Landmark based geometric morphometric analysis of wing shape in *Sibiricobombus* Vogt (Hymenoptera: Apidae: *Bombus* Latreille)

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Summary. *B.* (*Sibiricobombus*) *niveatus* and *B.* (*Sibiricobombus*) *vorticosus* are analyzed and compared with selected species of the subgenera *Melanobombus* and *Mendacibombus*. Different geometric morphometric methods based on wings landmark data show that there are no morphological differences between *B. niveatus* and *B. vorticosus*. These results support the hypothesis that *B. niveatus* and *B. vorticosus* should be regarded as conspecific. On the other hand, the wing shapes show significant differences among the bumblebee species and subgenera that are coherent with their specific and subgeneric status. This approach may help future studies not only by contributing towards solving problems in systematics but also in understanding the flight mechanism of bumblebees. It also supports studies on the evolution of crossvein structure and on aerodynamics of different wing shapes in insects.

Résumé. Analyse en géométrie morphologique des landmarks de forme d'ailes chez *Sibiricobombus* Vogt (Hymenoptera : Apidae : *Bombus* Latreille). La variabilité morphométrique de *Bombus (Sibiricobombus) sulfureus*. *B. (Sibiricobombus) niveatus* et *B. (Sibiricobombus) vorticosus* est analysée et comparée à une sélection d'espèces parmi les sous-genres *Melanobombus* et *Mendacibombus*. Différentes méthodes de morphométrie géométrique, basées sur des *landmarks* pris sur les ailes, montrent qu'il n'y a pas de différence morphologique entre *B. niveatus* et *B. vorticosus*. Ces résultats confortent l'hypothèse selon laquelle *B. niveatus* et *B. vorticosus* doivent être considérés comme conspécifiques. Par ailleurs, la forme des ailes des espèces et sous-genres de bourdons étudiés montre des différences significatives et cohérentes avec leur statut spécifique et subgénérique. A l'avenir, cette approche pourra non seulement contribuer à la résolution de cas litigieux en systématique, mais également à la compréhension des mécanismes de vol chez les bourdons. Elle pourra également servir aux travaux sur l'évolution de la structure de la nervation alaire et sur l'aérodynamique des différentes formes d'ailes chez les insectes.

Keywords: *Sibiricobombus*, Principal Component Analysis, Generalized Procrustes Analysis, Relative Warps Analysis, Geometric Morphometrics.

In taxonomical studies, species or populations may overlap when univariate characters are used. This is especially typical in the classification studies on males of bumblebees which may be very difficult to distinguish without extracting the genitalia (Richards 1968). In the same way, it is not possible to accurately identify all bumblebee species by using only their colour features (Rasmont 1983; Özbek 1983). There are still several unsolved problems in the taxonomy of bumblebees and this leads to search for alternative methods such as DNA studies (Koulianos & Schmid-Hempel 2000; Kawakita *et al*. 2003), allozyme based models (Pekkarinen 1979; Pekkarinen *et al*. 1979; Pamilo *et al*. 1987; 1996), sexual pheromone analysis (Valterová *et al*. 2002; Terzo *et al*. 2003; Rasmont *et al*. 2005) and morphometry both in cladistic (Williams 1985, 1994; Ito & Sakagami 1985) and traditional (Ito 1985; Aytekin *et al*. 2003) methods.

The Near East taxa of the subgenus *Sibiricobombus* Vogt are an example of such a confusing systematics. Three taxa are generally considered as clear species by most authors (Reinig 1971; Rasmont 1983; Özbek 1983; Baker 1996). *Bombus sulfureus* Friese 1905, *B. vorticosus* Gerstaecker 1872 and *B. niveatus* Kriechbaumer 1870. Williams (1991, 1994; 1998) agree with the specific isolation of *B. sulfureus* but he considers *B. vorticosus* as a morph of *B. niveatus*. Having studied the variability of the secretions of the male cephalic labial glands, Rasmont *et al*. (2005) also support the conspecificity of these taxa.

The complex shape of an organism cannot easily be summarized by using linear measurements as in

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traditional morphometrics (Pavlinov 2001). Because these measurements are highly correlated with size, much effort was spent for size correction (Zelditch *et al*. 2004). But there is no consensus on different size correction methods and several difficulties remain. For instance, the homologies of linear distances are difficult to assess and the same set of distance measures can be obtained from totally different shapes (Zelditch *et al*. 2004). In general, it is not possible to generate graphical representations of shapes from the linear distances (Adams *et al*. 2004). Therefore, some aspects of shape are always lost. Because of these and several other difficulties (*for details see* Zelditch *et al*. 2004), researchers explored alternative methods of quantifying and analyzing morphological shape. One of these methods is known as landmark based geometric morphometrics. The research field of this method involves studies on the structures that Cartesian coordinates can be taken (Pavlinov 2001). The main idea was proposed by D'Arcy Thompson at the beginning of the century (Lynch 2004). Thanks to the progress of computer technology, many new tools have been developed during the past decade which greatly facilitate the record of morphometric information (Rohlf 1990). The method of relative warps and shape coordinates (BC: Bookstein's Shape Coordinates) developed by Bookstein (1991) for the analysis of morphometric variation based on landmark data gives a special help to taxonomists. At the present time, geometric morphometric studies have gained significant support especially among anatomists (Lockwood *et al*. 2002) and taxonomists (Rohlf 1993; Alibert *et al.* 2001; Gumiel *et al*. 2003) by the use of various morphological characters. Readers unfamiliar with the techniques are directed to Bookstein (1991), Rohlf (1999), Pavlinov (2001) and especially to O'Higgins (2000), Zelditch *et al*. (2004) and Adams *et al.* (2004) which cover the more technical aspects of these methods.

It was Comstock (1893) who first popularized the use of insect wing venation for traditional classification (Kunkel 2004). Since the 1970's, several authors have begun to use the insect wings especially in 2D morphometrical studies in systematics and phylogeny (Plowright & Stephen 1973; Rohlf 1993; Klingenberg 2003; Gumiel *et al*. 2003). Because wings are solid or rigidly articulated structures they have become very useful tools for geometric morphometric studies (Pavlinov 2001). This methodology also affected the studies of bumblebees (Ito 1985; Aytekin *et al*. 2003). Morphometrical shape relationships among the subgenera of bumblebees have already been studied in a very basic way by Plowright & Stephen (1973). Klingenberg (2003) used landmarks on the bumblebee wings as a research tool for analysing the developmental instability by means of fluctuating asymmetry.

In the present paper we analyzed the morphometric variability of *B.* (*Sibiricobombus*) *sulfureus*, *B.* (*Sibiricobombus*) *niveatus* and *B.* (*Sibiricobombus*) *vorticosus*. They are compared with selected reference species from other subgenera. In this first use of landmark analysis for bumblebee taxonomy, we would like to test if a priori specific and subgeneric relationships could be supported by this approach. We also would like to assess if *B. vorticosus* and *B. niveatus* are morphometrically similar, as it would be expected if they were conspecific (Williams 1998; Rasmont *et al*. 2005).

Material and methods

Study area and sampling

Fifty two males belonging to six species and three subgenera were collected in Middle and East Anatolia between 2nd and 13th of August 2002, in Turkey, by M. Terzo, P. Rasmont, Y. Barbier, H. Hines and A. M. Aytekin. Subgenera are recognized by genitalia morphology (Richards 1968). The different species of *Sibiricobombus* are mainly identified by their color patterns (Özbek 1998). For *Sibiricobombus,* 26 specimens of *B. niveatus* (nive), 6 specimens of *B. vorticosus* (vort) and 3 specimens of *B. sulfureus* (sulf); for *Melanobombus,* 3 specimens of *B. erzurumensis* (erzu) and 8 specimens of *B. incertus* (ince) and for *Mendacibombus,* 6 specimens of *B. handlirschianus* (hand) were used for the analysis. The material has been collected in the following localities (WGS84 coordinates).

Aksaray: Agzikarahan, 38°27'N 34°09'E 1200 m (vort-511, vort-512, nive-515, nive-517); Kayseri: between Incili and Koccagiz, 38°32'N 35°44'E 1600 m (vort-527, vort-528, vort-534, vort-547, sulf-529, sulf-535, nive-530/nive-533, nive-536/nive-539); Erciyes Mnt, near the Tekir baraji, 38°28'N 35°30'E 2000 m (nive-525); near Cebir, 38°33'N 35°38'E 1900 m (sulf-552); Erzincan: Yeniyol-Ahmetli Road, 39°53'N 39°22'E 2120 m (nive-571, erzu-557, erzu-573, erzu-578); Sakaltutan Gecidi, 39°52 N 39°08'E 2180 m (ince-590); Artvin: Yalnızcam Gecidi, 41°03'N 42°17'E 2510 m (nive-655, hand-653, hand-671, hand-672, hand-679, hand-680, hand-692); Asiklar Village, 41°05'N 42°08'E 1480 m (nive-703/nive-712); Ardahan: Taslidere Village, 41°04'N 42°49'E 1960 m (nive-723, nive-724); Kars: Pasli Gecidi, 40°19' N 42°55'E 2030 m (nive-753, ince-751, ince-755/ince-757); Agri: Bayramagzi Village, 39°36'N 43°32'E 1820 m (ince-760, ince-766, ince-768).

Males were kept alive separately in small plastic vials and were killed by freezing and immediately dissected. Right front and right hind wings of the specimens were removed with forceps and mounted in entellane on labelled slides. All the specimens were screened - at the time they were collected - for the presence of known ecto- and/or endo-parasites to shield the morphometric data from possible traumatic variations (Mayr & Ashlock 1991; Aytekin *et al*. 2002). Their cephalic glands were also extracted for another study (Rasmont *et al*. 2005). All the specimens are conserved at the Laboratory of Zoology UMH (Belgium).

Data acquisition

All slides were photographed by using a Leica MZ-7.5 stereoscopic zoom dissection microscope and a DC-300 digital camera system. They are archived and labelled with unique codes. All specimens were scored by a single experimenter (BM; A. M. Aytekin). Photographs were first input to tps-UTIL1.28 (Rohlf 2004*a*). Twodimensional Cartesian coordinates of 20 landmarks from front wings (Fig. 1) and 6 landmarks from hind wings (Fig. 2) were digitized by tps-DIG1.40 (Rohlf 2004*b*). All wings were digitized twice in order to reduce the measurement error (ζ) (Arnqvist & Mårtensson 1998). The second session of the measurement was conducted after having removed the wing and re-placed it under the microscope in order to take the positioning error into account (Arnqvist & Mårtensson 1998; Alibert *et al*. 2001). No analogous systems were used during the whole procedure to keep the digital errors in minimum.

Statistical analysis

The coordinates were analyzed using tps-RELW1.34 (Rohlf 2004*c*) to calculate eigenvalues for each principal warp. The landmark configurations were scaled, translated and rotated against the consensus configuration by GLS Procrustes superimposition method (Bookstein 1991; Rohlf 1993; Rohlf 1999; Alibert *et al*. 2001). The consensus configurations and relative warps for each species were conducted. The variability in the shape space was assessed using the scores obtained for each individual on the first two relative warps which is technically a PCA. The relative warps correspond to the principal components and define a shape space in which individuals are replaced (Alibert *et al*. 2001). To better visualize the shape variation we only considered the mean configuration for each species in both front and hind wings. Thus, the consensus configurations per wings were subjected to relative warps analysis. The bending energies of all taxa were compared using tps-SPLINE 1.20 (Rohlf 2004*d*.). In this analysis *Melanobombus* and *Mendacibombus* were used as references to have a better approximation.

The coordinates of the landmarks obtained from tps-DIG were also used in *Morphologika* (O'Higgins & Jones 1999) to perform principal component analysis (PCA) of Procrustes registered landmark data in the tangent space to Kendall's shape space (Dryden & Mardia 1998) and to calculate centroid sizes (O'Higgins & Jones 1999) for each specimen. PCA graphs were performed using XLStat-pro 6.0 (Addinsoft 2003).

We also used Bookstein's shape coordinates (BC) as an alternative superimposition method for front wings (number of landmarks for hind wing was not enough) to *B. vorticosus* and *B. niveatus* to test the conspesificity. Landmarks 13 and 8 were chosen as baseline which allows more lineer orientation than those of others can. IMP software series (Sheets 2006) were conducted for the analysis. The data were first superimposed to BC by IMP CoordGen6. For testing significant differences in shape between the two compared species, we used the Hotelling's T^2 test that is provided by the software IMP TwoGroup.

The size morphometry of the examined species was investigated by using the centroid sizes of the front wings as estimator with nonparametric Kruskal-Wallis test (Sokal & Rohlf 1995). Centroid size is the square root of the sum of squared distances of a set of landmarks from their centroid or in other words it is the square root of the sum of the variances of the landmarks about that centroid in *x*- and *y*- directions (Bookstein 1991; Pavlinov 2001). Separate statistical comparisons were performed for *B. niveatus* and *B. vorticosus* using the nonparametric Mann-Whitney *U*-test (Sokal & Rohlf 1995). The calculated differences were then illustrated with graphs (Minitab v13.2).

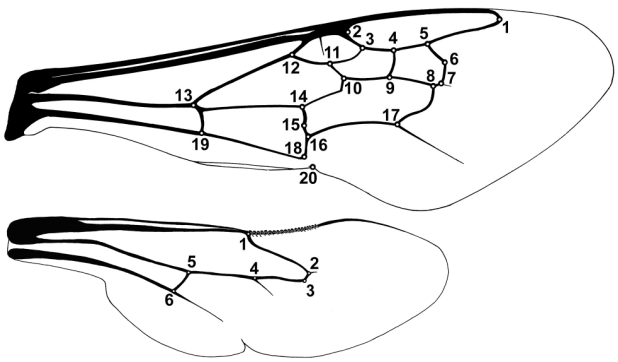

Figure 1-2
Location of the landmarks on the schematic wing of a bumblebee. 1 (top), 20 landmarks on the front wing; 2 (bottom), 6 landmarks on the hind wing.

Results

Relative warps

The relative warps are shown with the data obtained from front and hind wings, by using an orthogonal alignment projection method. Singular values explained by the first two relative warps for the front wing consensus were 0.051 and 0.038 respectively (total percentage of 88.91%). The landmarks 15 and 16 are determined as having the highest relative contributions. The landmarks 1, 13 and 19 associated with the highest variances for aligned species with values of s^2 = 0.2092, 0.1847 and 0.1979 respectively, whereas landmark 10 with the lowest (s^2 = 0.005). Relative positions of the average configurations of the subgenera are clustered together in the shape space defined by the first two relative warps (Fig. 3A). The relative positions of landmarks 13 and 19 in *Melanobombus* give a different basal shape to the wings than those of *Mendacibombus* and *Sibiricobombus*.

The same method is used for the hind wings. Singular values explained by the first two relative warps for the consensus data obtained from the hind wings are 0.104 and 0.032 respectively (total percentage of 97.95%). The landmarks 2 and 3 are determined as having the highest relative contributions. The landmark 1 is associated with the highest variance for aligned species with a value of s^2 = 0.3898 whereas landmark 4 is associated with the lowest variance (s^2 = 0.0094). Relative positions of the average configurations of the subgenera give the same clustering as those obtained

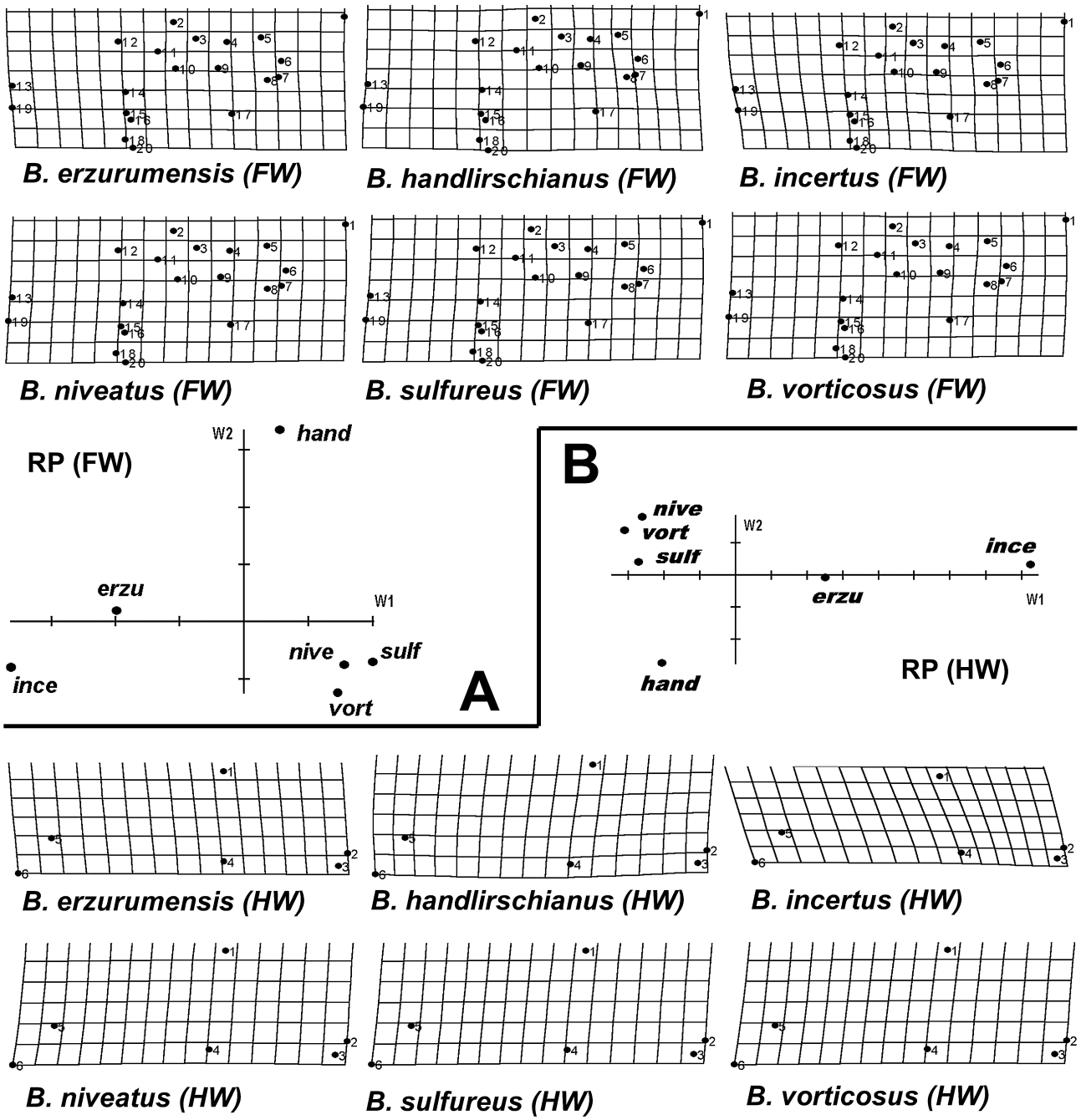

Figure 3
Relative positions of the mean configurations of the species for *Sibiricobombus* (nive-sulf-vort), *Mendacibombus* (hand) and *Melanobombus* (ince-erzu) in the shape space defined by the first two relative warps (x=1 y=2, D=0). **A, Front wing** (FW); **B, Hind wing** (HW) Filled circles indicate the position of the species. Deformation grids of these species superimposed on the consensus configuration are also shown for front wings (A) and hind wings (B). Circles with numbers indicate the landmarks.

Table 1. Bending energies, Procrustes distances (d), and angles (radians) among the examined species and reference.

Front-wing			
Studied *Bombus* species	**Energy (10^{-5})**	**Angle (10^{-5})**	**d (10^{-5})**
B. (Sibricobombus) niveatus Kriechbaumer 1870	3369	4802	4801
B. (Sibricobombus) vorticosus Gerstaecker 1872	3850	4783	4783
B. (Sibiricobombus) sulfureus Friese 1905	4004	5246	5245
B. (Mendacibombus) handlirschianus Vogt 1909	5073	5394	5393
B. (Melanobombus) erzurumensis (Özbek 1990)	2087	2279	2279
B. (Melanobombus) incertus Morawitz 1882	294	855	855

Hind-wing			
Studied *Bombus* species	**Energy (10^{-5})**	**Angle (10^{-5})**	**d (10^{-5})**
B. (Sibricobombus) niveatus Kriechbaumer 1870	1121	9634	9630
B. (Sibricobombus) vorticosus Gerstaecker 1872	1117	10362	10357
B. (Sibiricobombus) sulfureus Friese 1905	1299	9688	9684
B. (Mendacibombus) handlirschianus Vogt 1909	1816	9428	9425
B. (Melanobombus) erzurumensis (Özbek 1990)	289	4421	4420
B. (Melanobombus) incertus Morawitz 1882	61	1659	1659

from the front wings, in the shape space defined by the first two relative warps (Fig. 3B).

When the transformation grids on thin plate spline are examined in the hind wings, *B. sulfureus* shows expansion factors in landmarks 5 and 6 lower than 1.00 while they are more than 1.00 in the latter two *species (not illustrated)*. The bending energies, procrustes distances and angles calculated from the wing consensus data support the differences calculated by other methods used here (Tab. 1). The similarity in the means of these energies of the wing shapes for both hind and front wings between *B. niveatus* and *B. vorticosus* is remarkable. The values of bending energies, procrustes distance and angles for all taxa are also organised along a gradient.

Principal Component Analysis

PCA based on the data from the shape of the front and hind wings give similar results. The species are ordered along the first two principal components in a same range for front (Fig. 4) and hind wings (Fig. 5). The subgenera clustering pattern in both graphs are similar. However, *B.* (*Mendacibombus*) *handlirschianus* is distinguished by its scores on PC_2 in the front wing shape deformation, while *B.* (*Melanobombus*) *incertus* and *B.* (*Melanobombus*) *erzurumensis* are distinguished by their scores on PC_1 in the hind wing shape. *B.* (*Mendacibombus*) *handlirschianus* is well seperated from the species of *Sibiricobombus* by the PC_3 (*not illustated*).

BC

The mean shapes of the compared species *B. vorticosus* and *B. niveatus* are nearly identical (*not illustated*). Hotelling's T^2 on the data in Bookstein Two point registration yields $df_{niv} = 40$, $df_{vor} = 9$. The distance between means is very low (0.0279) which show no significant difference between taxa ($p > 0.05$).

Size morphometry

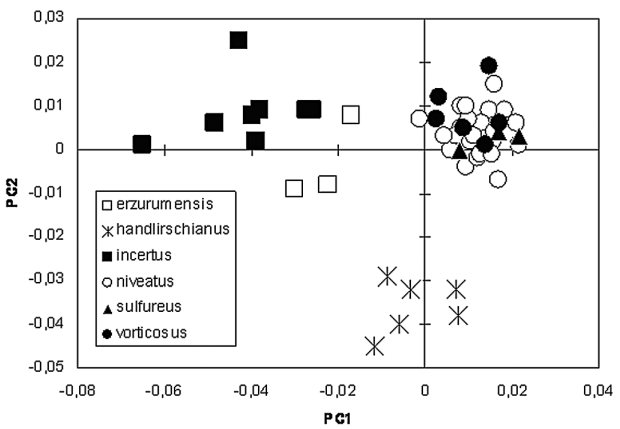

Figure 4
Distribution of the six different species of bumblebees from the subgenera *Sibiricobombus, Mendacibombus* and *Melanobombus* along the first two PC's. Principal component analysis of tangent space coordinates derived from GPA of the original coordinates that was conducted for the 20 landmarks digitized from the front wings. Horizontal axis, PC1; vertical axis PC2.

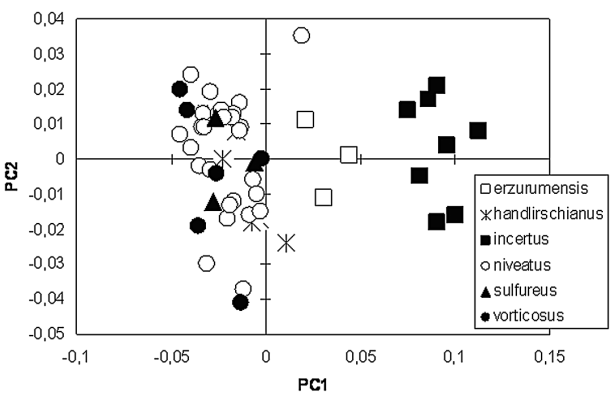

Figure 5
Distribution of the six different species of bumblebees from the subgenera *Sibiricobombus, Mendacibombus* and *Melanobombus* along the first two PC's. Principal component analysis of tangent space coordinates derived from GPA of the original coordinates that conducted for the 6 landmarks digitized from the hind wings. Horizontal axis, PC1; vertical axis PC2.

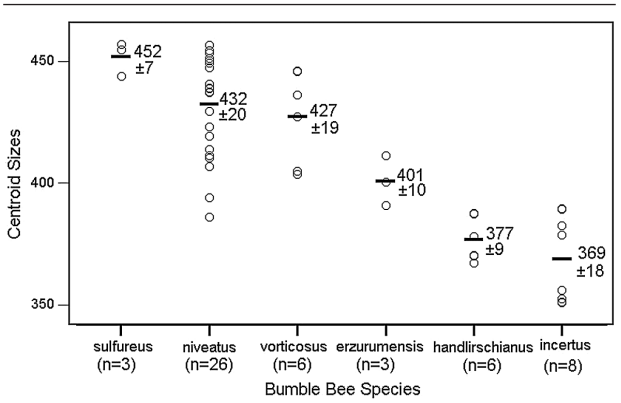

Figure 6.
Plot of the individuals of the bumblebees species examined from the data of centroid sizes from the front wings. Group means are indicated by lines and dotplots by open circles. The mean and the standart deviations which are rounded by two decimals also given inside the graph.

The Kruskal-Wallis test based on the centroid size data obtained from the front wings show that there is significant size differences (H = 34.75 at $P < 0.001$) among the examined species. The Mann-Whitney *U*-test shows that there is no significant difference between *B. vorticosus* and *B. niveatus* (W = 450.0 at $P > 0.05$). All the species show the expected classification schema on the species and subgenera levels by means of their centroid sizes (Fig. 6) except for *B. incertus* which have some extreme values that cause overlap with *B. handlirschianus*. No correlation is found between size and shape when principal component scores of the significant principal components are plotted *vs* centroid sizes (*not illustrated*).

Discussion

Sibling species are expected to show high morphological similarity. However, some differences in morphology that allow discrimination can be found when morphometric approaches are used (Moraes *et al*. 2004). Morphometrics – the quantitative description, analysis and interpretation of shape and shape variation in biology - is a fundamental area of research (Rohlf 1990). Unlike the analytical approaches, the geometric one is aimed at comparison of the shapes themselves (Pavlinov 2001). We used several different statistical methods for re-classification of some bumblebee species by this methodology. Our results show that there is no morphological (shape) or morphometrical (size) difference between wing shape and size of *B. niveatus* and *B. vorticosus* despite their different coat color pattern. This supports the hypothesis of Williams (1998) and Rasmont *et al*. (2005) who suggest that *B. niveatus* and *B. vorticosus* should be considered as conspecific. In this case the oldest available name is *Bombus niveatus* Kriechbaumer.

Because the front and hind wings in bumblebees (as in other holometabolous insects) develop from separate imaginal discs (Snodgrass 1956) they could be regarded as separate modules (Klingenberg 2003). If each wing comes from ontogenic processes then the variation should be homogenous and similar for each wing data and the results obtained from those data would be similar. Our results for phenetic similarity based on both wings are consistent with phylogenetic classification of the subgenera and species. All the methods used here for front and hind wings give similar results. Therefore we can say that for bumblebees, geometric morphometrics can be used as a powerfull tool in taxonomical studies, and has some advantages over other methods by being cheaper, faster and usable with old museum materials.

Our results also show that the wing shapes of both front and hind wings show significant differences among the bumblebee species, as has been found for Diptera (Rohlf 1993; Moraes *et al*. 2004) and Hemiptera (Gumiel *et al*. 2003). The origins of these differences are not clear at the present time. For bumblebees there can be some adaptive constrains such as the altitude, foraging behaviour, mate searching behaviour of the males, flying mechanism, pollen load, etc. The differences may also show strong phylogenetic patterns but it would be clear after making similar studies concerning workers and queens. The different schemas that result from the size morphometry (especially in the place of *B. incertus*) suggest that the size changes could be more adaptive than the shape itself. This can be interesting for the studies of flight. Previous studies of the flight mechanism of insects (Ellington 1984; Usherwood & Ellington 2002) were focusing on the kinematics of the whole wing area in terms of the aspect ratio ($AR = 4R^2/S$ where S is the total wing area and R is the single wing length) which is dependent on the *size* and not on the *shape*. In the present study, the use of the *Type I* landmarks (*see* Bookstein 1991 for details) which have more developmental and evolutionary sense is generally preferred to the use of the *Type II* landmarks (such as the wing tips) which are equivalent functionally (O'Higgins 2000). The latter is more important for the calculation of the *AR*'s. However, there are still very few studies that compare the forward flight of insects (Dudley & Ellington 1990; Usherwood & Ellington 2002). From our results we suggest that the venation shape may be at least as important as the aspect ratio in flying. These results may hopefuly help to the future studies for understanding the flight mechanism of bumblebees and the effects of different venation models on it.

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