

**PHYLOGENETIC RELATIONSHIPS OF DARK EUROPEAN
HONEYBEES *APIS MELLIFERA MELLIFERA* L.
FROM THE RUSSIAN URAL
AND WEST EUROPEAN POPULATIONS**

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Received 25 January 2011; Accepted 26 April 2011

S u m m a r y

A mitochondrial DNA region covering 5'-end of the gene of the second subunit NADH dehydrogenase (ND2), was PCR amplified in Dark European Honeybees *Apis mellifera mellifera* from the Urals (Russia) region and in the presumed *Apis mellifera macedonica* from the Ukraine. Phylogenetic analysis of sequenced DNA samples with GenBank (<http://www.ncbi.nlm.nih.gov>) data showed the existence of four evolutionary branches where the Ural honeybees were grouped with European *A. m. mellifera*, and Ukrainian honeybees with European *A. m. macedonica*. These results suggest the genetic relationship of the Russian Urals and the European populations of *A. m. mellifera*. These data have allowed us to put forward the hypothesis that the subspecies *A. m. mellifera*, is a unique specimen of evolutionary branch M.

Keywords: honeybees, Dark European Honeybees, *Apis mellifera mellifera*, mtDNA, genetic variation, ND2, sequencing, Urals, Russia.

INTRODUCTION

Apis mellifera has an extraordinarily huge natural distribution area spanning from Southern Scandinavia in the North to the Cape of Good Hope in the South; from Dakar in the West to The Altai Republic, the Oman coast, and western borders of China in the East (Ruttner et al., 1978; Sheppard and Meixner, 2003). It has been shown, that different types of honeybees in all occupied territories are subspecies and they are capable of crossing with each other, producing prolific descendants (Ruttner et al., 1978). Due to long geographical isolation and ecological adaptation there is a deep differentiation of honeybees leading to a big diversity of subspecies.

About 26 subspecies and numerous ecotypes have been described, based on behaviour, morphology, and molecular

evidence (Ruttner, 1992; Sheppard et al., 1997; Sheppard and Meixner, 2003; Meixner et al., 2010). Although Engel (1999) reported an existing 28 subspecies of honeybees, we have some doubts about it. The honeybee subspecies *A. m. major* (Ruttner, 1975) from Morocco and Northern Africa are not included in the nomenclature which has been accepted by Hepburn and Radloff (1996) as ecotype of *A. m. intermissa* (Maa, 1953). Ukrainian honeybees, earlier labeled as subspecies *Apis mellifera acervorum* (Scorikov, 1929), later have been referred to as *Apis mellifera sossimai* (Engel, 1999). Our recent data based on mtDNA analysis showed that the latter was not a separate subspecies, but only an ecotype of *Apis mellifera macedonica* (Ruttner, 1987). Also, subspecies *Apis mellifera ruttneri* (Sheppard et al., 1997)

from Malta, and *A. m. artemisia* (Engel, 1999), *A. m. taurica* (Alpatov, 1938), *Apis mellifera rodopica* (Petrov, 1990; 1991, 1995; Petrov and Ivanova, 2009; Ivanova, 2010; Ivanova et al., 2010 a; Ivanova et al., 2010 b) from Bulgaria have doubtful taxonomic status. In recent investigations, Ivanova et al. (2010 a; b) using polymorphisms of 4 allozyme loci (MDH, ME, EST and ALP) and RFLP of 3 mtDNA loci (16s rDNA, COI and ND5) showed that honey bee populations of Bulgaria were different from bee populations of Greece which previously were considered to belong to *A. m. macedonica* subspecies according to the research of Bouga et al. (2005), Ivanova (2010) and Ivanova et al. (2010). The recently discovered new subspecies *Apis mellifera pomonella* (Sheppard and Meixner, 2003) from Tien-Shan between Kazakhstan and the Chinese border could lengthen the modern list of *A. mellifera* subspecies.

Recently, our research has shown that among hybrid honeybees, at least four local populations of Dark European Honeybees *A. m. mellifera* were saved in the Urals (Ilyasov et al., 2006; Ilyasov et al., 2007). This research has shown the phylogenetic relationships of Russian and European populations of *A. m. mellifera*.

MATERIALS AND METHODS

We used honeybees of the *A. m. mellifera* subspecies from burzyanskaya (DQ181611-DQ181613) and tatyshlinskaya (DQ181614-DQ181616) populations - from the Bashkortostan republic of Russia, and visherskaya (DQ181617-DQ181619) and yuzhno-prikamskaya (DQ181620-DQ181622) populations - from the Perm region of Russia. These subspecies had earlier been discovered using polymorphism of intergenic locus COI-COII mtDNA (Ilyasov et al., 2007) (Fig. 1). We also analyzed honeybees from the Ukrainian apiary of Alexandr D. Komissar (DQ361088-DQ361090). The apiary is located near Kiev. Three individuals of

each of the 5 populations were analyzed. All 15 honey bee samples were sequenced. This amount was sufficient for reliable phylogenetic conclusions.

Genomic DNA extracted from honeybee thorax muscles was fixed in 96% ethanol. The total extraction of nucleic acids was conducted as described elsewhere (Chomzynski and Sacchi, 1987). Amplification was implemented in thermocycler Tercyc (DNA технологиya). PCR reactions were performed with corresponding 1 x buffer, 2 mM MgCl₂, 0.2 mM of each dNTP, 0.15 μM of primers forward 5'-TGATAAAAGAAATATTTTGA-3' and reverse

5'-GAATCTAATTAATAAAAAA-3' (Arias and Sheppard, 1996) and 1 unit of polymerase in a total volume of 30 μL. Reactions were submitted to an initial 5 min denaturation at 94°C, then 30 cycles of 94°C for 1 min, 42°C for 2 min, and 72°C for 2 min, and a final extension of 10 min at 72°C. Purified double-stranded PCR products were sequenced using the ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, USA) as recommended by the manufacturer (Amersham Pharmacia Biotech DYEnamic ET Terminator Cycle Sequencing Kit).

A fragment of 632 bp in mtDNA ND2 gene (from 502 to 1134 bp according to full mitochondrial DNA sequence of *Apis mellifera ligustica* Spinola from international GenBank NCBI (NC 001566) was sequenced in 15 honeybees. Sequenced fragments have been deposited to GenBank database (Ilyasov et al., 2005; Ilyasov et al., 2006). Phylogenetic analysis carried-out using fragments of sequences gene ND2 mtDNA, in size 416 bp, were aligned (502 to 918 bp of NC 001566) in program MEGA 3.1 (Kumar et al., 2004) using CLUSTAL W method. In phylogenetic analysis, we used sequences from a fragment of the ND2 gene mtDNA subspecies of *A. mellifera*: U35743-U35765, AY114484-AY114511, AY135560, AY136624 - AY136625, AY618910 - AY618911, AY618919 - AY618920.

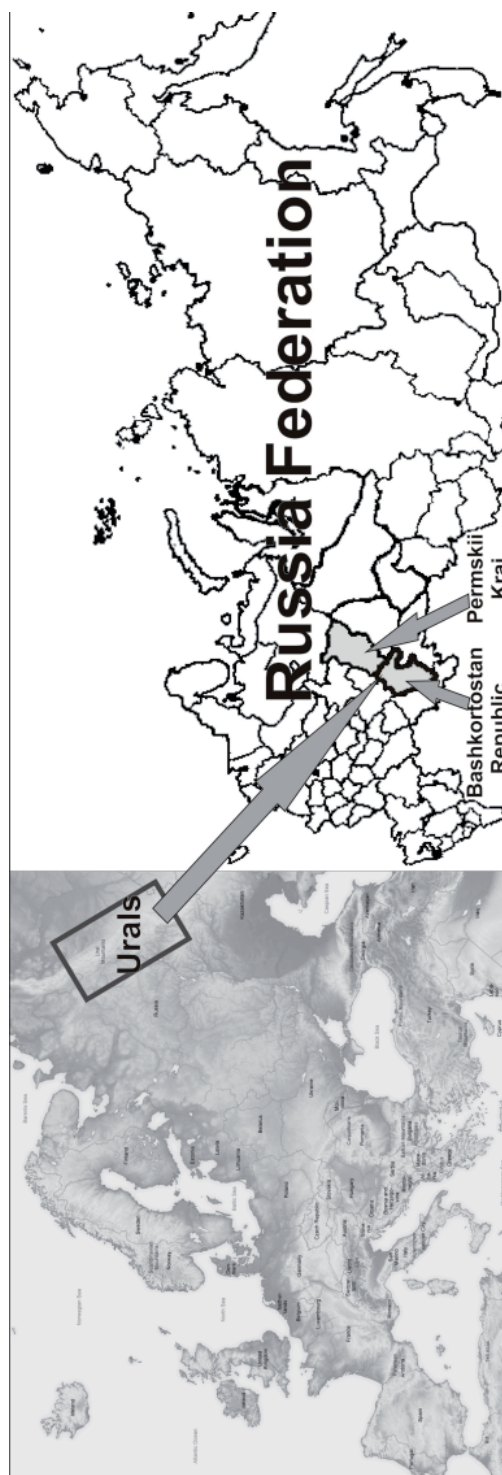


Fig. 1. Geographic location of areal honeybee *Apis mellifera mellifera* populations in the Russian Urals.

For rooting, the phylogenetic tree of subspecies was compared with sequences of a fragment of the ND2 gene mtDNA of honeybees of other species - *Apis cerana* AY849558 - AY849569 and *Mellipona bicolor* - AF466146 and NC004529.

Median network was constructed (Fig. 2) in program NETWORK (Fluxus Engineering). As a reference sequence, a fragment of mtDNA ND2 gene from the Near East subspecies *A. m. anatoliaca* (AY114499) was used.

RESULTS

Twelve nucleotide replacements were observed in sequenced mtDNA ND2 gene fragment in honeybees from the Russian Urals (Fig. 1) and Ukrainian populations, relative to the reference sequence of burzyan cave nesting honeybees (DQ181611). In examined honeybee populations from the Urals, 5 nucleotide substitutions were revealed where T>C replacement at position 536 in two honeybees DQ181614 and DQ181618 led to replacement in peptide aminoacid sequences in position 12 from Ile to Thr. Ukrainian honeybees differed by 8 nucleotide positions from the reference sequences, where replacement in position 987 was a transversion. The Ukrainian honeybees did not have strong differences. In the Ukrainian honeybees, only one nucleotide replacement in the ND2 gene fragment at position 1099 was detected.

DISCUSSION

All the samples on median network (Fig. 2) are grouped into four distinct clades (cognate to evolutionary branch A, C, M and O). The names of the clades are given according to Ruttner (1988), Arias and Sheppard (1996) and Franck et al. (2001)

The first group (cognate to evolutionary branch A) comprises mostly African honeybee subspecies. It is subdivided into two subgroups, one of which unites most of the African subspecies of honeybees to the south from the Sahara: *A. m. adansonii*

from Nigeria (U35743), *A. m. scutellata* (U35764), *A. m. capensis* (U35747) from the South Africa Republic, *A. m. monticola* (U35761) from Kenya, and africanized honeybees from Brazil (U35745-46). Other subgroups include *A. m. sahariensis* (U35762) and *A. m. inermis* (U35751) from Morocco, *A. m. sicula* (U35765) from Sicily, and *A. m. iberica* (U35750) from Portugal. The position of *A. m. sicula* samples in this group may possibly be explained by the hybrid origin of Sicilian honeybees where Franck et al. (2000b) demonstrated grouping of African and Mediterranean mitotypes. Such introgressive hybridization between samples of the evolutionary branches A and C has also been shown by Arias and Sheppard (1996), and Franck et al. (2001). Grouping of *A. m. iberica* with the African group is shown here for the first time and was quite unexpected. Earlier, Franck et al. (2001) showed possible intensive migratory behavior of African honeybees which could have been caused by their hybridization with *A. m. iberica* on the Iberian Peninsula. But, the anthropogenic factor should not be excluded as a possible reason for the hybridization of *A. m. iberica*. The ecocline structure of *A. m. iberica* in Spain from *A. m. mellifera*-like bees in the north to *A. m. inermis*-like, in the south is well known. An ecocline structure has also been described earlier based on morphological (Ruttner et al., 1978), allozyme data (Sylvester, 1982; Cornuet, 1983, 1986; Sheppard and Berlocher, 1984; Nunamaker et al., 1984), and mtDNA RFLP data (Smith, 1991; Garnery et al., 1995; Franck et al., 1998).

The subdivision of subspecies into southern and northern African groups is probably caused by the desertification processes in Africa, and expanding deserts which are serious geographical barriers for honeybees (Potts and Behrensmeier, 1992). It is possible that such barriers were not a complete and the honeybees were able to migrate, though to a lesser extent.

If true, such a migration possibility is probably the reason why differentiation is not as deep as believed before Ruttner (1988). Migrations of honeybees between southern and northern African groups could occur along the coast of Africa.

The second group (cognate to evolutionary branch O) included a small sample number of the subspecies *A. m. meda* (U35756) and *A. m. syriaca* (AY618920) from Syria, and *A. m. lamarckii* (U35753) from Egypt. Arias and Sheppard (1996), Franck et al. (2000 a) also attributed subspecies *A. m. lamarckii* to branch O instead of branch A of Ruttner et al. (1988). It is possible, that subspecies *A. m. lamarckii* in Egypt have been intensively hybridized both by African and Near-Eastern subspecies of honeybees which led to the discrepancies of this subspecies classification.

The third group (cognate to evolutionary branch M) comprises samples of *A. m. mellifera* from the Russian Urals (DQ181611-22) and the European populations: Switzerland (AY114495), France (U35758), Spain (U35759) and Norway (U35760). However, two samples of subspecies *A. m. sicula* (AY114493-94) from Sicily and *A. m. ligustica* (U35752 and AY114490) from Italy belong to this clad as well, which confirms hybridization with *A. m. mellifera*. It is known that there was constant hybridization between samples of evolutionary branches C and M in Italy, and between branches C, A and M in Sicily (Franck et al., 2000b). Absence of samples of subspecies *A. m. iberica* in clade M, does not confirm the hypothesis of some authors, that the evolutionary branch of M consists of at least two Western European subspecies - *A. m. iberica* and *A. m. mellifera*, which reached Western Europe from Morocco through the Iberian peninsula. A similar result has been shown by Arias and Sheppard (1996) where *A. m. iberica* was grouped separately from *A. m. mellifera*. Franck et al. (2001) using microsatellite loci and intergenic locus COI-COII mtDNA mitotypes, and

Garner et al. (1992) using mtDNA of *A. m. iberica* from the Iberian peninsula, have shown clinal changes from *mellifera*-like bees in the north, to *intermissa*-like in the south. These authors have assumed these changes are a consequence of recent contacts of honeybees from evolutionary branches A and M. Thus, *A. m. mellifera* is a unique representative of evolutionary branch M which is shown by our data.

The fourth group (cognate to evolutionary branch C) consists of a large number of subspecies and comprises honeybees from the Mediterranean, the Near East and the Caucasus: *A. m. ligustica* from Italy, *A. m. carnica* from Germany, Austria and Slovenia, samples *A. m. macedonica* from Greece and the Ukraine, *A. m. cecropia* from Greece, *A. m. sicula* from Sicily, *A. m. cypria* from Cyprus, *A. m. adami* from Crete, *A. m. caucasica* from the Caucasus (some researchers delegate this subspecies to evolutionary branch O), *A. m. meda* from Syria, *A. m. anatoliaca* from Turkey, *A. m. syriaca* from Syria and *A. m. pomonella* from Tien Shan. This clade has a typical star like phylogeny showing quite recent expansion. Median network demonstrates that in evolutionary branch C, a very high frequency of subspecies with mitotype C - 20 in honeybees samples of 7 subspecies, was observed. It is also necessary to stress the fact that mtDNA intergenic locus COI-COII comprises only one element Q in samples from evolutionary branch C, whereas samples from other evolutionary branches comprise from one to four Q elements with the addition of element P. It may seem that mitotype of evolutionary branch C is very similar to ancestral forms of *A. mellifera*. Yet, evolutionary branches A, M, C and O had almost identical amounts of mutational changes from the common ancestor. Therefore, evolutionary branch C is not an ancestor form, just very similar to ancestral forms of *A. mellifera*.

Samples of *A. m. macedonica* (DQ361088-90) from the Ukraine are united on median network with European samples of *A. m. macedonica*

(U35755, AY114503-04). This unity proves that at least a part of the native honeybees from the Ukraine belong to the subspecies *A. m. macedonica*, but not to *A. m. acervorum* as was earlier believed (Skorikov, 1929).

Samples of the *A. cerana* bee species also show considerable differentiation from *A. mellifera*. Geographical differentiation of *A. cerana* has been marked Tilde et al. (2000) on the basis of morphological characters, Smith et al. (2000, 2004), Sihanunthavong et al. (1999) on the basis of mtDNA and Sittipraneed et al. (2001) on the basis of microsatellite loci. *Mellipona bicolor* samples in honeybee sequence comparisons allowed us to determine the correct root and make conclusion about relationships of *Apis* species where *Apis cerana* and *Apis mellifera* are sisterly species.

Sequence comparisons of ND2 gene mtDNA from all bee samples with DNASTAR program between *Mellipona bicolor*, and each of the species *Apis* - *A. mellifera* and *A. cerana*, revealed 22.18% and 23.02% of differences accordingly. Between *A. mellifera* and *A. cerana* the value is 15.54 %. DeSalle et al. (1987) has shown that differences for *Drosophilla* in mtDNA sequence, is 2% which corresponds to the time of divergence between two species in 1 million years (Arias and Sheppard, 1996; Arias and Sheppard, 2005). Using this rate, we calculated a divergence time between *Mellipona bicolor* and *A. mellifera* that would be equal to about 11.09 million years, between *Mellipona bicolor* and *A. cerana* it would be equal to about 11.5 million years and between *A. mellifera* and *A. cerana* it would be equal to about 7.77 million years, which is similar to the divergence time between *Apis* species designed by Arias and Sheppard (2005). Species *A. mellifera* and *A. cerana* separated from *Mellipona bicolor* about 11.3 million years ago, and separated from the common ancestor of *Apis* about 3.89 million years ago. *A. cerana* was separated from the common

ancestor about 0.42 million years earlier than *A. mellifera*. The evolutionary age of *A. mellifera* is about 3.47 million years, and for *A. cerana* it is about 4.31 million years.

CONCLUSIONS

Thus, our research has shown the close genetic relationship of the Russian Urals and the Western European populations of *A. m. mellifera*. Honeybees *A. m. mellifera* living widely throughout Eurasia are characterized as belonging to a single subspecies which is the base of the evolutionary branch M. Ukrainian honeybees from the apiary of Alexandr D. Komissar, located near Kiev which we have confirmed as belonging to honeybee subspecies *A. m. macedonica*. This conclusion contradicts the hypotheses reported by Gubin (1975) which considered the Ukrainian honeybees as populations of *A. m. carnica* and Engel (1999), who catagorized them as subspecies *A. m. sossimai*.

ACKNOWLEDGMENTS

We gratefully acknowledge grand RFBR OFI №06-04-08183 and Agidel for supporting our investigations and to Dr. Alexandr D. Komissar for providing samples of Ukrainian honeybees from his apiary near Kiev (DQ361088, DQ361089, DQ361090). In the future we also plan to research the molecular taxonomy of honey bee populations in Eastern Europe.

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**FILOGENETYCZNE ZWIĄZKI PSZCZOŁY
ŚRODKOWOEUROPEJSKIEJ *APIS MELLIFERA MELLIFERA* L.
I POPULACJI URALSKICH
ORAZ ZACHODNIOEUROPEJSKICH**

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S t r e s z c z e n i e

Region mitochondrialnego DNA pszczoły środkowoeuropejskiej *Apis mellifera mellifera* z Uralu (Rosja) oraz *Apis mellifera macedonica* z Ukrainy, obejmujący koniec 5' genu drugiej podjednostki dehydrogenazy NADH (ND2), został amplifikowany za pomocą reakcji PCR. Analiza filogenetyczna sekwencjonowanych próbek DNA z danymi GenBank (<http://www.ncbi.nlm.nih.gov>) wykazała istnienie czterech odnóg ewolucyjnych, gdzie pszczoły z Uralu zostały zgrupowane z europejską pszczolą *A.m.mellifera*, natomiast pszczoły ukraińskie z europejską pszczolą *A.m.macedonica*. Wyniki te sugerują związek genetyczny populacji *A.m.mellifera* z Uralu i z Europy. Dane te umożliwiły nam wysunięcie hipotezy, że podgatunek *A.m.mellifera* jest unikatowym przykładem gałęzi ewolucyjnej M.

Słowa kluczowe: pszczoła miodna, pszczoła środkowoeuropejska, *Apis mellifera mellifera*, mtDNA, zmienność genetyczna, ND2, sekwencjonowanie, Ural, Rosja.

