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# Reevaluation of honeybee (*Apis mellifera*) microtaxonomy: a geometric morphometric approach

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**Abstract** – In the present study, the microtaxonomy of honeybee (*Apis mellifera* L.) subspecies was reevaluated based on a geometric morphometric method. Wing images of honeybee subspecies, obtained from the Morphometric Bee Data Bank in Oberursel, Germany, were assigned to four honeybee lineages from the individual images, and 40 Cartesian coordinates were obtained. Honeybee lineages were significantly different based on individual and colony consensus average wing shapes of honeybee subspecies ( $P < 0.001$ ). According to the discriminant function analysis of honeybee lineages, the A lineage and M lineage showed some degree of overlap. Multivariate statistical analysis displayed that *Apis mellifera intermissa*, a member of M lineage, seemed to belong to the A lineage. In addition, *Apis mellifera sicula*, a member of C lineage; *Apis mellifera sahariensis*, a member of M lineage; and *Apis mellifera syriaca*, a member of O lineage, were located closer to the A lineage rather than to their own lineages. In the previous studies, the results of principal component analysis of morphometric data and the mtDNA analysis of honeybee subspecies supported these subspecific affinities. Thus, geometric morphometric analysis of wing shape could be used as a reliable tool to discriminate among honeybee subspecies and may have advantages over standard morphometry.

*Apis mellifera* / geometric morphometric / landmark / subspecies / discriminant function analysis

## 1. INTRODUCTION

*Apis mellifera* L. subspecies occur naturally throughout almost all of Africa and Europe and parts of Asia, and are distinguishable based on behaviour, physiology and morphology (Ruttner 1988). Across this large area, the species occupies quite varied ecological regions, from deserts to tropical rainforests and from mountainous regions to swamps, and in these regions, more than two dozen *A. mellifera* subspecies have been described (Ruttner 1988; Sheppard et al. 1997; Engel 1999; Sheppard and Meixner 2003). Traditional morphometry has been widely

used to identify and classify *A. mellifera* (Alpatov 1929; Daly et al. 1982; Ruttner 1988). Ruttner (1988) described 36 morphometric characters (distances, angles, classes of pigmentation) for the discrimination of honeybee subspecies, and these characters have since become widely used in honeybee subspecies classification. These variables are typically combined and analysed through multivariate statistical analysis. In particular, wing shape has been characterized using angles and vein length measurements (Ruttner 1988). The usefulness of wing angles in phylogenetic studies was well shown by Diniz-Filho et al. (2000).

Using standard morphometry, Ruttner (1988, 1992) hypothesized the existence of four evolutionary lineages within the species: M in Western Mediterranean and Northern Africa, A in Africa,

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C in Central Mediterranean and Southeastern Europe, and O in Western Asia. In addition to the discrimination of honeybee subspecies or lineages with standard morphometry, biochemical and molecular methods, including analyses of isozymes, mitochondrial DNA polymorphism, nuclear DNA and microsatellites, have been used to evaluate microevolutionary processes in the honeybee (reviewed in Sheppard and Smith 2000). These relationships have been largely confirmed by studies based on mitochondrial DNA (Smith 1991; Garnery et al. 1992; Arias and Sheppard 1996; Smith et al. 1997; Palmer et al. 2000; Franck et al. 2000, 2001) and SNPs (Whitfield et al. 2006).

An alternative morphometric method known as ‘geometric morphometrics’ (GM) has been developed that utilizes the analysis of shape rather than distances and angles (Bookstein 1991). GM uses landmark coordinates which later are superimposed by translation, scaling and rotation so that the effect of size is removed. After superimposition, the landmark configurations differ only in shape and can be analysed by multivariate statistical methods (Rohlf and Marcus 1993; Zelditch et al. 2004). Wing shape variation of insects at different taxonomic levels have been studied using GM (De La Riva et al. 2001; Pretorius and Scholtz 2001; Monteiro et al. 2002; Houle et al. 2003; Schachter-Broide et al. 2004; Pretorius 2005; Aytekin et al. 2007; Sadeghi et al. 2009). In addition, wing shape variation based on GM has been used to discriminate and identify honeybee subspecies (Francoy et al. 2008; Tofilski 2008), the heritability of wing shape (Monteiro et al. 2002) and the influence of hybridization on fluctuating asymmetry (Schneider et al. 2003). Traditional morphometry including both wing and body characters has provided substantial insight into and good discrimination among honeybee subspecies and populations (Diniz-Filho et al. 2000; Ruttner et al. 2000; Kandemir et al. 2000), although standard morphometry of wing characters alone provides less resolution than geometric morphometry (Tofilski 2008). GM is also less time-consuming than standard mor-

phometric procedures (Francoy et al. 2008; Tofilski 2008).

The aim of this study was to reevaluate the microtaxonomy of honeybee subspecies based on geometric morphometrics and compare the results obtained in the present study to those obtained by using the same data set of 24 *A. mellifera* subspecies in a previous standard morphometric study (Kauhausen-Keller et al. 1997).

## 2. MATERIALS AND METHODS

Pictures of worker bee forewings were obtained from the Morphometric Bee Data Bank in Oberursel, Germany (Table 1). Each wing image was archived and labelled with unique subspecies codes in the computer. The subspecific groups and the honeybee lineages were previously determined (Kauhausen-Keller et al. 1997). A total of 1,792 worker bee wings of *A. mellifera* belonging to 186 colonies and 24 subspecies were used in geometric morphometry. All colonies were described by means of 10 workers. For geometric morphometric analysis, a total of 20 landmarks (venation intersections) on the forewings were identified according to Zelditch et al. (2004) classification (Figure 1), and wing photographs were first processed by tpsUtil 1.40 (Rohlf 2008a). Then, two-dimensional  $x, y$  Cartesian coordinates of the identified landmarks were digitized using tpsDig 2.11 (Rohlf 2008b). The coordinates of the landmarks obtained from tpsDig were used in Morphueus (Slice 2002) to perform the procedure of geometric morphometry (superimposition method). Thus, landmark configurations were scaled, translated and rotated against the consensus configuration by the generalized procrustes superimposition method (Bookstein 1991). After superimposition, multivariate analysis of variance (MANOVA) and pairwise tests were also applied using Morphueus. Aligned landmark coordinates were used as the data set for the discriminant function analysis of honeybee lineages and subspecies, and cross-validation tests were used to check the accuracy of the equations in identifying the colonies. Differences in wing shape among different honeybee lineages were visualised by deformation grids using thin plate splines (Slice 2002). An unweighted pair group method with arithmetic mean (UPGMA) analysis (Rohlf 2004) was performed on Mahalanobis distances computed from the discriminant

**Table I.** *A. mellifera* subspecies obtained from Oberursel Database used in the analysis.

Subspecies	Sample size	
	Colony	Bee
Tropical African		
<i>A. m. adansonii</i>	11	108
<i>A. m. capensis</i>	3	30
<i>A. m. lamarckii</i>	7	69
<i>A. m. litorea</i>	6	53
<i>A. m. monticola</i>	8	77
<i>A. m. scutellata</i>	14	136
<i>A. m. unicolor</i>	7	67
<i>A. m. yemenitica</i>	14	126
Western Mediterranean–Northern African		
<i>A. m. iberica</i>	2	18
<i>A. m. intermissa</i>	8	78
<i>A. m. mellifera</i>	14	136
<i>A. m. sahariensis</i>	2	19
Central Mediterranean–Southeastern European		
<i>A. m. carnica</i>	15	146
<i>A. m. cecropia</i>	9	87
<i>A. m. ligustica</i>	11	107
<i>A. m. macedonica</i>	2	20
<i>A. m. sicula</i>	4	39
Middle Eastern		
<i>A. m. adami</i>	5	50
<i>A. m. anatoliaca</i>	5	50
<i>A. m. armeniaca</i>	6	54
<i>A. m. caucasica</i>	12	119
<i>A. m. cypria</i>	4	39
<i>A. m. meda</i>	8	79
<i>A. m. syriaca</i>	9	85

function analysis to construct a dendrogram showing the relationships among honeybee subspecies based on wing shape.

### 3. RESULTS

#### 3.1. Honeybee lineages

Differences between honeybee lineages were tested with MANOVA for forewing shape and

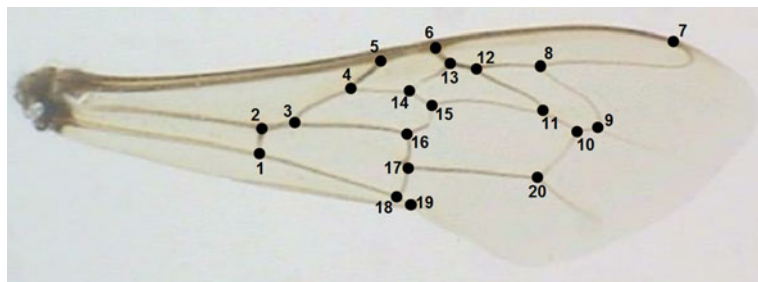
were statistically significant (Wilks'  $\lambda=0.000$ ,  $P<0.001$ ). Pairwise comparisons were followed, and all honeybee lineages were found to be statistically different from one another (Wilks'  $\lambda=0.000$ ,  $P<0.001$ ).

##### 3.1.1. Discrimination of individuals

Analysis of variance (ANOVA) of Cartesian coordinates of the landmarks on forewing showed that all of the Cartesian coordinates of landmarks displayed statistically significant differences among individuals of lineages (Figure 2). Results of the discriminant function analysis of individuals indicated that the first, the second and the third axes explained 56.7%, 24.4% and 15.9% of the total variation, respectively, and 100% of the total variation was explained by the first three discriminant functions. The A lineage and M lineage showed partial overlapping, whilst the C lineage and O lineage formed an apparent non-overlapping cluster (Figure 2a). More than 89% (89.9%) of the individuals were correctly classified to their original groups.

##### 3.1.2. Discrimination of colonies

For better visualization and simplicity, measurements of the 10 individuals per colony were averaged and the same analyses were performed on colonies. The ANOVA showed that 33 out of 40 Cartesian coordinates of landmarks displayed statistically significant differences among colonies of lineages ( $df=3, 182$ ;  $P<0.05$ ). According to the results of the discriminant function analysis of colonies, the first, the second and the third axes explained 49.2%, 33.9% and 16.9% of the total variation, respectively, and 100% of the total variation was explained by the first three discriminant functions. Scatter plots of individuals and colonies demonstrated the same discrimination results (Figure 2a, b). All colonies were assigned to their original group with a high probability (97.3%). Cross-validation tests based on discriminant functions correctly classified 95.2% of the colonies (Table II).



**Figure 1.** Location of landmarks on *A. mellifera* worker fore wing.

Finally, the aligned landmark coordinates were used as a data set for grouping honeybee subspecies to their assigned honeybee lineages. According to these analyses, all honeybee subspecies were correctly classified 100% to their original groups.

### 3.2. Honeybee subspecies

The differences between honeybee subspecies were similarly tested with MANOVA as in honeybee lineages (Section 3.1), and the differences were found to be statistically significant (Wilks'  $\lambda=0.000$ ,  $P<0.001$ ). Pairwise comparisons were also followed, and all honeybee subspecies were found to be statistically different (Wilks'  $\lambda=0.000$ ,  $P<0.001$ ).

#### 3.2.1. Discrimination of colonies

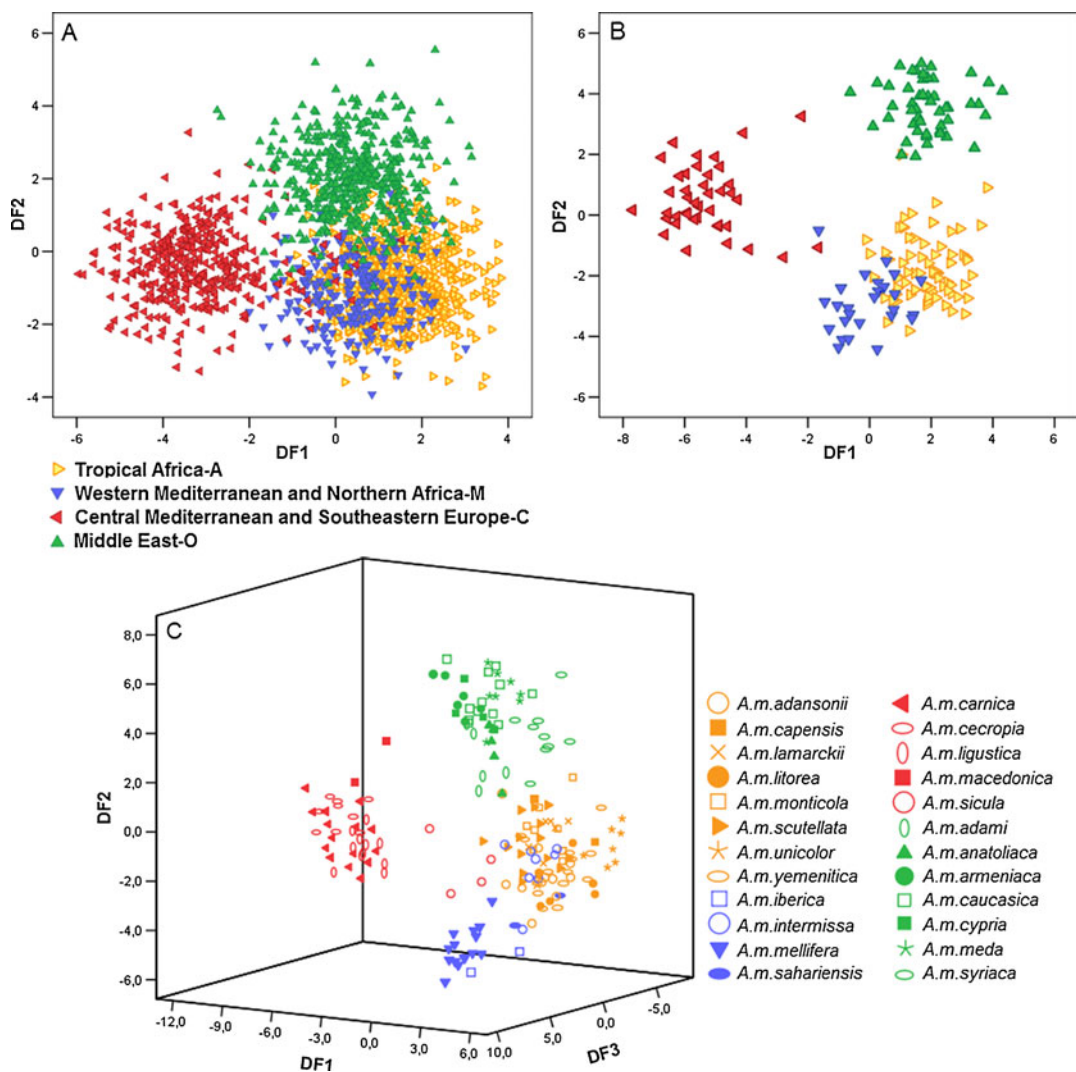
ANOVA of Cartesian coordinates of the landmarks showed that 38 out of 40 Cartesian coordinates of landmarks displayed statistically significant differences among colonies of subspecies ( $df=21, 162$ ;  $P<0.05$ ). Discriminant function analysis resulted in the clear separation of colonies of subspecies when forewing shape was utilized (Figure 2c). However, A lineage and M lineage honeybee subspecies showed some degree of overlap. Almost all of the honeybee subspecies were correctly classified (99.5%) to their original groups. Cross-validation tests based on discriminant functions correctly classified 79.0% of the colonies.

The resulting scatter plots (Figure 3a–d) obtained by rotating Figure 2c show the

distributions of each lineage (A, M, C and O, respectively) on the three discriminant functions. Lineage A (Figure 3a) consists of subspecies of tropical Africa: *Apis mellifera adansonii*, *Apis mellifera capensis*, *Apis mellifera lamarckii*, *Apis mellifera litorea*, *Apis mellifera monticola*, *Apis mellifera scutellata*, *Apis mellifera unicolor* and *Apis mellifera yemenitica*. Members of the A lineage were completely distinguishable from the other lineages (M, C and O).

Lineage M (Figure 3b) consists of four subspecies (*Apis mellifera iberica*, *Apis mellifera intermissa*, *Apis mellifera mellifera* and *Apis mellifera sahariensis*). Among them, *A. m. intermissa* seems to belong to the A lineage rather than M lineage and *A. m. sahariensis* is also located close to the A lineage. Similarly, the C lineage (Figure 3c) includes *Apis mellifera carnica*, *Apis mellifera cecropia*, *Apis mellifera ligustica*, *Apis mellifera macedonica* and *Apis mellifera sicula*. The members of this lineage do not overlap with the other groups, except *A. m. sicula* which has a close proximity to the A lineage. On the other hand, in the O lineage (Figure 3d), only *Apis mellifera syriaca* seems to be closer to A lineage; other members of this lineage (*Apis mellifera adami*, *Apis mellifera anatoliaca*, *Apis mellifera armeniaca*, *Apis mellifera caucasica*, *Apis mellifera cypria*, *Apis mellifera meda* and *Apis mellifera syriaca*) were quite distinguishable.

Wing shape differences between the lineages were illustrated by deformation grids on the thin plate spline (Figure 4a–f). The thin plate spline showed that the highest differences were seen in



**Figure 2.** Scatter plot of four honeybee lineages based on DFA for Cartesian coordinates of the landmarks on fore wing discrimination of individuals (A), discrimination of colonies (B) and scatter plot of honeybee subspecies based on DFA for Cartesian coordinates of the landmarks on fore wing (C).

pairs with C honeybee lineages. Figure 5 shows the phenetic relationships among honeybee subspecies based on Mahalanobis distances computed from the discriminant function analysis. The phenogram resulted in two main clusters. The first cluster consisted of members of the C lineage; however, the second cluster consisted of two subgroups: One of the subgroups was formed by members of M lineage and the other

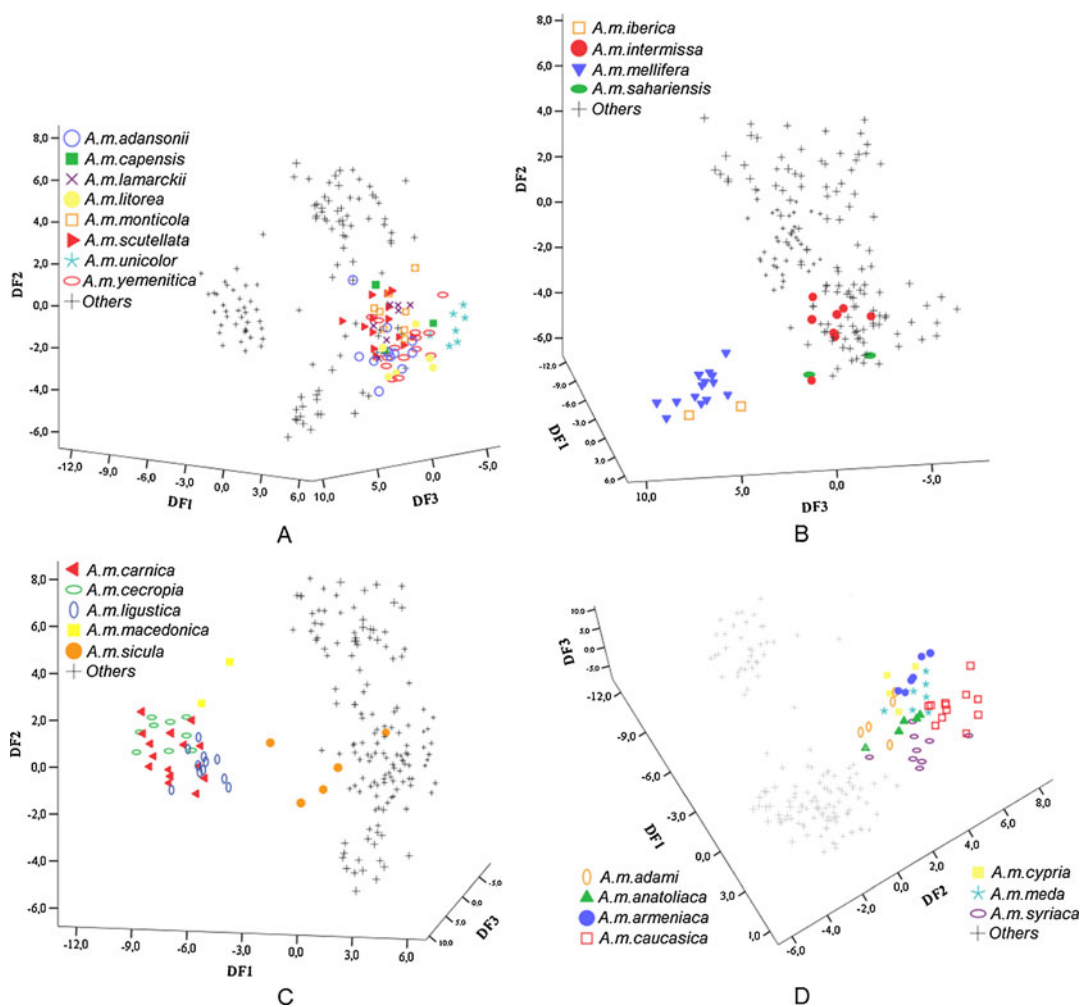
consisted of members of the A lineage and members of the O lineage. At 102.86 phenon line, four lineages were clearly visualised.

#### 4. DISCUSSION

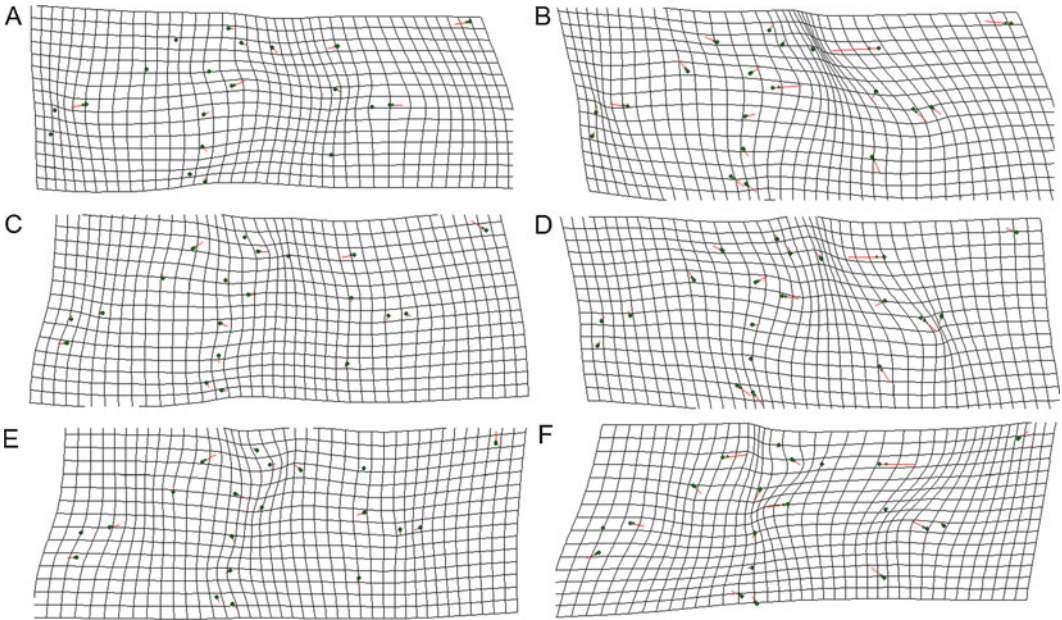
In the present study, geometric morphometric discrimination of honeybee lineages and 24 subspecies was comparable to standard mor-

**Table II.** Colonies of honeybee lineage classification with cross-validation.

Group	A lineage	M lineage	C lineage	O lineage
A lineage	66	2	1	0
M lineage	1	24	1	0
C lineage	0	0	38	0
O lineage	3	0	1	49
Total N	70	26	41	49
Proportion	94.3	92.3	92.7	100.0



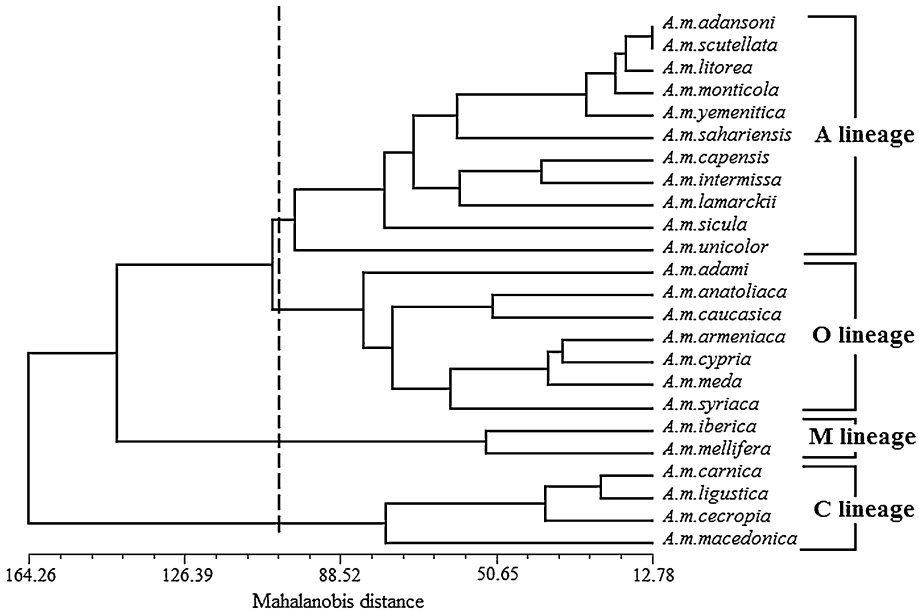
**Figure 3.** Three-dimensional scatter plots of tropical African subspecies (A), Western Mediterranean and Northern African subspecies (B), Central Mediterranean and South Eastern European subspecies (C) and Middle Eastern subspecies (D).



**Figure 4.** Comparison of the deformation grids of honeybee lineages: A with M (A), A with C (B), A with O (C), M with C (D), M with O (E) and C with O (F).

phometry, as previously reported for several honeybee subspecies or populations (Francoy et

al. 2008, 2009; Tofilski 2008). The new methodology was shown to be very reliable, efficient



**Figure 5.** UPGMA phenogram showing the geometric morphometric relationship among honeybee subspecies based on Mahalanobis distances computed from the discriminant function analysis.



and fast for the identification of honeybee subspecies.

Discriminant function analysis of geometric morphometric data demonstrated that *A. m. intermissa* belongs to the A lineage rather than the M lineage, as previously postulated (Figure 3b). *A. m. sahariensis* was also found to group with the A lineage rather than M, as had been shown in a principal component analysis of morphometric data (Kauhausen-Keller et al. 1997). mtDNA studies have also supported the relationship between subspecies *A. m. sahariensis* and *A. m. intermissa* and the A lineage (Arias and Sheppard 1996; Franck et al. 2001).

Additional findings at the subspecies level showed congruence between standard morphometry and geometric morphometrics. *A. m. sicula*, originally considered a member of the C lineage, was shown to exhibit a close affinity to the A lineage (Figure 3c). Standard morphometry, mtDNA studies and now GM have shown support for this relationship (Arias and Sheppard 1996; Kauhausen-Keller et al. 1997; De La Rua et al. 2009). Similarly, the affinity for *A. m. syriaca* of the O lineage towards the A lineage subspecies (Figure 3d) was noted in both the present study and in Kauhausen-Keller et al. (1997). Thus, both methodologies provided similar resolution and the same outcome for subspecies discrimination and lineage associations.

Discriminant function analysis of subspecies was also supported by the UPGMA clustering based on Mahalanobis distances. The subgroup represented by the members of lineage A consisted of *A. m. intermissa*, *A. m. sahariensis* and *A. m. sicula* (Figure 5). The results of UPGMA clustering of geometric morphometric data strongly support the existence of four honeybee lineages, as concluded by previous standard morphometric (Ruttner 1988) and molecular studies (Arias and Sheppard 1996; Franck et al. 2000; Whitfield et al. 2006).

Geometric morphometric analysis allowed the visualization of the differences among lineages or subspecies to be plotted as vectors and deformation grids. The deformation grid identifies the

landmark regions that contribute most to the discrimination. When differences between the lineages were illustrated by the deformation grids, the highest differences were seen in pairs with C honeybee lineages (Figure 4b, d, f).

In the present study, we found that a geometric morphometric approach provided consistent discrimination among four honeybee lineages and 24 honeybee subspecies. Tofilski (2008) demonstrated that geometric morphometrics yielded marginally better discrimination of three honeybee subspecies (*A. m. mellifera*, *A. m. carnica* and *A. m. caucasica*) than standard morphometry. Our studies and previous studies (Tofilski 2008; Francoy et al. 2008, 2009; Miguel et al. 2011) provide a robust demonstration of the usefulness of geometric morphometrics for honeybee microtaxonomy, including the discrimination of honeybee lineages and subspecies. Moreover, Miguel et al. (2011) found that GM and microsatellite data also supported similar interpretations at this taxonomic level. A recent paper by Bloch et al. (2010) used GM analysis to classify a single honeybee wing remnant from an archaeological site to its probable subspecies origin. Together with previous studies, our findings demonstrate that geometric morphometry can be a powerful tool to evaluate and resolve the subspecific taxonomy of honeybees.

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**Ré-évaluation de la microtaxonomie de l'abeille *Apis mellifera* par une approche de morphométrie géométrique**

***Apis mellifera* / morphométrie géométrique / sous-espèce / élément du paysage / analyse fonctionnelle discriminante**

## Re-evaluation der Mikrotaxonomie der Honigbienen (*Apis mellifera*) Ein geometrisch-morphometrischer Ansatz

### *Apis mellifera* / geometrisch-morphometrisch / Landmarken / Unterarten / Diskriminanz-funktionsanalyse

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