USING DNA MICROSATELLITE MARKERS TO EXPLAIN THE DIFFICULTY IN DISTINGUISHING THE CAUCASIAN BEES FROM THE CARNIOLAN BEES BASED ON WING VENATION

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Introduction

Most of honey bees reared in Poland belong to three subspecies: *A. m carnica*, *A. m. caucasica* and *A. m. mellifera*. Breeders declare affiliation of bees to one of these subspecies and their racial purity is verified by testing configuration of 19 points on the forewing, which gives a high confidence of classification (Gerula et al. 2009; Bouga et al. 2011). However, classification it is not always suitable, especially in distinguishing some Caucasian (*A. m. caucasica*) and Carniolan (*A. m carnica*) bees. It was shown that the range of metric variability of the forewings in *A. m carnica* and *A. m. caucasica* strongly overlap, making classification of these bees very unreliable (Gerula et al. 2009). Therefore, additional criteria for distinguishing these races should be used. The molecular analysis can provide better insight into the origin of honey bees. Traditionally, analyses of mitochondrial DNA (mtDNA) were used to investigate the source of breeding material in honey bees (i.e., Garnery et al. 1992; Franck et al. 1998). The fact that the mtDNA is inherited down the maternal line is both an advantage and a disadvantage, depending on the application: mtDNA sequences are useful for studying phylogeography (Garnery et al. 1992) or biological invasions (Pinto et al. 2004), but they are less applicable for assessing admixture levels of individuals. Biparentally inherited nuclear loci are better suited for this purpose. Among them, microsatellites are one of the most widely used markers because of their high variability allowing resolution of contemporary population processes (for examples, see De La Rúa et al. 2003; Jensen et al. 2005; Muñoz et al. 2009; Oleksa et al. 2011). In this study, we used microsatellites and Bayesian clustering methods to study hybridization between Carniolan and Caucasian bees kept outside their aboriginal range.

Materials and Methods

Bees from the two subspecies as well reference samples of *A. m. mellifera* were examined. Both bees with typical and atypical wing venation for their own subspecies were studied. Samples of Caucasian bees were taken from 7 colonies (queens) belonging to the 5 strains and Carniolan bees from 12 colonies (queens) belonging to 3 strains. 16 worker bees from each colony were analysed. Insects were taken directly from hive frames and conserved in 90% ethanol. DNA was extracted from thoraces with Insect Easy DNA Kit (Omega Bio-Tek) according to the manufacturer instructions.

In the study we used eight nuclear microsatellite loci: Ap55, Ap43, Ap66, A24, A7, A88, A28 and A113 (for primer sequences, see Solignac et al. 2004), amplified in one multiplex reaction with Multiplex PCR Kit (Qiagen) following the kit instructions. PCR was performed using the PTC200 thermocycler (MJ Research) under the following conditions: an initial denaturation at 94°C for 5 min, followed by 35 cycles of (1 min at 94°C, 30 s at 58°C and 1 min at 72°C) and a final extension of 10 min at 72°C after the cycles. The separation of fragments was carried out on automated sequencer ABI PRISM 3130xl (Applied Biosystems) using the internal size standard (LIZ 600, Applied Biosystems). Resulting electropherograms were scored using GeneScan ver. 3.7 and Genotyper ver. 3.7 software (Applied Biosystems).

In order to assign genotypes to specific subspecies, we used a Bayesian statistical method implemented in the STRUCTURE ver. 3.2.1 (originally described in Pritchard et al. 2000). The program implements a model-based clustering method for inferring population structure using genotype data. The model assumes that there are K populations, each of which is characterized by a set of allele frequencies at each locus. Individuals in the sample are probabilistically assigned to one of the populations, or jointly to two or more populations if their genotypes indicate they are admixed. This procedure accounts for the presence of Hardy–Weinberg and linkage disequilibrium by introducing population structure, and attempts to find population groupings that (as far as possible) are not in disequilibrium. The Markov Chain Monte Carlo method can allow the posterior probability distribution to be computed for estimated parameters. We used the admixture model, which assumes that each individual (i) has inherited some fraction of its genome from ancestors in all K populations, and the correlated allele frequency model. Such assumptions seem to be the most reliable for detecting structure between closely related or admixed populations (Falush et al. 2003).

A burn-in of 50,000 iterations, followed by an MCMC (Markov Chain Monte Carlo algorithm) of 1,000,000 iterations was applied. In the analysis only queen genotypes, inferred from workers genotypes, were included. Mean individual admixture proportions, qi, and their 90 % credible limits were estimated for each individual.

Results and Discussion

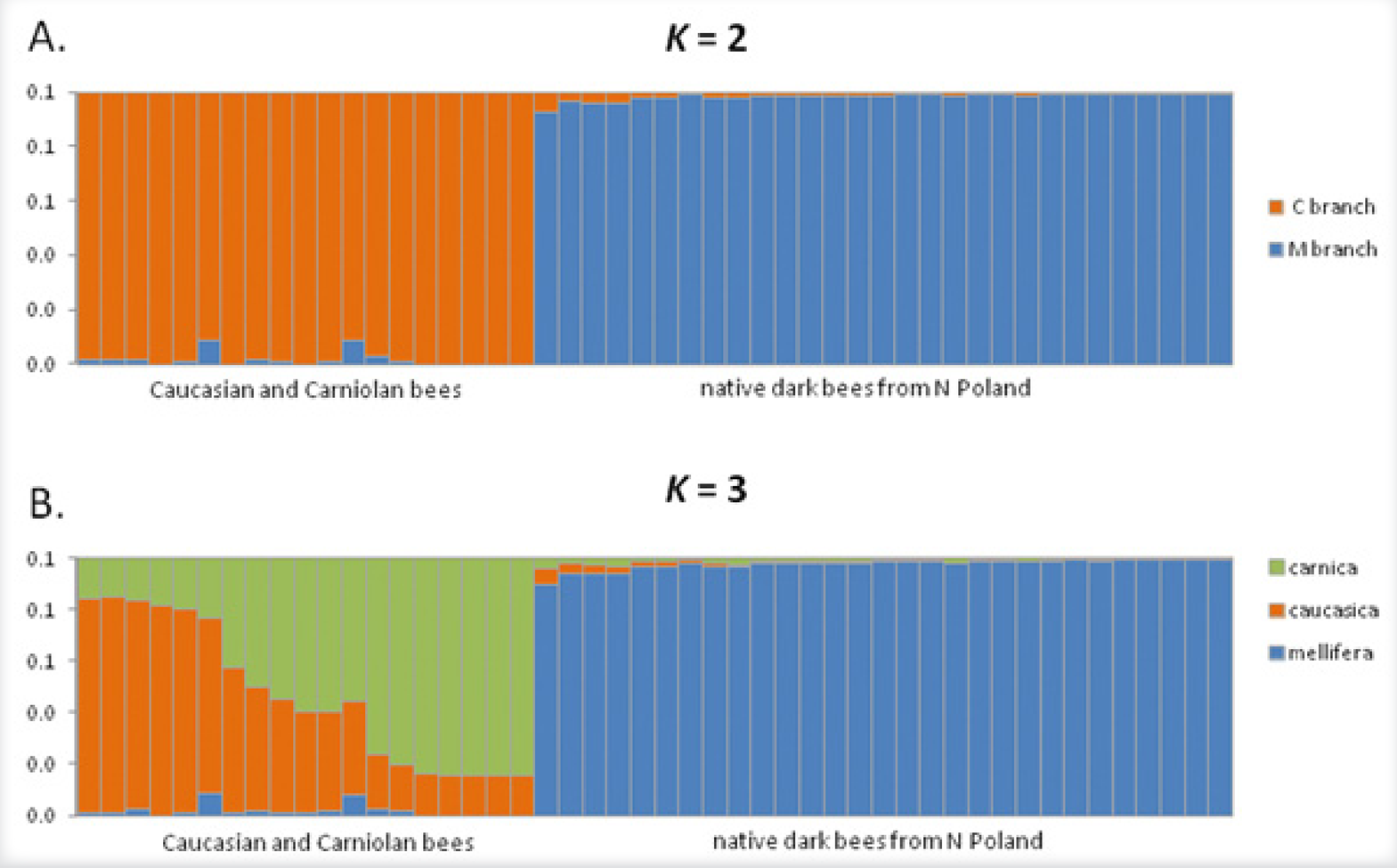

Fig. 1. Results of STRUCTURE clustering. Each vertical bar represents one mother bee, and its parts – proportions of genes from putative ancestral populations. (A) two groups assumed (evolutionary branches M and C), (B) three groups assumed (*A. m. mellifera*, *A. m. carnica* and *A. m. caucasica*).

Under assumption of two groups (M and C evolutionary branch) and including native Polish dark bees as a reference, all putative Carniolan and Caucassian queens were classified as belonging to the evolutionary branch C. A further allocation of this group into two subgroups (possibly corresponding to *A. m carnica* and *A. m. caucasica*) indicated that all tested queens could be hybrids of the two subspecies. In all genotypes admixture of genes from the other subspecies was estimated to be at least over a dozen of percentage.

It should be noted however that all assignment coefficients had wide credible intervals as a result of small differences in allele frequencies between Caucasian and Carniolan bees. While the tested bees from breeding lines *A. m carnica* and *A. m caucasica* showed a large differentiation from the subspecies A. m. melliefera (F_{ST} = 0.2), differentiation between them were much smaller ($F_{ST} \approx 0.05$). This resulted in a low power of discrimination. Further research on the identification of bees can cope with this problem in two ways: 1) by increasing the number of studied loci; 2) by including in the analyses reference samples of *A. m. carnica* and *A. m. caucasica* from their indigenous area of occurrence.

The results indicate low racial purity of Caucasian bees bred in Poland. Their genetic composition reflects the admixture of Carniolan bees and only slight admixture of native dark bees *A. m. mellifera*. Microsatellites may provide a useful complement to the morphological criteria that could be routinely used to study the origin of bees for breeding programs.

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