2A, spot 47, 48; Table 1). As well, 11 different forms of MRJP2 (Figure 2A, spots 38−44, 49−52) were observed with M_r 51.45 kDa and p*I* value of 6.83 (Table 1). Among them, spots 39−44 were the majority and spots 38 and 49−52 were the minority. The 21 identified different forms of MRJP3 showed (Figure 2A, spot 12−23, 30−36, 45−46) M_r value from 61.47 to 65.66 kDa and p*I* of 6.47−6.87 (Table 1). Six different forms of MRJP4 were found showing M_r value of 52.88−52.90 kDa and p*I* of 5.89−5.90 (Figure 2A, spots 24−29). In addition, 6 different forms (M_r 70.19 kDa, p*I* 5.95) of MRJP 5 (Figure 2A, spot 1−2 and 5−8) were identified of which 3 in forms of GOD, 1 PRX2540 and 1 GST S1, which were exclusively documented in *A. mellifera ligustica* by previous reports.

The proteins identified in RJ sample of *A. cerana cerana* were assigned as 6 protein spots of GOD, 22 protein spots of MRJP1, 9 protein spots of MRJP2, 18 protein spots of MRJP3, 3 protein spots of MRJP4, 2 protein spots of MRJP7. GOD is found in the RJ of *A. cerana cerana* for the first time through this study. It is also clear that MRJPs are the dominant proteins in RJ in *A. cerana cerana* (Figure 3). However, it is quite different from the RJ sample of *A. mellifera ligustica* in that there were 2 major MRJP1 (Figure 2B, spots 41, 42) and 20 minor (Figure 2B, spots 7, 8, 30−40, 43−47, 58, 60). Likewise, the M_r and p*I* from *A. cerana cerana* MRJPs were found varied ranging 49.32−49.48 kDa and 5.10−5.50, respectively, and the reasons of variation are similar with *A. mellifera ligustica*. The identified 9 forms of MRJP2 showed M_r of 52.83−53.03 kDa and p*I* 8.02−8.27 (Figure 2B, spots 48−55, 59). Eighteen different forms of MRJP3 (Figure 2B, spots 9−18, 22−29) were observed with M_r of 61.97−69.65 kDa and p*I* of 6.47−8.60. The 3 isoforms of MRJP4 (Figure 2B, spots 19−21) showed the same M_r of 55.29 kDa and p*I* of 6.00 accordingly. Two MRJP7 (Figure 2B, spots 56, 57) were uniquely identified with M_r of 24.91 kDa and p*I* of 5.54. Six forms of

Table 1. Proteins Identified in Royal Jelly Sample of Italian Bees (*A. mellifera ligustica*)[a]

spot number	theoretical p*I*/M_r (kDa)	experimental p*I*/M_r (kDa)	sequence coverage	identified peptides	score	protein name	accession number
1	5.95/70.19	5.81/65.37	26.0%	10	83	Major royal jelly protein 5 [*Apis mellifera*]	gil58585138
2	5.95/70.19	6.02/65.19	18.0%	9	86	Major royal jelly protein 5 [*Apis mellifera*]	gil58585138
3	5.88/25.23	6.22/64.99	61.0%	11	81	Peroxiredoxin 2540 [*Apis mellifera*]	gil66535082
4	5.40/17.69	6.44/64.89	52.0%	9	128	Glutathione S transferase S1 [*Apis mellifera*]	gil66534655
5	5.95/70.19	6.59/64.83	18.0%	11	84	Major royal jelly protein 5 [*Apis mellifera*]	gil58585138
6	5.95/70.19	6.67/64.76	26.0%	14	99	Major royal jelly protein 5 [*Apis mellifera*]	gil58585138
7	5.95/70.19	6.79/64.69	20.0%	12	87	Major royal jelly protein 5 [*Apis mellifera*]	gil58585138
8	5.95/70.19	6.90/64.59	29.0%	16	93	Major royal jelly protein 5 [*Apis mellifera*]	gil58585138
9	6.48/67.90	6.59/68.61	26.0%	13	86	Glucose oxidase [*Apis mellifera*]	gil58585090
10	6.48/67.90	6.66/68.32	35.0%	14	120	Glucose oxidase [*Apis mellifera*]	gil58585090
11	6.48/67.90	6.73/68.62	27.0%	9	87	Glucose oxidase [*Apis mellifera*]	gil58585090
12	6.47/61.97	7.04/64.53	28.0%	16	137	Major royal jelly protein 3 [*Apis mellifera*]	gil58585142
13	6.47/61.97	7.18/64.58	25.0%	14	125	Major royal jelly protein 3 [*Apis mellifera*]	gil58585142
14	6.50/61.97	7.35/64.41	28.0%	8	90	Major royal jelly protein 3 [*Apis mellifera*]	gil58585142
15	6.47/61.97	7.53/64.00	22.0%	12	122	Major royal jelly protein 3 [*Apis mellifera*]	gil58585142
16	6.47/61.97	7.75/63.86	19.0%	10	105	Major royal jelly protein 3 [*Apis mellifera*]	gil58585142
17	6.50/61.97	7.98/63.50	21.0%	8	87	Major royal jelly protein 3 [*Apis melifera*]	gil58585142
18	6.47/61.97	6.56/64.68	19.0%	12	101	Major royal jelly protein 3 [*Apis mellifera*]	gil58585142
19	6.47/61.97	6.65/64.70	18.0%	8	92	Major royal jelly protein 3 [*Apis mellifera*]	gil58585142
20	6.47/61.97	6.75/64.67	19.0%	10	84	Major royal jelly protein 3 [*Apis mellifera*]	gil58585142
21	6.47/61.97	6.91/64.63	17.0%	8	85	Major royal jelly protein 3 [*Apis mellifera*]	gil58585142
22	6.47/61.97	6.59/60.52	19.0%	8	83	Major royal jelly protein 3 [*Apis mellifera*]	gil58585142
23	6.47/61.97	6.71/60.54	28.0%	14	108	Major royal jelly protein 3 [*Apis mellifera*]	gil58585142
24	5.90/52.88	5.96/59.66	25.0%	8	90	Major royal jelly protein 4 [*Apis mellifera*]	gil58585170
25	5.90/52.88	6.18/60.28	18.0%	6	100	Major royal jelly protein 4 [*Apis mellifera*]	gil58585170
26	5.89/52.88	6.35/60.41	22.0%	10	97	Major royal jelly protein 4 [*Apis mellifera*]	gil58585170
27	5.89/52.88	6.50/61.19	22.0%	10	97	Major royal jelly protein 4 [*Apis mellifera*]	gil58585170
28	5.89/52.88	6.61/58.63	21.0%	10	85	Major royal jelly protein 4 [*Apis mellifera*]	gil58585170
29	5.89/52.88	6.68/58.01	18.0%	9	88	Major royal jelly protein 4 [*Apis mellifera*]	gil58585170
30	6.47/61.97	6.72/60.55	29.0%	16	121	Major royal jelly protein 3 [*Apis mellifera*]	gil58585142
31	6.47/61.97	6.81/60.25	26.0%	12	104	Major royal jelly protein 3 [*Apis mellifera*]	gil58585142
32	6.47/61.97	6.93/60.21	43.0%	21	138	Major royal jelly protein 3 [*Apis mellifera*]	gil58585142
33	6.47/61.97	7.06/60.21	29.0%	15	106	Major royal jelly protein 3 [*Apis mellifera*]	gil58585142
34	6.47/61.97	7.19/60.28	22.0%	10	108	Major royal jelly protein 3 [*Apis mellifera*]	gil58585142
35	6.47/61.97	7.36/60.19	22.0%	12	108	Major royal jelly protein 3 [*Apis mellifera*]	gil58585142
36	6.47/61.97	7.62/59.12	22.0%	12	100	Major royal jelly protein 3 [*Apis mellifera*]	gil58585142
37	5.10/48.86	5.25/48.35	46.0%	19	180	Major royal jelly protein 1[*Apis mellifera*]	gil58585098
38	6.83/51.45	6.85/47.09	23%	11	111	Major royal jelly protein 2 [*Apis mellifera*]	gil58585108
39	6.83/51.45	6.96/47.02	23%	10	92	Major royal jelly protein 2 [*Apis mellifera*]	gil58585108
40	6.83/51.45	7.09/47.02	23%	10	93	Major royal jelly protein 2 [*Apis mellifera*]	gil58585108
41	6.83/51.45	7.25/47.03	43%	20	116	Major royal jelly protein 2 [*Apis mellifera*]	gil58585108
42	6.83/51.45	7.41/46.92	43%	20	116	Major royal jelly protein 2 [*Apis mellifera*]	gil58585108
43	6.83/51.45	7.59/46.83	46.0%	20	117	Major royal jelly protein 2 [*Apis mellifera*]	gil58585108
44	6.83/51.45	7.75/46.78	23.0%	11	109	Major royal jelly protein 2 [*Apis mellifera*]	gil58585108
45	6.87/65.66	6.79/46.19	24.0%	12	108	Major royal jelly protein 3 [*Apis mellifera*]	gil56422035
46	6.47/61.97	6.88/46.20	23.0%	9	91	Major royal jelly protein 3 [*Apis mellifera*]	gil58585142
47	5.10/48.86	5.76/42.96	19.0%	10	88	Major royal jelly protein 1[*Apis mellifera*]	gil58585098
48	5.10/48.86	5.94/43.03	19.0%	7	85	Major royal jelly protein 1 [*Apis mellifera*]	gil58585098
49	6.83/51.45	6.77/40.42	32.0%	15	125	Major royal jelly protein 2 [*Apis mellifera*]	gil58585108
50	6.83/51.45	7.37/43.07	23.0%	10	110	Major royal jelly protein 2 [*Apis mellifera*]	gil58585108
51	6.83/51.45	7.38/43.09	26.0%	11	98	Major royal jelly protein 2 [*Apis mellifera*]	gil58585108
52	6.83/51.45	7.76/43.10	23.0%	9	93	Major royal jelly protein 2 [*Apis mellifera*]	gil58585108

[a] Spot number corresponds to the number of protein spots in Figure 2A. Theoretical molecular weight (M_r) and isoelectric point (p*I*) of the identified proteins were retrieved from the protein database of NCBInr. Experimental M_r and p*I* were calculated with PDQuest Software and internal standard molecular mass markers. Sequence coverage is the ratio of the number of amino acids in every peptide that matches with the mass spectrum divided by the total number of amino acids in the protein sequence. Identified peptide is the number of paring an experimental fragmentation spectrum to a theoretical segment of protein. Mascot score is search against the database NCBInr. Protein name is given when proteins were identified by MALDI-TOF/MS and LC-Chip/ESI-QTOF MS. Accession number is the unique number given to mark the entry of a protein in the database NCBInr.

GOD observed the same M_r of 67.90 kDa and p*I* of 6.48 (Figure 2B, spots, 1−6). However, MRJP5 were missing in this sample.

Analysis of RJ Protein Abundances. To compare the protein abundances between the two RJ samples, protein profiles were visualized and mean ± SE of the normalized values were presented (Figure 4). Accordingly, only MRJP7 were uniquely detected (Figure 2B, spots 56, 57) in the RJ sample from *A. cerana cerana*. On the other hand, 3 protein species, MRJP5 (Figure 2A, spots 5−8), PRX2540 (Figure 2A, spot 3), and GST S1 (Figure 2A, spot 4), were exclusively identified in the RJ sample from *A. mellifera ligustica*. Among the identified proteins in the two RJ samples, only GOD from *A. mellifera*

Table 2. Proteins Identified in Royal Jelly Sample of Chinese Bees (*Apis cerana cerana*)[a]

spot number	theoretical p*I*/M_r (kDa)	experimental p*I*/M_r (kDa)	sequence coverage	identified peptides	score	protein name	accession number
1	6.48/67.90	6.23/69.05	12.00%	14	482	glucose oxidase [*Apis mellifera*]	gil58585090
2	6.48/67.90	6.39/68.97	12.00%	9	300	glucose oxidase [*Apis mellifera*]	gil58585090
3	6.48/67.90	6.53/68.80	12.00%	10	387	glucose oxidase [*Apis mellifera*]	gil58585090
4	6.48/67.90	6.59/68.61	12.00%	11	372	glucose oxidase [*Apis mellifera*]	gil58585090
5	6.48/67.90	6.66/68.32	12.00%	15	389	glucose oxidase [*Apis mellifera*]	gil58585090
6	6.48/67.90	6.73/68.62	12.00%	10	359	glucose oxidase [*Apis mellifera*]	gil58585090
7	5.50/49.39	4.56/65.62	33.00%	11	146	major royal jelly protein 1 precursor [*Apis cerana*]	gil40557703
8	5.50/49.39	4.63/65.56	44.00%	19	217	major royal jelly protein 1 precursor [*Apis cerana*]	gil40557703
9	8.60/69.65	6.44/64.89	13.00%	8	84	major royal jelly protein 3 [*Apis cerana*]	gil56422037
10	8.60/69.65	6.59/64.83	27.00%	11	143	major royal jelly protein 3 [*Apis cerana*]	gil56422037
11	8.60/69.65	6.67/64.76	21.00%	11	83	major royal jelly protein 3 [*Apis cerana*]	gil56422037
12	8.60/69.65	6.79/64.69	21.00%	11	83	major royal jelly protein 3 [*Apis cerana*]	gil56422037
13	8.60/69.65	6.90/64.59	13.00%	8	82	major royal jelly protein 3 [*Apis cerana*]	gil56422037
14	8.60/69.65	7.04/64.53	15.00%	9	97	major royal jelly protein 3 [*Apis cerana*]	gil56422037
15	8.60/69.65	7.35/64.41	29.00%	20	145	major royal jelly protein 3 [*Apis cerana*]	gil56422037
16	8.60/69.65	7.53/64.00	26.00%	15	106	major royal jelly protein 3 [*Apis cerana*]	gil56422037
17	8.60/69.65	7.75/63.86	29.00%	20	145	major royal jelly protein 3 [*Apis cerana*]	gil56422037
18	8.60/69.65	7.98/63.50	29.00%	19	125	major royal jelly protein 3 [*Apis cerana*]	gil56422037
19	6.00/55.29	6.18/60.28	18.00%	8	82	major royal jelly protein 4 precursor [*Apis cerana*]	gil42601244
20	6.00/55.29	6.35/60.41	21.00%	9	82	major royal jelly protein 4 precursor [*Apis cerana*]	gil42601244
21	6.00/55.29	6.50/61.19	27.00%	12	81	major royal jelly protein 4 precursor [*Apis cerana*]	gil42601244
22	6.47/61.97	6.58/60.34	29.00%	15	111	major royal jelly protein 3 [*Apis mellifera*]	gil58585142
23	6.47/61.97	6.65/60.23	31.00%	17	128	major royal jelly protein 3 [*Apis mellifera*]	gil58585142
24	6.87/66.06	6.72/60.55	20.00%	9	82	major royal jelly protein 3 [*Apis mellifera carnica*]	gil56422035
25	6.87/66.06	6.81/60.25	29.00%	15	100	major royal jelly protein 3 [*Apis mellifera carnica*]	gil56422035
26	6.47/61.97	6.93/60.21	22.00%	12	82	major royal jelly protein 3 [*Apis mellifera*]	gil58585142
27	8.60/69.65	8.66/63.14	25.00%	17	156	major royal jelly protein 3 [*Apis cerana*]	gil56422037
28	8.60/69.65	8.79/63.18	31.00%	21	157	major royal jelly protein 3 [*Apis cerana*]	gil56422037
29	8.60/69.65	8.95/63.00	28.00%	19	124	major royal jelly protein 3 [*Apis cerana*]	gil56422037
30	5.50/49.40	3.23/49.50	42.00%	19	130	major royal jelly protein 1 precursor [*Apis cerana*]	gil40557703
31	5.10/49.32	3.48/50.22	22.00%	9	84	major royal jelly protein 1 [*Apis mellifera*]	gil58585098
32	5.10/49.32	4.10/50.55	20.00%	8	88	major royal jelly protein 1 [*Apis mellifera*]	gil58585098
33	5.10/49.32	4.14/50.90	18.00%	8	97	major royal jelly protein 1 [*Apis mellifera*]	gil58585098
34	5.50/49.40	4.20/50.38	29.00%	14	84	major royal jelly protein 1 precursor [*Apis cerana*]	gil40557703
35	5.50/49.48	4.28/50.05	20.00%	11	84	major royal jelly protein 1 [*Apis cerana cerana*]	gil33358394
36	5.50/49.40	4.35/49.57	38.00%	15	162	major royal jelly protein 1 precursor [*Apis cerana*]	gil40557703
37	5.50/49.39	4.51/48.60	53.00%	23	167	major royal jelly protein 1 precursor [*Apis cerana*]	gil40557703
38	5.50/49.40	4.64/48.46	38.00%	16	139	major royal jelly protein 1 precursor [*Apis cerana*]	gil40557703
39	5.50/49.40	4.78/48.37	39.00%	16	125	major royal jelly protein 1 precursor [*Apis cerana*]	gil40557703
40	5.50/49.40	4.87/48.20	33.00%	14	83	major royal jelly protein 1 precursor [*Apis cerana*]	gil40557703
41	5.50/49.48	5.14/47.81	36.00%	18	127	major royal jelly protein 1 [*Apis cerana cerana*]	gil33358394
42	5.50/49.40	5.60/47.20	48.00%	19	136	major royal jelly protein 1 precursor [*Apis cerana*]	gil40557703
43	5.50/49.39	5.82/47.37	38.00%	17	134	major royal jelly protein 1 precursor [*Apis cerana*]	gil40557703
44	5.50/49.48	6.66/47.00	31.00%	13	158	major royal jelly protein 1 [*Apis cerana cerana*]	gil33358394
45	5.50/49.48	6.75/47.13	30.00%	13	88	major royal jelly protein 1 [*Apis cerana cerana*]	gil33358394
46	5.50/49.40	6.85/47.09	42.00%	20	150	major royal jelly protein 1 precursor [*Apis cerana*]	gil40557703
47	5.50/49.40	6.96/47.02	33.00%	16	101	major royal jelly protein 1 precursor [*Apis cerana*]	gil40557703
48	8.02/52.83	7.09/47.02	41.00%	15	151	major royal jelly protein 2 [*Apis cerana*]	gil46358503
49	8.02/52.84	7.25/47.03	32.00%	14	89	major royal jelly protein 2 [*Apis cerana*]	gil46358503
50	8.02/52.84	7.41/46.92	32.00%	14	89	major royal jelly protein 2 [*Apis cerana*]	gil46358503
51	8.02/52.84	7.59/46.83	34.00%	12	85	major royal jelly protein 2 [*Apis cerana*]	gil46358503
52	8.02/52.84	7.81/46.87	33.00%	12	91	major royal jelly protein 2 [*Apis cerana*]	gil46358503
53	8.02/52.84	7.96/46.90	29.00%	12	118	major royal jelly protein 2 [*Apis cerana*]	gil46358503
54	8.02/52.83	8.68/46.78	27.00%	11	112	major royal jelly protein 2 [*Apis cerana*]	gil46358503
55	8.02/52.84	8.93/46.83	21.00%	11	115	major royal jelly protein 2 [*Apis cerana*]	gil46358503
56	5.54/24.91	4.76/46.70	78.00%	28	909	major rojal jelly protein 7 [*Apis cerana*]	gil57546160
57	5.54/24.91	4.94/46.65	79.00%	48	1018	major rojal jelly protein 7 [*Apis cerana*]	gil57546160
58	5.50/49.39	6.21/45.76	60.00%	26	90	major royal jelly protein 1 precursor [*Apis cerana*]	gil40557703
59	8.27/53.03	6.75/42.51	44.00%	41	1149	major royal jelly protein 2 [*Apis cerana*]	gil46358503
60	5.10/48.86	6.89/40.09	12.00%	35	177	major royal jelly protein 1 [*Apis mellifera*]	gil58585098

[a] Spot number corresponds to the number of protein spots in Figure 2A. Theoretical molecular weight (M_r) and isoelectric point (p*I*) of the identified proteins were retrieved from the protein database of NCBInr. Experimental M_r and p*I* were calculated with PDQuest Software and internal standard molecular mass markers. Sequence coverage is the ratio of the number of amino acids in every peptide that matches with the mass spectrum divided by the total number of amino acids in the protein sequence. Identified peptide is the number of paring an experimental fragmentation spectrum to a theoretical segment of protein. Mascot score is search against the database NCBInr. Protein name is given when proteins were identified by MALDI-TOF MS and LC-Chip/ESI-QTOF MS. Accession number is the unique number given to mark the entry of a protein in the database NCBInr.

ligustica showed significant lower abundance than from *A. cerana cerana* (Figures 2 and 4). However, the major MRJP1 (Figure 2A, spot 37) of *A. mellifera ligustica* was significantly higher than that of *A. cerana cerana* (Figure 2B, spot 42). Even though RJ from *A. cerana cerana* had 22 protein spots of MRJP1, its total abundance was significantly lower than *A. mellifera ligustica* (Figure 4), and this was validated by Western blot (Figure 5). The RJ from the two samples contains higher

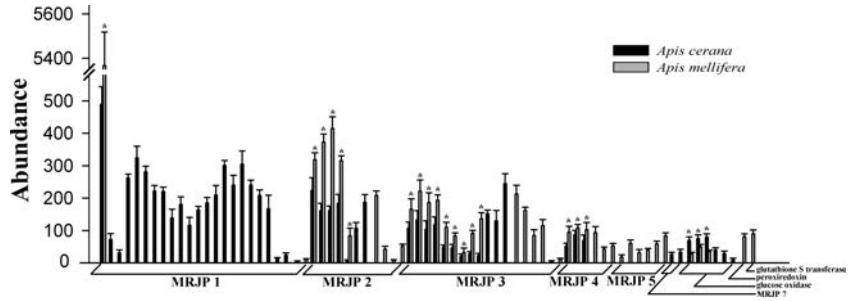

Figure 4. Comparison of each identified protein abundance between royal jelly samples from Italian bees (*Apis mellifera ligustica*) (gray) and Chinese bees (*Apis cerana cerana*) (black). Asterisks indicate the significant differences ($p < 0.05$) between the protein abundance of the two bees by mean ± SE ($n = 3$).

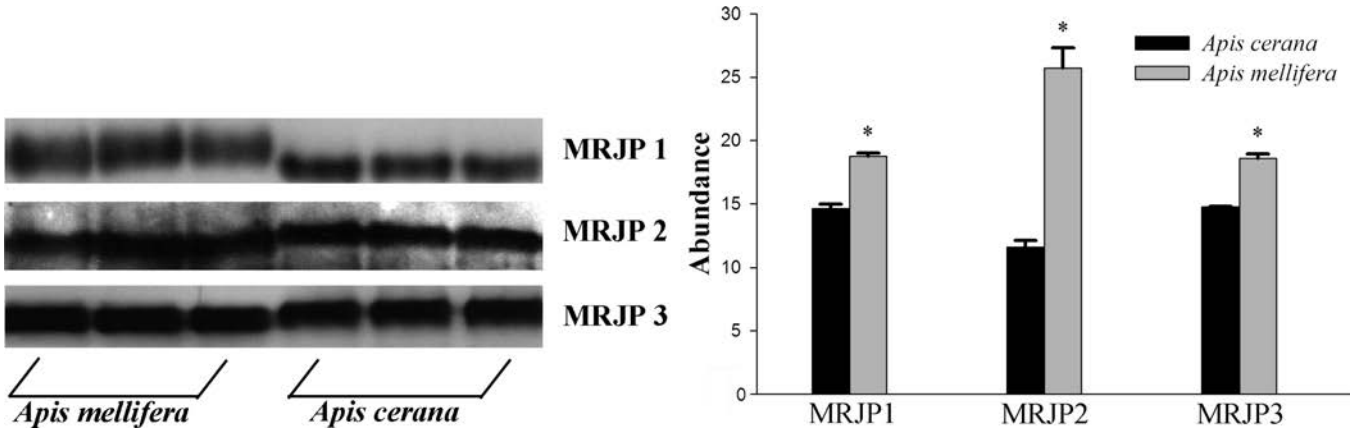

Figure 5. Western blot analysis of major royal jelly protein1 (MRJP1), MRJP2, and MRJP3 from Italian bees (*Apis mellifera ligustica*) and Chinese bees (*Apis cerana cerana*). The extracted proteins from royal jelly samples were subjected to SDS-PAGE followed by Western blot analysis. MRJP1, 2, and 3 were detected using polyclonal antibody anti-MRJP1, 2, and 3, respectively. Equal loading of lanes was maintained by performing a total protein assay. Volume of the detected bands was normalized by dividing the sum of band volumes for all detected bands. Asterisks indicate the significant differences ($p < 0.05$) between the sample at mean ± SE ($n = 3$).

numbers of protein spots of MRJP2 and MRJP3. Nevertheless, comparing their total abundance, they were significantly higher in the RJ from *A. mellifera ligustia* as compared with RJ from *A. cerana cerana* (Figure 4), and this was also confirmed by Western blot analysis (Figure 5). Regarding the MRJP4, we also observed significantly higher abundance of this protein in the RJ from *A. mellifera ligustia* than the RJ from *A. cerana cerana* (Figure 4).

Discussion

This study comprehensively compared RJ produced by *A. mellifera ligustica* and *A. cerana cerana* in production, protein profiles, and abundances using proteomic approaches. The differences in the protein profiles were validated by Western blot analysis. A previous study used the shortest 7 cm IPG strip for protein electronic focusing, only 15 MRJPs identified in the *A. cerana cerana* RJ, and less polymorphism of MRJP2, 3 compared with European bees.[15] The present study used 17 cm IPG strip and complementary strategy of MALDI-TOF and LC-Chip/ESI-QTOF MS, which assisted in the identifications of 60 proteins in the RJ from *A. cerana cerana* RJ. Apart from enabling large protein number identification, qualitative and quantitative RJ protein differences between the bee species of *A. cerana cerana* and *A. mellifera ligustica* were successfully evaluated, and this has further extended our knowledge on RJ concerning the two bee species. The MRJPs recorded M_r range from 48.86 to 70.19 kDa (Tables 1) and 24.91 to 69.95 kDa (Table 2) for *A. mellifera ligustica* and *A. cerana cerana,* respectively, were generally consistent with the previously reported results.[11,15,18,19]

The most striking difference in abundance and number of MRJP1 was observed between the two RJ samples (Figure 3). In the RJ from *A. cerana cerana,* there were about 22 MRJP1 spots outnumbering MRJP3, and it was the most variant protein. This is also agree with the expressed sequence tags library from the hypopharyngeal glands of *Apis cerana* previously reported at transcriptional level.[20,21] Even if there is a report on the MRJP1 from RJ of *Apis mellifera* as to its glycosylation sites that contribute to the variable forms of MRJP1,[19] this study is the first of its kind in presenting a report on MRJP1 isoforms at the protein level. In this study, RJ from *A. cerana cerana* has shown higher number of MRJP1 forms than *A. mellifera ligustica.* This might be attributed to other post-translational modifications such as phosphorylation or sialic acid modification that may cause variability in molecular mass and p*I* of MRJPs[22] and/or due to genetic polymorphism. Concurrent to these, different forms of MRJP1, MRJP2, MRJP3, MRJP4, and MRJP5 were already demonstrated in *Apis mellifera*[11,12,18,19,23] and lower number of isoforms of MRJP2, MRJP3, and MRJP4 in *A. cerana cerana* with the species polymorphism as probable reason.[20] Similar to our investigation, less forms of MRJP2 and MRJP3 in the RJ from *A. cerana cerana* as compared with RJ from *A. mellifera ligustica* was reported.[15] Interestingly, although there was a report on the successful cloning of the gene encoding MRJP5 from the head of *A. cerana cerana,*[20] it was neither found in the previous

report[15] nor in this study. This might be due its relatively lower abundance or it not being sufficient enough to be identified by protocol we employed at present.

RJ serves mainly as nutrition for the development of honeybee larvae and queens, in which the most abundant proteins, the major MRJPs, do it.[6] *A. mellifera ligustica* bee species is much bigger than *A. cerana cerana* both at individual bee body size and at the colony level. Hence, it demands more protein containing nutrition than that of *A. cerana cerana* to feed developing larvae and queen. Therefore, the significantly higher abundance of MRJP1, MRJP2, MRJP3, MRJP4, and MRJP5 in the RJ from *A. mellifera ligustica* (Figures 4 and 5) appears to be from the natural selection of the species. These different honeybee species (*Apis mellifera, Apis cerana, Apis dorsata, Apis florae*) selection for more efficient nutrition of queens and larvae have been reported.[24] In addition, the extra biological mission of minor homologues of MRJPs in the honeybee colony have been reported. Recent record on MRJP2 showed its new function resulting from various post-translational modifications of major proteins.[6] This ignites further study if minor MRJP1 isoforms might possess biological activity that can perform medical functions in the RJ from *A. cerana cerana*.

As a natural preservative, existence of the protein called GOD has been reported in RJ and the hypopharyngeal gland of Africanized and European bees.[11,12,18,19,23] Like other bees, *A. cerana cerana* produces RJ to feed their larvae and queens in which GOD should also play a necessary biological preservative role. However, it was not reported so far in the RJ from *A. cerana cerana*, and hence, GOD is a novel finding of this work in the species RJ.

Like other animals, honeybees possess a suite of enzymes to reduce damage by these life-shortening oxidative byproduct. Similar to our previous results,[11,18] we also found antioxidant enzymes, PRX2540 (Figure 2A, spot 3) and GST S1 (Figure 2A, spot 4), in the RJ from *A. mellifera ligustica*. This antioxidant defense system, conserved throughout evolution, has already been identified in the hypopharyngeal gland,[25] venom glands,[26] and spermatozoa of *A. mellifera*.[27−29] However, some enzymes, such as glutathionereductase and glutathione peroxidase, are absent in insects, and thioredoxin reductase[30] and thioredoxin peroxidase[31] are present. Cognizant to this fact, the current study did not find GST S1 and PRX2540 in RJ from *A. cerana cerana*. This could likely mean either unique adaptations among different taxonomic groups (some enzymes are absent in some insect species) or they are too low in their abundances to be detected by our present method.

MRJP7 has been identified in the RJ from both Africanized honeybees (*A. mellifera* L.) and *A. cerana cerana*.[15,19] However, information on its biological function in the bees is only limited to the recent report that showed its increased expressions together with MRJP1, 2 in nurse bee's brain in relation to caste determination during the honeybee larvae differentiation.[22] For that matter, MRJP7 was not identified in *A. mellifera ligustica* RJ in this study, and this might be due to its lower abundances. Rather, it is more expressed in the RJ from *A. cerana cerana*, suggesting an evolutionary strategy of the species to compensate the lower abundance of all other MRJPs to feed its developing larvae and queens as it is already recognized.[32]

These days, medicinal properties of RJ in which different forms of proteins in the RJ plays specific roles have been well recognized in promoting health. For instance, MRJP1 is known to support liver regeneration and to protect hepatocytes from damages, MRJP2 acts as antibacterial activity[6,33] and stimulates tumor necrosis factor alpha release,[34] and MRJP3 exhibits antiallergic and immunoregulatory activity.[23,35] Therefore, the importance of RJ or its medicinal value could be judged based on both the number and types of protein forms it contains. Accordingly, the lower number of protein variants and low abundances of MRJPs in the RJ from *A. cerana cerana* indicates its quality is less than that of RJ from *A. mellifera ligustica*. Also in terms of volume of production, RJ from *A. cerana cerana* is 25-fold less than that of *A. mellifera ligustica*. From this, it can be tentatively concluded that RJ harvesting using an *A. mellifera ligustica* honeybee colony is more recommended both for large scale or commercial production and for medical demands.

In conclusion, this proteomic approach study comprehensively compared RJ produced by *A. mellifera ligustica* and *A. cerana cerana* and came up the conclusion that RJ produced by *A. mellifera ligustica* exceed that of *A. cerana cerana* in terms of production, protein profiles, and abundances. Identifications of RJ protein called GOD in *A. cerana cerana* and discovery of MRJP1 isoforms at the protein level are novel findings of this work. Therefore, this study not only helped us improve our understanding of RJ from the two honeybee species but also greatly improved our knowledge of RJ at the global proteomic level.

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