Original Article

MATERNAL INHERITANCE IN HYBRIDS OF THREE HONEY BEE SUBSPECIES

Paweł Węgrzynowicz[1]*
Dariusz Gerula[1]
Adam Tofilski[2]
Beata Panasiuk[1]
Małgorzata Bieńkowska[1]

[1] Research Institute of Horticulture, Apiculture Division, Puławy, Poland
[2] University of Agriculture, Department of Pomology and Apiculture, Kraków, Poland
*corresponding author: pawel.wegrzynowicz@inhort.pl

A b s t r a c t

The identification of honey bee (*Apis mellifera*) subspecies is often based on the measurements of workers' fore-wings. The interpretation of the measurements can be difficult because the phenotype of workers is affected by both genetic and environmental factors. Moreover, it is not clear how the phenotype is affected by maternal inheritance. We have used the methodology of geometric morphometrics to verify if hybrids of honey bee subspecies and their backcrosses are more similar to either the father or mother colony. The comparison was based on fore-wing venation of three honey bee subspecies: *A. m. carnica, A. m. caucasica, A. m. mellifera*. First generation hybrids and backcrosses of those subspecies were obtained through instrumental insemination. Workers of the hybrids were compared with their parental colonies. The shape of wing venation was more similar to the maternal than to parental colony. This phenomenon was particularly visible in first generation of hybrids but it was also present in backcrosses. There were also symptoms of genetic dominance of some subspecies but this effect interacted with maternal inheritance and was difficult to interpret.

Key words: bee queens, hybrids, maternal inheritance, morphometric analysis, subspecies, wing venation

INTRODUCTION

The identification of honey bee (*Apis mellifera*) subspecies is essential for breeding programs as a basic criteria for the register of queens in the herd books is the confirmation of their affiliation to the subspecies declared by the breeder. Moreover, the identification of the subspecies is important for their conservation (De la Rúa et al. 2009). Such identification is often based on measurements of various body parts including legs and sternite, but wing measurements only are usually sufficiently precise (Kandemir, Özkan, & Fuchs, 2011; Francoy et al., 2008; Tofilski, 2008; Gerula et al., 2009; Nawrocka et al., 2018). There is a significant correlation between morphometric and molecular methods (Miguel et al., 2011; Oleksa & Tofilski, 2015).

Until 2008 the honey bee subspecies used for breeding in Poland had been identified through the method developed by Gromisz (1981). It is based on the microscopic measurements of the width of 4th tergite, length of proboscis and cubital index, i.e. the ratio of two forewing vein lengths (Ałpatow, 1948). In 2009, a new method was introduced which was based on geometric morphometrics of the fore-wing (Gerula et al., 2009) and the identification of nineteen landmarks located at forewing vein junctions. It is recognized by the Polish National Animal Breeding Centre, and the measurement results are one of the criteria for bee herd books registration. The wings measurements are performed yearly for selected breeding lines and serve to confirm their subspecific identity. However, there is a lack of information on the morphometric

characteristics of the hybrids between the subspecies, which makes the interpretation of wing measurements difficult when a colony represents values intermediate between two or more subspecies. Hybrids between two honey-bee subspecies are generally believed to have a wing venation similar to the average shape of the two subspecies and this has been confirmed by some studies (Rinderer et al., 1990). In particular, the phenotype of hybrids of *A. m. carnica*, *A. m. caucasica* and *A. m. mellifera* was intermediate between the phenotypes of subspecies from which they were obtained (Gromisz et al., 1974). However, other study showed that the hybrids between Italian bees (*A. m. ligustica*) and Africanized bees were more similar to the later once (Francoy et al., 2012). On the other hand, in other study where European bees were crossed with Africanized bees, the shape of wing venation was affected by racial identity of maternal side (Schneider et al., 2003). The phenotype of hybrids does not need to be intermediate between parents because a genetic dominance can be present, as reported for the defensive response of honey bees (Guzman-Novoa & Page, 1994, Guzman-Novoa et al., 2002). Moreover, in social insects a stronger expression of one of the genotypes can be related to behavioral dominance (Page & Robinson, 1991; Paleolog, 2009), which is present when parental genotypes differ in threshold for the performance of a particular task. In this situation, the task is mainly performed by one of the genotypes. This explains why the behavior of workers in colonies created through the mixing of bees from two subspecies or breeding lines was often not intermediate between the colonies of origin (Paleolog et al., 2003; Paleolog, 2009).

In this study, we have used the methodology of geometric morphometrics to verify if hybrids of the subspecies *A. m. carnica*, *A. m. caucasica*, and *A. m. mellifera* as well as backcrosses of these hybrids are more similar to either the father or mother colony. Because we do not assume any causal influences leading to the presence of the higher similarity of hybrids to either of them, we call this phenomenon "maternal inher-

itance" and not "maternal effects" (Wof & Wade, 2009). Furthermore, we have expected the presence of maternal inheritance and a higher similarity of the hybrids to the mother colonies. The comparison was based on fore-wing venation of three honey bee subspecies and their hybrids. Moreover, we investigated the genetic dominance of any of the subspecies and if hybrids were more similar to one of the subspecies used for crossing.

MATERIAL AND METHODS

The research was carried out at the Institute of Horticulture, Apiculture Division in Puławy, Poland in the years 2013-2015. We used for this study three honey-bee subspecies: *A. m. carnica, A. m. caucasica, A. m. mellifera*. The parental generation consisted of fifteen bee queens: five sister queens of *A. m. carnica*, five sister queens of *A. m. caucasica* and five sister queens of *A. m. mellifera*. Each queen was instrumentally inseminated with semen collected from brother drones. The above mentioned inbred queens were introduced into regular bee colonies before winter, and in the spring of the next year one queen of each subspecies was chosen for interracial crossing. The three selected queens are later referred to as mother queens. The selection of mother queens was based on the lowest variation of their daughter workers' wing measurements.

In order to obtain the first generation of hybrids, the daughter queens were produced from the three mother queens. Each of these daughter queens was instrumentally inseminated with semen of one drone which was the son of one of the two other mother queens representing the remaining subspecies. In this way six groups of first generation of hybrids were produced (second row in Fig. 1) which represented all possible hybrid combinations. In order to estimate maternal effects, two groups of hybrids were produced for each pair of subspecies. For example, in one group of hybrids *A. m. carnica* queen was inseminated with *A. m. mellifera* drone, and in another group *A. m. mellifera* queen was inseminated with
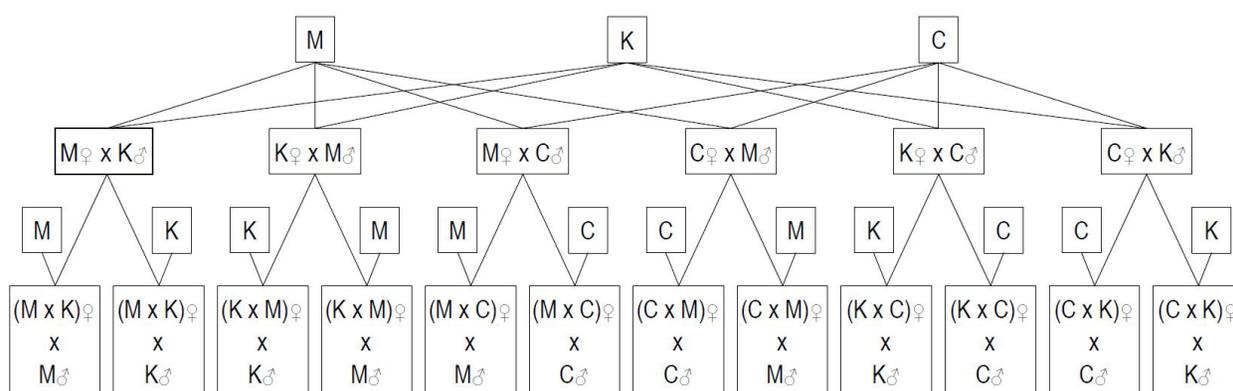
Fig. 1. Scheme of crossing performed to obtain hybrids and backcrosses. Letters in the rectangles represent subspecies: M - *A. m. mellifera*, K - *A. m. carnica*, C - *A. m. caucasica*. In the first-generation hybrids (depicted in the second row) the first letter (before x) indicates maternal colony and the last letter (after x) indicates paternal colony. In the backcrosses, (depicted in the bottom row) the first two letters separated by x, which are enclosed in parentheses, represent maternal hybrid colony and the last letter represents parental inbred colony.

A. m. carnica drone. There were ten to eighteen colonies in each group of first generation hybrids.

In order to obtain backcross hybrids, granddaughter queens were reared from the daughter queens and then instrumentally inseminated with semen of one drone originating from the mother queens. In this way, twelve groups of backcrosses were produced (bottom row in Fig. 1). There were four to nineteen colonies in each group of backcrosses.

The instrumentally inseminated daughter queens and granddaughter queens were introduced into small colonies managed in Mini-Plus polystyrene hives (30x30x60cm) in order to obtain workers which were their offspring. Combs with sealed brood before emergence were moved from experimental colonies into incubators with a temperature of 35°C and relative humidity of 65% just before bee emergence to ensure that bees collected for further analysis were of a specific origin.

Bees for morphometric analysis were taken directly from incubated combs shortly after emergence and stored in plastic containers filled with ethyl alcohol. Only workers and not queens were used for wing measurements. The right wings were dissected from each worker. The wings were mounted in a glass photographic frame and scanned with Nicon Coolscan 500 ED scanner (image resolution 2400 dpi, grayscale).

Wings images were analyzed with the use of the DrawWing software to determine the coordinates of nineteen landmarks (Tofilski, 2008; Gerula et al., 2009). These coordinates were aligned using generalized orthogonal least-squares procedures (Rohlf & Slice, 1990). Each queen was represented by forty workers. The data were averaged within colonies and the mean values were used in further analysis. The aligned coordinates were analyzed in order to obtain the first two canonical variates (Gerula et al., 2009). The differences in wing shape between colonies were measured with Mahalanobis distances (MD). The Mahalanobis distances between a colony and its maternal and paternal sides were analyzed with paired T-test. In cases where the measurements could not be paired, T-test was used. The calculations were done using Statistica v13 software.

RESULTS

The difference in wing venation between colonies of the three subspecies used to produce the first generation of hybrids was highest between *A. m. carnica* and *A. m. mellifera* (Mahalanobis distance, MD = 6.37) and smallest between *A. m. mellifera* and *A. m. caucasica* (MD = 5.41). The difference between *A. m. carnica* and *A. m. caucasica* was intermediate (MD = 5.64).

Table 1.
Difference in fore-wing venation between workers in colonies from the first generation of hybrids and their parental colonies. The difference was measured using Mahalanobis distance (MD). $P_{parents}$ indicates significance of differences (based on paired t-test) between each of the hybrid colonies and their parental colonies. $P_{subspecies}$ indicates significance of differences (based on t-test) between hybrids and their mothers (or fathers in separate column) when parental roles of subspecies were swapped.

Mother	Father	N	MD to mother	MD to father	$P_{parents}$
carnica	*mellifera*	18	4.38	5.19	**0.000**
mellifera	*carnica*	12	4.41	4.85	**0.044**
$P_{subspecies}$			0.878	**0.009**	
mellifera	*caucasica*	12	4.89	5.40	**0.010**
caucasica	*mellifera*	10	4.17	4.13	0.775
$P_{subspecies}$			**0.006**	**0.000**	
caucasica	*carnica*	14	4.58	4.85	0.234
carnica	*caucasica*	17	4.26	4.63	**0.024**
$P_{subspecies}$			0.086	0.203	

Table 2.
Difference in fore-wing venation between workers in colonies with hybrids obtained through backcrossing and their parental colonies. The difference was measured using Mahalanobis distance (MD). $P_{parents}$ indicates significance of differences (based on paired t-test) between each of the hybrid colonies and their parental colonies.

Mother	Father	N	MD to mother	MD to father	$P_{parents}$
carnica x mellifera	*carnica*	17	3.66	3.75	0.624
carnica x mellifera	*mellifera*	19	3.53	3.58	0.720
mellifera x carnica	*carnica*	4	3.93	4.24	0.494
mellifera x carnica	*mellifera*	5	3.77	4.16	**0.049**
mellifera x caucasica	*mellifera*	10	4.20	4.33	0.597
mellifera x caucasica	*caucasica*	4	3.83	4.34	0.135
caucasica x mellifera	*mellifera*	5	4.58	4.16	0.218
caucasica x mellifera	*caucasica*	8	4.26	4.55	0.231
caucasica x carnica	*caucasica*	10	3.91	3.99	0.665
caucasica x carnica	*carnica*	6	4.01	4.20	0.287
carnica x caucasica	*caucasica*	4	4.05	4.71	0.079
carnica x caucasica	*carnica*	6	3.97	4.15	0.513

In five of the six first-generation hybrids groups, workers were more similar to their mother colony than to the father colony, and in four of the groups the differences were statistically significant (Tab. 1). Only in the group where *A. m. caucasica* queens were inseminated with *A. m. mellifera* drones, workers in the hybrid colonies were more similar, although not statistically significantly, to the father colony (Tab. 1). When the data from all first-generation hybrid groups were combined, workers in hybrid colonies were significantly more similar to the mother colony (MD = 4.44) than the father colony (MD = 4.87) (paired t-test: t = -6.22; P<0.0001; N = 83) (Tab.1).

Because the distance to the mother and father colonies differed, we analyzed them separately in order to verify if there was dominance of any of the subspecies genotypes. Hybrids between *A. m. carnica* and *A. m. mellifera* were more similar to *A. m. carnica* in case of both distances, but only the latter were statistically significant (Tab. 1). Hybrids between *A. m. mellifera* and *A. m. caucasica* were significantly more similar to *A. m. mellifera* in case of the distance to the father but the opposite was true in case of that of the mother (Tab. 1). A similar pattern was observed in hybrids between *A. m. carnica* and *A. m. caucasica*, even though in this case the differences were not significant (Tab. 1).

Backcross hybrid workers were more similar to workers of their mother colony in eleven out of twelve groups, but the differences were marginally statistically significant only in one of the groups (Tab. 2). One group of backcross hybrid workers were more similar, although not of statistical significance, to workers of their father colony. The mother colony in this group was a first generation hybrid between *A. m. caucasica* and *A. m. mellifera,* representing the group which was more similar to its father colony in the first generation crossing. When data from all backcross hybrids were combined, the workers were significantly more similar to workers from the mother than the father colonies (paired t-test: t = -2.4; P = 0.017; N = 99). Canonical variate analysis shows that both the first generation of hybrids (Fig. 2A) and
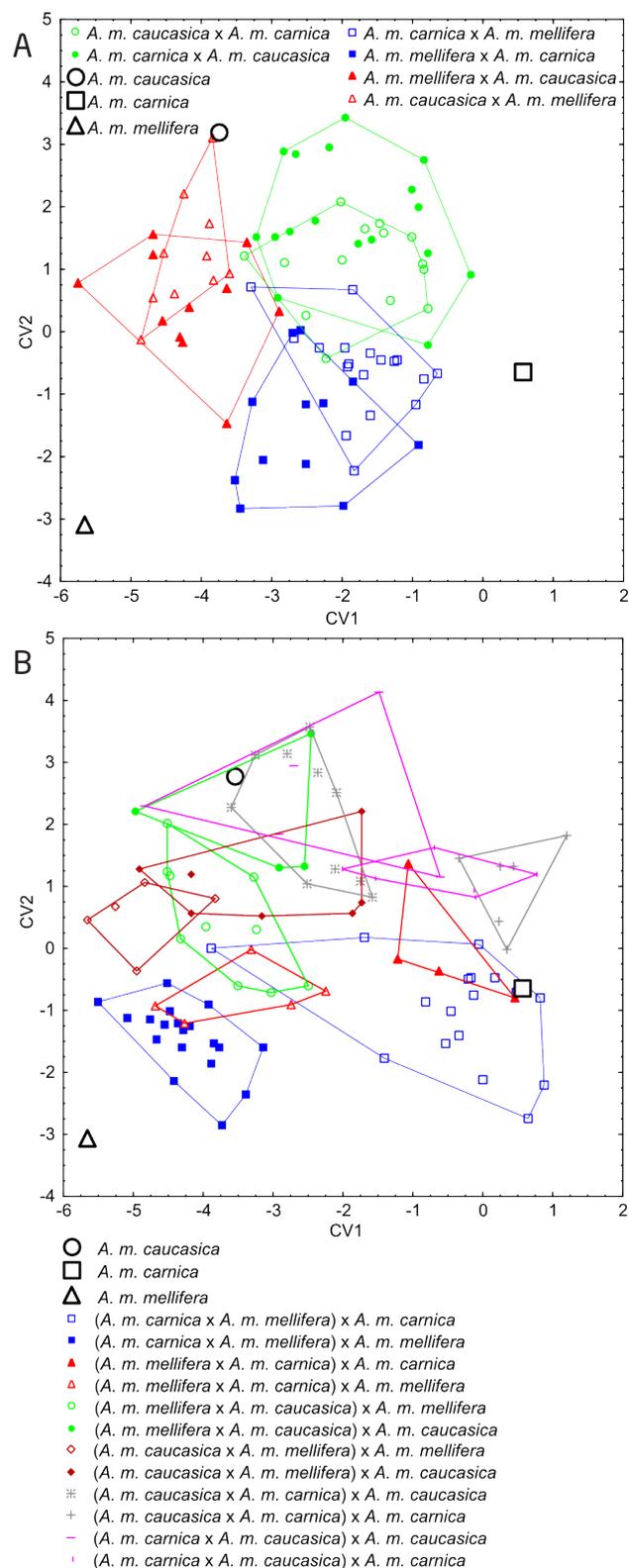

Fig. 2. Variation of the fore- wing venation of first-generation hybrids among three honey bee subspecies A and their backcrosses B. Each colony is represented by one marker in a two-dimensional space of canonical variates used for identification of the subspecies (Gerula et al., 2009). Parental colonies used to obtain the hybrids were marked with large black markers.

backcrosses (Fig. 2B) are very variable in terms of fore-wing venation. The first generation hybrids tend to be closer to their maternal colony than to their paternal colony (Fig. 2A). It is remarkable that the hybrids are not located mid-way between the parental colonies but tend to be further away from *A. m. mellifera* and closer to *A. m. caucasica* (Fig. 2AB).

DISCUSSION

The data presented here show that worker bees of interracial hybrids in terms of wing venation were more similar to the maternal colonies than the paternal once. This effect was clearly visible for first generation hybrids, however for back-crosses the results were similar but were not confirmed statistically for each of the groups separately. The lack of statistical differences can be related to small differences between groups and inadequate number of colonies used. In the earlier studies, it was not verified if there is maternal inheritance on wing venation, that is if hybrids are more similar to the mother than to the father. Gromisz et al. (1974) showed that cubital index was more similar to the mother colony than to father one but did not confirm statistically that the differences were sig-nificant. In other studies, experimental design did not allow such a comparison to be made (Francoy et al., 2012) or the study was focused on the identification of hybrids (Rinderer et al., 1990). Although in earlier studies maternal inheritance of wing venation in honey bees was not investigated there are some reports about presence of maternal inheritance in case of hygienic behavior (Unger & Guzman-Novoa, 2010) and duration of larval develop-ment (Moritz, 1985). The presence of maternal effects can be related to mitochondrial DNA which is inherited only from the mother. The mi-tochondrial genome contains a small number of genes which are not directly related to morpho-genesis but can affect the wing shape indirectly. More research is required to explain the genetic background of the observed maternal effects. Although the maternal effects are present, the difference in the similarity of hybrids to maternal and paternal colony is relatively small. It had been reported that hybrids of two honey bee subspecies inherited intermediate values of the wing venation of parental subspecies (Gromisz et al., 1974; Rinderer et al., 1990). Although the results of earlier studies agreed to a large extent, there were some differences between them. Francoy et al. (2012) stressed the closer similarity of the hybrids to one of the paternal subspecies, Rinderer et al. (1990) showed that different traits can be more similar to one or the other parental subspecies. Those differ-ences can be related to methodologies used in earlier studies which were based on cubital index (Gromisz et al., 1974), angles between veins (Rinderer et al., 1990) or geometric mor-phometrics (Francoy et al., 2012).

Apart from a higher similarity to the maternal colony, we found that hybrids tended to be more similar to one of the subspecies used to produce them. Those effects were statistically significant only in case of some subspecies com-binations (Tab. 1, $P_{subspecies}$), which can be related to genetic and behavioral dominance (Guzman-Novoa & Page, 1994; Paleolog, 2009). However, the interpretation of the data is complicated by a different response when the maternal colony represents one or the other subspecies. A different experimental setup is required to study this problem and verify if genetic or behavioral dominance is present. In earlier studies, a higher similarity of hybrids to one of the subspecies used for crossing was observed in *A. m. ligustica* and Africanized bees where the hybrids were more similar to Africanized bees (Francoy et al., 2012).

The Identification of honey bee subspecies used for breeding in Poland is based on geometric morphometrics (Gerula et al., 2009). The data presented here will help to better interpret the results of identification. Unfortunately, the hybrids and backcrosses do not form distinct groups of points (Fig. 2) and instead are relatively spread out in the space of the first two canonical variates, which makes the identi-fication of hybrids based on fore-wing venation difficult and imprecise.

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