

NOVEL ASPECTS IN CHARACTERISATION OF CARNIOLAN HONEY BEE (*Apis mellifera carnica*, Pollmann 1879)

Janko BOŽIČ¹, Dušan KORDIŠ², Igor KRŽAJ², Adrijana LEONARDI², Robert MOČNIK¹, Mitja NAKRST^{3,4}, Peter PODGORŠEK³, Janez PREŠERN³, Simona SUŠNIK BAJEC⁵, Minja ZORC⁵, Jelena ZURC⁵, Peter DOVČ^{5,6}

ABSTRACT

Apis mellifera carnica is a honey bee subspecies, first described by Pollmann in 1879. Its original habitat since the last glaciation is Balkan Peninsula with neighbouring regions. Slovenia is often mentioned as a homeland of Carniolan honey bee although it is evident that natural habitat of *A. m. carnica*, is much bigger and that Slovenia sits at the western edge of its areal. Due to its soft temper, adaptation to extreme low temperatures, good honey production in spring and summer (mainly produced on coniferous trees), Carniolan honey bee is in many countries well accepted and popular honey bee variety. In this study we present a different approaches for characterization of Carniolan honey bee in its home country, Slovenia and compare some traits with populations in the neighbouring countries. In this review we present genetic data (microsatellites and mtDNA sequences), bioinformatics analysis of available DNA sequence data, phenotypic characterisation using cubital index and lengths of tibia and proboscis, proteomic analysis of royal jelly as well as analysis of dancing behaviour.

Key words: Carniolan honey bee, *Apis mellifera carnica*, characterisation, population structure

1 INTRODUCTION

Apis mellifera carnica is a honey bee subspecies, first described by Pollmann in 1879. Its original habitat since the last glaciation is Balkan Peninsula with neighbouring regions. Slovenia is often mentioned as a homeland of Carniolan honey bee. When considering distribution of *A. m. carnica*, however, it is evident that Slovenia sits at the western edge of its areal (Rihar, 2013). However, due to the vivid traffic with Carniolan queen bees since the 18th century, Carniolan honey bee is spread worldwide, today. Due to its soft temper, adaptation to extreme low temperatures, good honey production in spring and summer (mainly produced on coniferous trees), Carniolan honey bee is in many countries well accepted and popular honey bee variety. In the western part of Slove-

nia, in Primorska region, populations of *A. m. carnica* and *A. m. ligustica* are in close contact, representing the western border of the natural habitat of Carniolan honey bee. Both subspecies can be frequently found in this region, so it seems that territory of Italian honey bee partially overlaps with that of Carniolan bee. The question remains how closely the current distributions resemble the natural distribution and what is the contribution of human activities (Gogala, 1999). Slovenia is the only EU member which protected genetic material of the indigenous honey bee subspecies in the accession treaty (Act... 2003). Additionally, the subspecies is protected with local laws (e.g. ReZKČ, Official Gazette of Republic of Slovenia, 18/2014 and earlier, 18/2002, 110/2002, 45/2004 ...), which permit beekeeping solely with the colonies of indigenous subspecies. In addition to these official reasons

1 University of Ljubljana, Biotechnical faculty, Department of biology, Večna pot 111, SI-1000 Ljubljana, Slovenia

2 Institute Jožef Stefan, Jamova c. 39, SI-1000 Ljubljana, Slovenia

3 Agricultural Institute of Slovenia, Hacquetova ul. 17, SI-1000 Ljubljana, Slovenia

4 New address: Čebelarstvo Cvet, Žeje 30, SI-1233 Dob, Slovenia

5 University of Ljubljana, Biotechnical faculty, Department of Animal Science, Groblje 3, SI-1230 Domžale, Slovenia

6 Corresponding author, e-mail: peter.dovc@bf.uni-lj.si

there are also practical reasons for local beekeepers to prefer Carniolan honey bee, mainly due to its adaptation to the local environment.

In Slovenia we have more than 9800 registered beekeepers, taking care of about 140000 bee colonies. However, only a small proportion of them are professional beekeepers. About 4000 beekeepers are involved in the national honey bee breeding program. Slovenian selection is based on the selection index:

$$Si = H + 0.81a + 2.23s,$$

where H represents honey production in kg, a is the score for gentleness and s is the score for swarming. The average honey production in Slovenia in the last years is slightly above 20 kg per family, however oscillations among years are substantial due to fluctuations in weather conditions and consequently in vegetation. In addition, the presence of the parasite *Varroa destructor* severely hampers bee keeping in Slovenia in the last decades. In order to develop national strategy for prevention of *A. m. carnica* in its home country, which should include preservation of genetic variability and improvement of its production and health traits, it is necessary to have an insight into the population structure and special traits which characterize this population.

In order to approach this goal, we summarize in this review genetic, proteomic, phenotypic and behavioural data characterizing population of *A. m. carnica* in Slo-

venia and in the neighbouring countries. In addition to classical genetic markers, in this study represented by microsatellite loci and COI-COII region of the mitochondrial DNA, as well as phenotypic data representing cubital index, tibia and proboscis length, we include also bioinformatics, proteomics and behavioural data. Using the bioinformatics approach, we searched for potential differences among subpopulations at genomic, transcriptomic and epigenetic level. Further, the two dimensional electrophoresis analysis of royal jelly proteins revealed the first insight in the regional differences among royal jellies from different locations and finally, our behavioural study of communication between honey bees belonging to *A. m. carnica* and *A. m. ligustica* subspecies revealed some genetically determined differences.

2 GENETIC CONSTITUTION OF THE *A. m. carnica* POPULATION

The population structure of *A. m. carnica* in Slovenia and neighbour countries has been performed using 23 microsatellite markers and sequencing of the COI-COII region of the mtDNA. The microsatellite data revealed considerable genetic diversity of Slovenian population of *A. m. carnica*, however no significant population structure could be observed between different regions. The Croatian population, however, is clearly differentiated from Slovenian population, probably due to geographi-

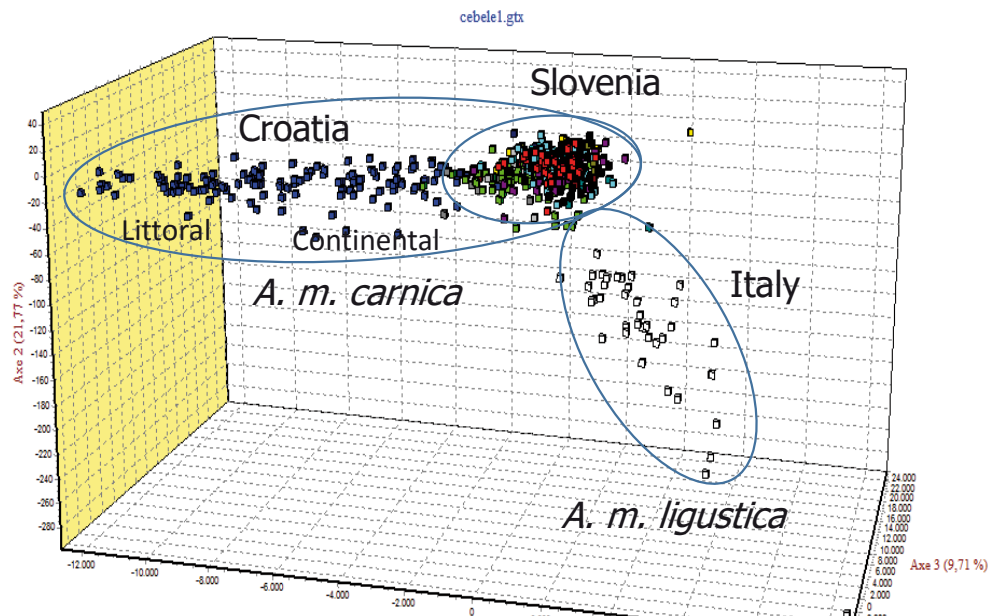


Figure 1: Principal component analysis (PCA) using microsatellite (MS) data from 23 MS loci. The left upper part of the graph represents Croatian samples reaching from littoral samples on the left side to the continental samples on the right side, all Slovenian samples are concentrated in the central cluster (Slovenian and Croatian samples belong to *A. m. carnica*), Italian samples (*A. m. ligustica*) are shown on the lower right side of the graph.

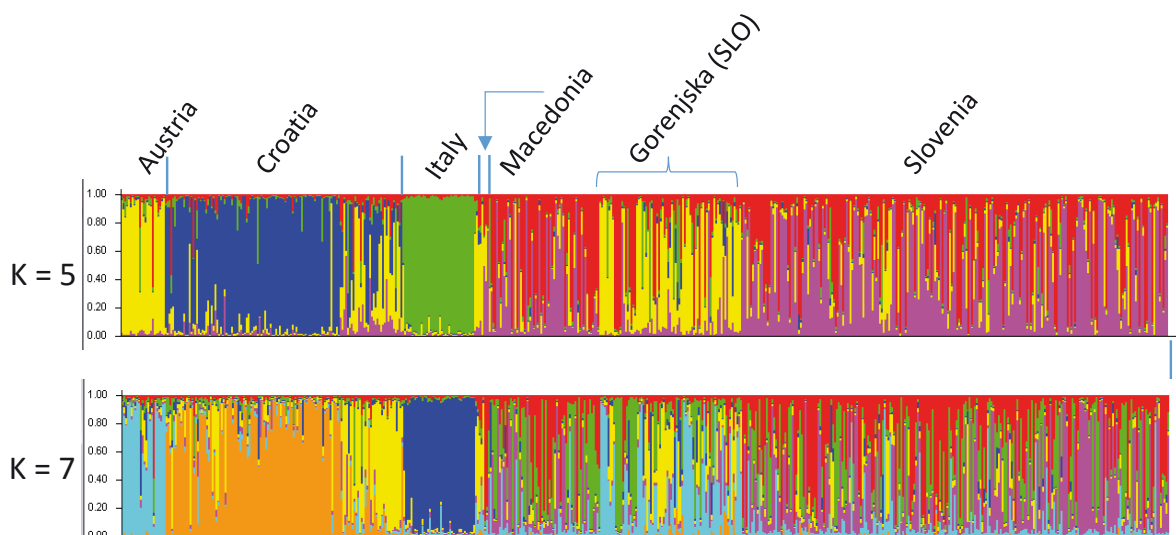


Figure 2: Structure analysis of MS data for samples from Slovenia, Austria, Croatia, and Macedonia. For $K = 5$ and $K = 7$ we got similar clusters showing differentiation of Austrian, Croatian and Italian samples from samples from Slovenia. In the group of Slovenian samples is a cluster of samples from Gorenjska region, which shows some similarity with samples from Austria.

cal obstacles, with the indication that in Croatia the continental population can be differentiated from the littoral population, which also includes honey bee populations from the islands.

The microsatellite data show that Slovenian population is relatively compact in terms of genetic variability and distinct from Croatian and Italian population. This data suggest that in some cases the introduction of *A. m. carnica* from other sources to the Slovenian population could theoretically be detected, however, no private alleles, which could be used as a strict genetic marker could not be found for any of the analysed populations. The analysis of allele frequencies at MS loci showed considerable allelic richness and high degree of heterozygosity in all populations. It seems that biology of the honey bee with characteristic promiscuous mating behaviour of queen bees makes this species much less sensitive to inbreeding.

The analysis revealed considerable variability in Slovenian population, which is probably a consequence of high initial variability, but partly also a consequence of introduction of genetic material from other sources in the past. It is interesting that Croatian and Austrian samples build their own groups and that within the Slovenian group there is a cluster of haplotypes which are similar to Austrian haplotypes. This could be explained by the traffic between Gorenjska region and Carinthia (probably in both directions, not excluding the possibility of crossbreeding in nature due to the high density of honey bee colonies) in the past but also more recently. The surprising similarity between Slovenian and Macedonian samples could be explained by a very low number

of Macedonian samples analysed and by the assumption that Macedonian honey bees were introduced to Slovenian population in the second half of the last century.

In addition to MS analysis, we sequenced mitochondrial DNA (the region between genes for COI and COII) from 280 individuals of *A. m. carnica* from Slovenia, Austria and Macedonia and *A. m. ligusta* from Italy. In our material we found 9 different haplotypes in Slovenian population of *A. m. carnica*, which were represented in more than one specimen. The most frequently represented haplotypes were c2c (33 %), c1a (27 %) and c2j (26 %). The following six haplotypes were present, at much lower frequencies (Fig. 3).

These results clearly show that population structure of *A. m. carnica* in Slovenia is much more complex than assumed initially (Sušnik *et al.*, 2004). Instead of only one mtDNA haplotype, c2c, which was the only mtDNA haplotype identified in the above mentioned study, there are also present two other, almost equally abundant haplotypes (c1a and c2j), and six less abundant mtDNA haplotypes (Fig. 3). Our recent results reveal some additional haplotypes, but some of them were found only in one specimen. The phylogenetic relationships among all mtDNA haplotypes found in Slovenian population of *A. m. carnica* and some reference haplotypes are shown in Fig. 4.

3 BIOINFORMATIC ANALYSIS

In honey bees, usually only a limited number of specific genes are responsible for the particular trait, and

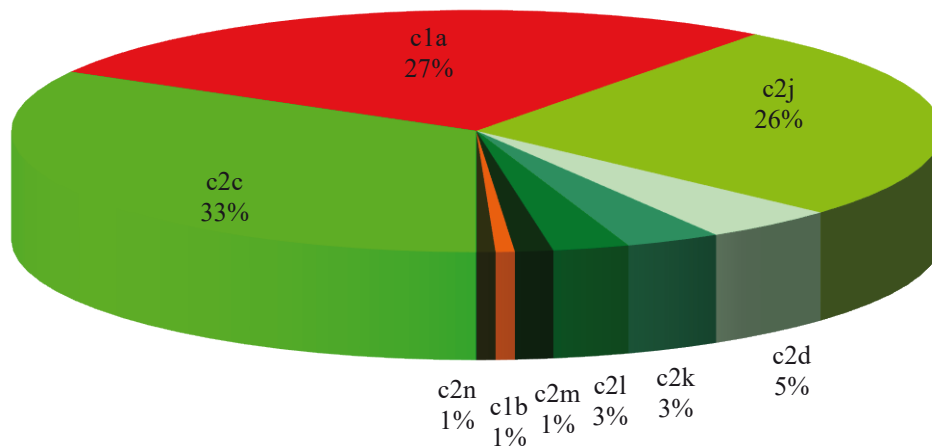


Figure 3: Relative abundance of mtDNA haplotypes in Slovenian population of *A. m. carnica*

very often, numerous genes are epigenetically modified or are involved in the various genetic regulatory networks and in protein-protein interactions (Robinson *et al.*, 2005; Zayed and Robinson, 2012). Carniolan honey bee differs from other subspecies in their behaviour (they are not aggressive) and physiological adaptations to cold winters, which allows them better and longer survival. The complete genome sequence is available for the Italian bee (*Apis mellifera ligustica*) (Honey Bee Genome Sequencing Consortium, 2006; Elsiik *et al.*, 2014). However, three partial genomes of the worker bees of Carni-

olan honey bee have recently become publicly available (Harpur *et al.* 2014), covering from 30 to 40 % of the reference honey bee genome, but the individual nucleotide sequences are very short (~ 50 bp). These genome data were used as a resource to identify potential differences between the two closely related honey bee subspecies, both belonging to the mtDNA haplotype group C. Candidate genes, which could be responsible for the behavioural and physiological characteristics (Robinson *et al.*, 2005; Smith *et al.*, 2008; Zayed and Robinson, 2012) of the Carniolan bee (e. g. vitellogenin, erk7, Mrjp4, dopa-

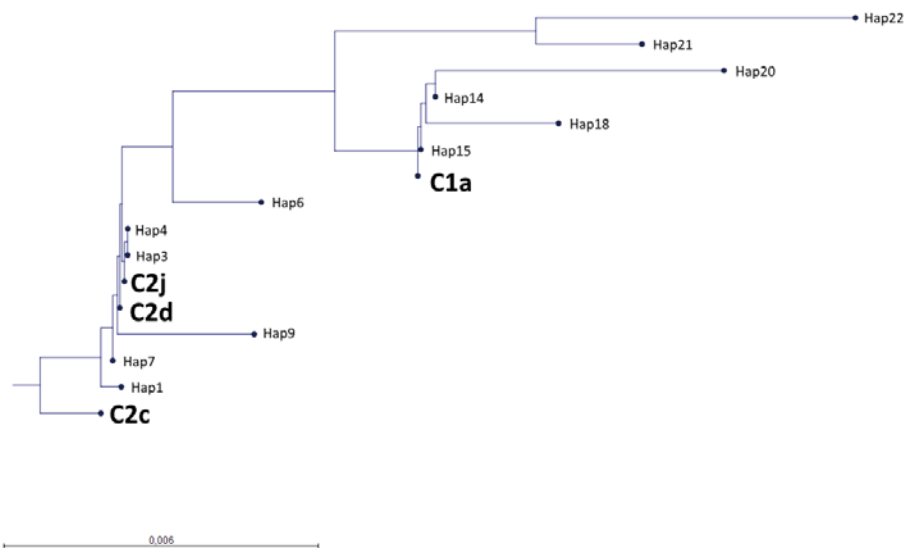


Figure 4: Phylogram showing relationships among mtDNA haplotypes found in Slovenian population of *A. m. carnica*. The most common haplotypes (*c1a*, *c2c*, *c2j* and *c2d*) are printed in bold. The haplotype *c1a* is also one of the most common haplotypes in the Italian population of *A. m. ligustica*.

mine receptor (dop3) tyramine receptor (TyrR), octopamine receptor (OA1), circadian protein period (Per), NMDA receptor 1 (Nmdar1), melitin, trehalase and a number of other genes) have therefore been analysed in the partial genomes of the Carniolan bees. However, the investigated genes showed at the nucleotide level no differences between the Carniolan and Italian subspecies of honey bees.

Further we were interested in possible differences in regulatory regions of genes which are associated with phenotypic and behavioural traits. Since subspecies of honey bees are subjected to different selection pressures the consequences could be the modification of binding sites for transcription factors, which are formed by *cis*-regulatory evolution and the selection for certain adaptive traits (Alaux *et al.*, 2009; Harpur *et al.*, 2014). We analysed the *cis*-regulatory regions in the promoter regions of candidate genes in the partial genomes of the *A. m. carnica*. We were searching for DNA sequence differences in these regions between the two closely related honey bee subspecies. For each candidate gene from 2000 to 2500 bp of the promoter region have been analysed. We found that in the investigated candidate genes there are no differences at the *cis*-regulatory level between the Carniolan and Italian honey bee subspecies.

Honeybee workers are well suited for examination of the relation between gene expression profiles in individual brains and behaviour, because they exhibit strong and stable differences in behaviour between individuals. Behavioural differences arise as a part of the system of age-related, socially regulated division of labour common to species of social insects (Whitfield *et al.*, 2003). Numerous transcriptomic data for *A.m. carnica* and *A.m. ligustica* are publicly accessible (NCBI SRA, GEO, etc.). These data are separated by source (for instance *A. m. carnica* from China and Germany and *A. m. ligustica* from different countries), tissue (e.g. brain), sex and social function (queen bee, worker bee and drone). In our study, the same candidate genes same as in the genomic analysis, have been analysed to find differences between the Carniolan and Italian honeybees at the transcriptomic level. Similarly, as shown at the genome level, also at the transcriptomic level the collected data showed no difference between the transcripts from both subspecies.

Since only a limited number of genes is responsible for particular characteristics, and very often, some of them are epigenetically modified (Herb *et al.*, 2012; Zayed and Robinson, 2012), we decided to search publicly available data bases for informative epigenetic changes in Carniolan honey bees. Unfortunately, we could not find any differences in epigenetic marks, which would provide an insight into epigenetic modifications of the candidate genes that could be responsible for behav-

oural and physiological traits. In conclusion, it is quite surprising that neither at the genomic nor at the transcriptomic level any differences between both subspecies of honey bees could be found. This finding indicates a limited value of candidate gene analysis approach for honey bee subspecies comparisons. The main reason for that might be the fact that these two subspecies separated only ~ 25000 years ago (Wallberg *et al.*, 2014). However, analysis of whole genome sequences of honey bee subspecies, when they will be available, might shed a new light in subspecies differentiation at genomic and transcriptomic level.

4 PHENOTYPIC TRAITS IN *A. m. carnica*

A. m. carnica is often described as the grey honeybee. Its hairs are brownish – gray and short, giving overall gray perception. *Carnica*'s abdomen is free of »yellow rings« typical, for example, for *A. m. ligustica*, with the exception of the first abdominal segment, which is often leather coloured. Also, brownish spots could be present laterally on the 2nd tergite (Dews and Milner, 2004). Ruttner *et al.* (1978) measured 33 traits in their morphometric study of *Apis mellifera* species and performed multivariate analysis of the results. Among the characteristics contributing significantly to variability were hairiness and wing venation.

Poklukar (1992) discussed the length of worker bees' legs as a possible selection parameter. Milne and Pries (1984) suggested that large tibia could increase the pollen intake (and indirectly improve honey harvest) due to the larger surface of the corbicular area. Figure 5 shows the distribution of tibia's length from 31 apiaries across Slovenia. The median length of tibia is 3.23 mm (n = 6470). The second property, often discussed as an important morphological trait, but harder to measure due to the more complicated nature of preparation, is the proboscis length. Nakrst (2008) showed that median length of the proboscis of *A. m. carnica* is 6.54 mm (n = 1387), which is longer, compared to some other subspecies like *A. m. mellifera* (~ 6 mm) and *A. m. ligustica* (6.3–6.6 mm), but shorter compared to *A. m. caucasica* (~ 7 mm). However, the most popular trait for morphological determination is wing venation, already used by Ruttner *et al.* (1978) and improved by Tofilski (2008). The special focus is on so-called cubital index (CI), the ratio between segments' length of the bottom vein of third cubital cell on forewing (labeled as RK1 and RK2 in Fig 3C top). Tofilski (2008) mentions that RK1 is usually large in *A. m. carnica* workers and Rihar (2013) claimed that RK1 is at least twice the length of RK2. CI of selection hives of certified queen breeders in Slovenia must be

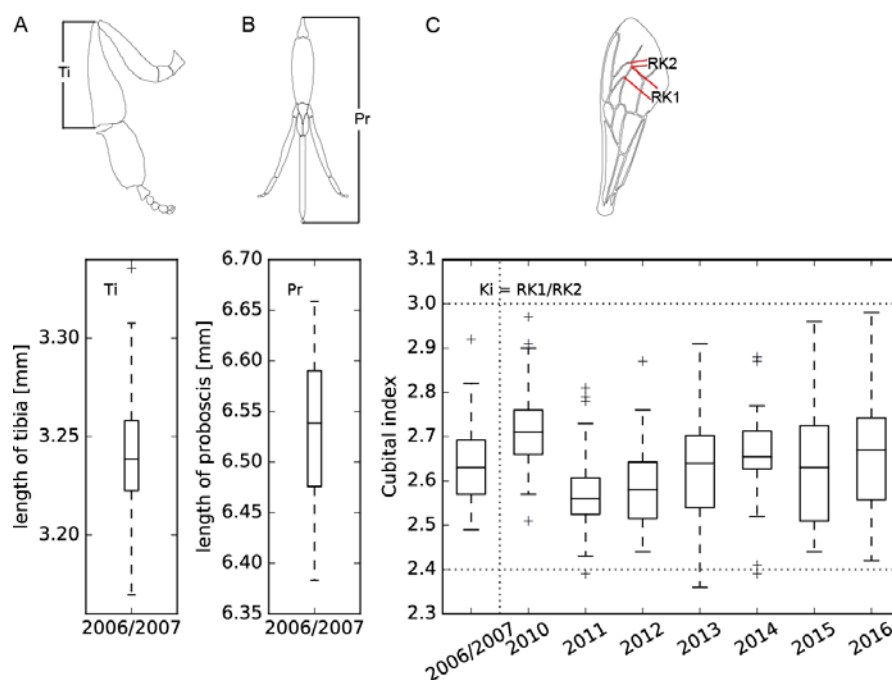


Figure 5: Lengths of worker tibia from 31 apiaries around Slovenia. 6470 tibias were measured by Nakrst (A). Lengths of proboscis from 25 apiaries around Slovenia. 1370 measurements were made (B). Cubital index of worker bees over several years. Top panel shows the two vein segments measured. The computed ratios are shown in the graph below. Data from 25 pseudorandomly selected apiaries are shown. Horizontal dotted lines show limits of acceptable values (C). Measurements in A and B and 2006/2007 in C from Nakrst (2008).

between 2.4 and 3.0. Figure 5 shows distribution of CI from 25 pseudorandomly selected apiaries all over Slovenia over several years. Not all of the samples complied with the prescribed expectations; it is evident, however, that the majority of measurements falls between the two limits (horizontal dotted lines). Verbič (2015) provided CIs measured recently on a sample of honey bees from Italy (Špeter, Italy). The author determined median CI less than 2, which is far below the expectations for Carniolan honey bee and low even for Italian subspecies.

Since the unambiguous morphological characterization and determination of subspecies is not always possible, it is easier, faster and probably also cheaper for a beekeeper or queen breeder to obtain a simple morphological parameter like CI and use it together with molecular determination.

5 PROTEOMIC ANALYSIS

Proteomic approach was another approach that we employed to define novel genetic markers to differentiate between local variations or ecotypes of Carniolan honey bee (*Apis mellifera carnica*) on the basis of molecular correlation and for its clearer distinction from the related subspecies.

Local living conditions can influence expression of some genes or type and level of their post-translational modifications. Induced by environmental factors, it has been shown that differences between specimens living in different environments appear only on the phenotypic level while on the genetic level these individuals are identical. A good example for this phenomenon is differentiation ecotypes of the nose-horned viper (*Vipera ammodytes ammodytes*). In the case of this snake the correlation between the polypeptide composition of the venom and the environment, in which a particular specimen was living, was clearly established in the study of geographically separated populations of the snake in the vicinity of Krapina, Karlovac and Slunj in Croatia (Lang Balija *et al.*, 2005; Halassy *et al.*, 2011). Based on this example we assume that polypeptide composition of the venom of the Carniolan honey bee population may reflect differences in local ensemble of their predators. Sampling of venom locally, from individual bee populations, turned to be a huge problem, however, it was much easier to obtain samples of royal jelly from the local beekeepers. It is well known that royal jelly carries out also antimicrobial function. Our assumption was therefore that differences in local nutrition sources and microbiomes, to which individual bee populations have been exposed, are reflected in specific polypeptide compositions of their

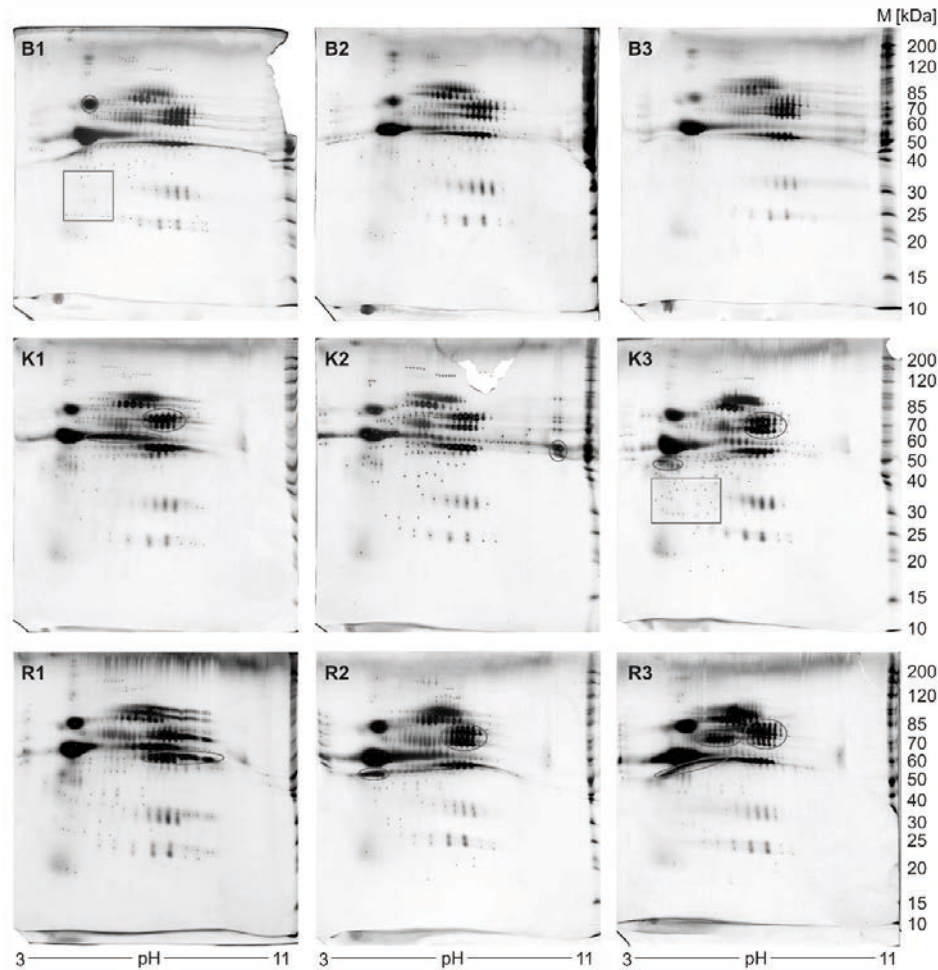


Figure 6: Comparative proteomics of *Apis mellifera carnica* royal jelly different Slovenian regions. Samples B and K originate from Gorenjska (Bohinj and Radovljica), while sample R is from Dolenjska region (Raka). High molecular mass protein components of the samples were separated using two-dimensional polyacrylamide gel electrophoresis. For each analysis, 50 μg of royal jelly was dissolved in the rehydration buffer (30 mM Tris, 7 M urea, 2 M thiourea, 0.002% (m/v) bromophenol blue, 2.5% (m/v) CHAPS, 0.25% (m/v) ABS-14 (alkilamidofobetaïn-14), 1% (v/v) ampholytes (pH 3–11), 1.2% (v/v) DeStreak Reagent), and in this solution 7-cm long electrophoretic strip, immobilized with a non-linear 3–11 pH gradient (GE Healthcare, USA), was immersed overnight. Analyses were performed as detailed in Leonardi *et al.* (2012). Groups of protein spots that specifically differ between samples are encircled.

royal jelly. For these reasons we decided to comparatively analyse polypeptide composition of royal jellies of Carniolian honey bees from different geographical locations in Slovenia. Samples were obtained in September 2015 from two regions, from Raka in Dolenjska (R) and from two places in Gorenjska, Bohinj (B) and at Kozinc apiaries around Radovljica (K). On each site, three samples of royal jelly were separately collected, each from a different open queen cell (R1–R3, B1–B3 and K1–K3). The queen cells at each location represent three different hives.

Two-dimensional polyacrylamide gel electrophoresis (2DE) analyses of royal jelly samples are displayed in Fig. 6. In each gel we determined the number of all protein spots that were clearly separated and observed

differences in the representation of high molecular mass (> 10 kDa) protein components in the analysed samples. The highest number of discrete protein spots (271) has been counted in the sample K3 from Gorenjska, while the lowest (230) in the sample B2 from the same Slovenian region but from another place. The number and the position of the most intense protein spots in samples collected at different locations were not significantly diverse. On the contrary, such differences are clearly apparent among low abundance proteins. However, as it has been already published (Schönleben *et al.*, 2007), the main problem for accuracy of analysis of the low abundance proteins in royal jelly is due to the high abundance proteins. The most similar 2DE protein patterns were ob-

tained with royal jellies B1–B3 from Gorenjska region. In this group of samples, the B1 sample contained more low abundance proteins (inside square in Fig. 6) and one more intense spots at acidic pH around 80 kDa. Based on the 2DE protein spot patterns and the intensity of some protein spots in the K series of samples (marked in Fig. 6), it is possible to clearly distinguish between individual K samples, and, in addition, between K and B samples. Specific differences are evident between three R samples from Dolenjska region; however, their 2DE patterns are clearly distinguishable from those of samples originating from Gorenjska region. Obviously, the high molecular mass protein composition of the *A. m. carnica* royal jelly is much richer than that of its venom. With about 250 individual protein spots per 2DE gel, royal jelly represents much more complicated and therefore reliable protein fingerprint to possibly define individual ecotypes of Carniolan honey bee than the venom with only about 49 spots (Peiren *et al.*, 2005). The detailed analysis of 2D profiles shows that individual samples of royal jelly differ as well within the same location as between locations. This clearly shows that environmental factors have an important influence (different locations), but also possible genetic contribution (differences between samples from the same location) seems to play a role.

By the means of mass spectrometry and bioinformatics, we will structurally identify in the next step components of the Carniolan honey bee royal jelly, which abundance in samples from different locations characteristically varies, in order to define specific environmental factors which may significantly define the composition of royal jelly from different locations. However, determination of environmental and possibly even genetic factors underlying these differences remains a significant challenge for further research.

6 BEHAVIOURAL CHARACTERISTICS

Behaviour differences between subspecies of honeybees were observed mainly at the level of colonies, related to the development, swarming and overwintering (Page *et al.*, 1995; Brillet *et al.*, 2002). At individual level it is harder to measure specific parameters that can be linked to the subspecies specificity. Best documented are defense behavior (aggression) (Alaux *et al.* 2009) and dance communication (Rinderer and Beaman, 1995). It is interesting that evolutionary closely related subspecies, *A. m. ligustica* and *A. m. carnica* are the most distinctive in the dance communication, at least while they are reporting larger distance to foragers, above 100 m (Boch, 1957). It has been recently reported that there might be only one genetic locus responsible for the observed dif-

ferences in dance pattern formation at shorter distances (Johnson *et al.* 2002). Carniolan bees do not perform sickle dance during the change from round to waggle dance and they do that change much slower than it was reported for Italian and other subspecies. We set out to check reported differences in bees from authentic regions of both subspecies and from colonies maintained at the border region.

Dance communication experiment was performed in two frame observation hive that was set up with one brood frame and the queen from the Carniolan colony, maintained before at the border area with Italy (west of Kobarid) in June 2014. In addition, 1000 Italian and 1000 Carniolan bee workers were added, each from two different colonies maintained at the border region and populated with queens from certified breeders of Carniolan and Italian subspecies from Slovenia and Italy, respectively. Observations were performed within one month with additional repopulation of the family with both subspecies after 10 days. Experiments were done at 25, 40, 55, 70 and 91 meters distances of the artificial feeder from the beehive. Bees were individually marked with colour marks and after departure their behaviour was recorded inside the hive. Behaviours of returned bees were later evaluated with replay of video recordings using computer video viewers during slow and frame by frame replay. Bees that did two consecutive full turns of the body were recognized as a bee dancers. Number of video-frames was used to estimate duration of one dance turn and the number of body positions at up, down, left and right direction was used to evaluate orientation of the body toward food site using vector's calculations. Duration of the dance turn against the distance was evaluated using linear regression analysis and body orientations were tested with Z-test. For each distance and subspecies were analysed about 10 individuals with two dances each.

In this experiment, we were not able to distinguish well sickle dance from round dance. Instead of trying to determine shape of the dance we rather checked dependence of duration of one complete turn from the distance of the food source. We were not able to detect significant regression coefficients for all included distances. When we exclude the first 25 m distance, which has high variability in the duration, we got significant regression coefficient for Italian bees but not for Carniolan bees (Fig. 6). In addition, we also observed that regression coefficients were also related to general higher values of full turn time in Carniolan bees than in Italian ones. Estimated regression line crossed at around 110 m distance. Although Carniolan bees didn't show significant dependence of turn time on the distance they showed better direction orientation at this short distances that Italian bees. Orientation tendency – flights pointed at food source direc-

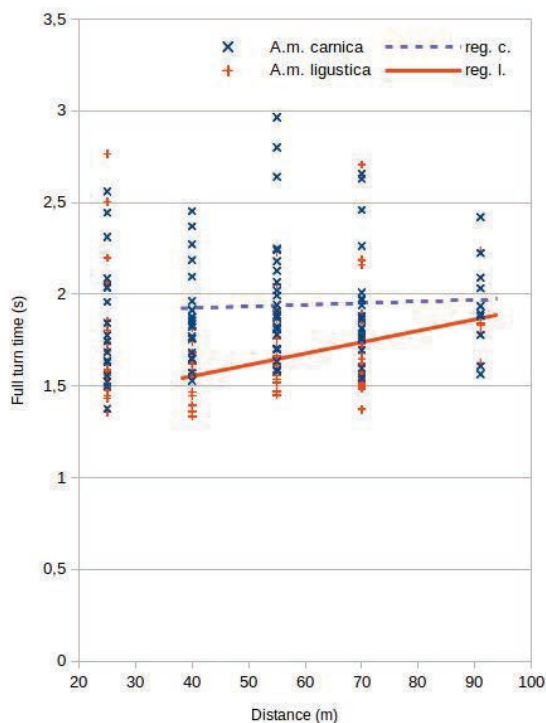


Figure 7: Dependence of full turn time in the dances of Carniolan (*A. m. carnica*) and Italian (*A. m. ligustica*) bees. Only for Italian bees it was possible to fit regression line (reg. coef. 0.0062 ± 0.0018 , $p < 0.001$, solid line), but not for Carniolan bees ($p = 0.683$) at the distance range 40 to 91 m. The shortest tested distance 25 m was excluded from the final regression model.

tion, measured in 138 dances, was significantly lower in Italian bee (29.45 %) compared to Carniolan bee (47.15 %, »z« test = 4.30; $p < 0.0001$).

The reported differences in transition from round dance to waggle dance between Italian and Carniolan bees (Boch, 1957; Rinderer and Beaman, 1995; Johnson *et al.*, 2002) might be caused by different offset of duration of the complete turn in round dance at forage a few meters away from the hive and degree of increase in one complete turn duration during increase of the forage distance. That might be the base of possible body turns and the form of the dance. Carniolan bees might do better orientation of the dances at short distance due to the higher offset close to zero distance. Well oriented dances were observed also on the base of the body orientation during producing of the dance sounds (Kirchner *et al.*, 1988). Taking into account that behavioural traits are often determined by a low number of genes in honey bee (Zayed and Robinson, 2012), the observed differences between Italian and Carniolan bee dances might be based on simple modification of neurotransmission inside of central excitatory state for performing communication dance. It would not be surprising if that could have some

connection with other well known differences between this two subspecies.

7 REFERENCES

- Act concerning the conditions of accession of the Czech Republic, the Republic of Estonia, the Republic of Cyprus, the Republic of Latvia, the Republic of Lithuania, the Republic of Hungary, the Republic of Malta, the Republic of Poland, the Republic of Slovenia and the Slovak Republic and the adjustments to the Treaties on which the European Union is founded - Final act - III. Other Declarations - M. Declarations by the Republic of Slovenia - 42. Declaration by the Republic of Slovenia on the Slovenian indigenous bee *Apis mellifera carnica* (kranjska čebela). (2003). European Parliament.
- Alaux, C., Sinha, S., Hasadsri, L., Hunt, G. J., Guzmán-Novoa E., DeGrandi-Hoffman, G., Uribe-Rubio, J. L., Southey, B.R., Rodriguez-Zas, S., Robinson, G. E. (2009). Honey bee aggression supports a link between gene regulation and behavioral evolution. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 15400–15405.
- Boch, R. 1957. Rassenmassige unterschiede bei den tanzen der honigbiene (*Apis mellifica* L.). *Zeitschrift für Vergleichende Physiologie*, 35, 136–167

- Brillet, C., Robinson, G. E., Bues, R., Le Conte, Y. (2002). Racial Differences in Division of Labor in Colonies of the Honey Bee (*Apis mellifera*). *Ethology*, 108, 115–126. doi:10.1046/j.1439-0310.2002.00760.x
- Dews, J. E., Milner, E. (2004). Breeding better bees. 4th edition. British Isles Bee Breeders Association.
- Gogala, A. (1999). Bee fauna of Slovenia: checklist of species (Hymenoptera: Apoidea). *Scopolia*, 42, 1–79.
- Elsik, C. G., Worley, K. C., Bennett, A. K., Beye M., Camara F., Childers C. P., de Graag D. C. et al. (2014). Finding the missing honey bee genes: lessons learned from a genome upgrade. *BMC Genomics*, 15, 86.
- Halassy, B., Brgles, M., Habjanec, L., Lang Balija, M., Kurtović, T., Marchetti Deschman, M., Križaj, I., Allmaier, G. (2011). Intraspecific variability in *Vipera ammodytes ammodytes* venom related to its toxicity and immunogenic potential. *Comparative Biochemistry and Physiology-Part C*, 153, 223–230.
- Harpur, B. A., Kent, C. F., Molodtsova, D., Lebon, J. M., Alqarni, A. S., Owayss, A. A., Zayed, A. (2014). Population genomics of the honey bee reveals strong signatures of positive selection on worker traits. *Proceedings of the National Academy of Sciences of the United States of America*, 111, 2614–2619.
- Herb, B. R., Wolschin, F., Hansen, K. D., Aryee, M. J., Langmead, B., Irizarry, R., Amdam, G. V., Feinberg, A. P. (2012). Reversible switching between epigenetic states in honeybee behavioral subcastes. *Nature Neuroscience*, 15, 1371–1373.
- Honey Bee Genome Sequencing Consortium. (2006). Insights into social insects from the genome of the honey bee *Apis mellifera*. *Nature* 443, 931–949.
- Johnson, R.N., Oldroyd, B. P., Barron, A. B., Crozier, R. H. (2002). Genetic control of the honey bee (*Apis mellifera*) dance language: segregating dance forms in a backcrossed colony. *Journal of Heredity*, 93, 170–173.
- Kirchner, W. H., Lindauer, M., Michelsen, A. (1988). Honeybee dance communication: Acoustical indication of direction in round dances. *Naturwissenschaften*, 75, 629–630. doi:10.1007/BF00366482
- Lang Balija, M., Vrdoljak, A., Habjanec, L., Dojnović, B., Halassy, B., Vranešić, B., Tomašić, J. (2005). The variability of *Vipera ammodytes ammodytes* venoms from Croatia: Biochemical properties and biological activity. *Comparative Biochemistry and Physiology-Part c*, 140, 257–263.
- Leonardi, A., Biass, D., Kordiš, D., Stöcklin, R., Favreau, P., Križaj, I. (2012). *Conus consors* snail venom proteomics proposes functions, pathways, and novel families involved in its venom system. *Journal of Proteome Research*, 11, 5046–5058.
- Milne, C.P., Pries, K.J. (1984). Honeybee corbicular size and honey production. *Journal of Apicultural Research*, 23(1), 11–14.
- Nakrst, M. 2008. Morphological characterization of honeybee (*Apis mellifera carnica*) workers and queens in queen rearing apiaries. Graduation thesis. (68 p), University of Maribor.
- Official Gazette of Republic of Slovenia. 18/2014. Resolucija o zaščiti kranjske čebele.
- Official Gazette of Republic of Slovenia. 45/2004. Uredba o programih kmetijske strukturne politike in kmetijske politike razvoja podeželja za leti 2005 in 2006.
- Official Gazette of Republic of Slovenia. 110/2002. Zakon o urejanju prostora.
- Official Gazette of Republic of Slovenia. 18/2002. Zakon o živinoreji.
- Page, R. E., Robinson, G. E., Fondrk, M. K., Medhat, E. N. (1995). Effects of worker genotypic diversity on honey bee colony development and behaviour (*Apis mellifera* L.). *Behavioral Ecology and Sociobiology*, 36, 387. doi:10.1007/BF00177334
- Peiren, N., Vanrobaeys, F., de Graaf, D. C., Devreese, B., Van Beeumen, J. Jacobs, F. J. (2005). The protein composition of honeybee venom reconsidered by a proteomic approach. *Biochimica et Biophysica Acta*, 1752(1), 1–5.
- Poklukar, J. (1992). Genetski parametri površine tibije domaće pčele (*Apis mellifera carnica*) i proizvodnja meda. Dissertation (116 p.), Sveučilište u Zagrebu.
- Rihar, J. (2013). Vzrejamno boljše čebele. 4th ed., Ljubljana: Pansan, 272 p.
- Rinderer, T. E., Beaman, L. D. (1995). Genic control of honey bee dance language dialect. *Theoretical and Applied Genetics*, 91, 727–732. doi:10.1007/BF00220950
- Robinson, G. E., Grozinger, C. M., Whitfield, C.W. (2005). Sociogenomics: social life in molecular terms. *Nature Reviews Genetics*, 6, 257–270.
- Ruttner, F., Tassencourt, L., Louveaux, J. (1978). Biometrical-statistical analysis of the geographic variability of *Apis mellifera* L. I. Material and Methods. *Apidologie*, 9(4): 363–381.
- Schönleben, S., Sickmann, A., Mueller, M. J., Reinders, J. (2007). Proteome analysis of *Apis mellifera* royal jelly. *Analytical and Bioanalytical Chemistry*, 389, 1087–1093.
- Smith, C.R., Toth, A.L., Suarez, A.V., Robinson, G.E. (2008). Genetic and genomic analyses of the division of labour in insect societies. *Nature Reviews Genetics*, 9, 735–748.
- Sušnik, Bajec S., Kozmus, P., Poklukar, J., Meglič, V. (2004). Molecular characterisation of indigenous *Apis mellifera carnica* in Slovenia. *Apidologie*, 35, 623–636.
- Tofliski, A. (2008). Using geometric morphometrics and standard morphometry to discriminate three honeybee subspecies. *Apidologie*, 39, 558 – 563.
- Verbič, J. (2015). The basic morphological characterization of honeybee workers obtained from beekeepers with their own breeding of queen bees in wider region of Dolenjska. Graduation thesis (25 p.) University of Ljubljana.
- Wallberg, A., Han, F., Wellhagen, G., Dahle, B., Kawata, M., Haddad, N., Simões, Z.L., Allsopp, M.H., Kandemir, I., De la Rúa, P., Pirk, C.W., Webster, M.T. (2014). A worldwide survey of genome sequence variation provides insight into the evolutionary history of the honeybee *Apis mellifera*. *Nature Genetics*, 46, 1081–1088.
- Whitfield, C.W., Cziko, A.M., Robinson, G.E. (2003). Gene expression profiles in the brain predict behavior in individual honey bees. *Science*, 302, 296–299.
- Zayed, A., Robinson, G. E., (2012). Understanding the relationship between brain gene expression and social behavior: lessons from the honey bee. *Annual Review of Genetics*, 46, 591–615.