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Applications of Morphometrics to the Hymenoptera, Particularly Bumble Bees (*Bombus*, Apidae)

Robin E. Owen
Department of Chemical & Biological Sciences, Mount Royal University, Calgary, Alberta, Canada

1. Introduction

I will first briefly review the types and range of morphometric studies of the Hymenoptera, and will discuss the characters used. Wing venation characters are very commonly employed and I will briefly discuss wing development and functional aspects of hymenopteran wings in this context. The chapter will be partly a selected review of work in this area by myself and others but will also include some original work of my own not previously published.

The Hymenoptera are an extremely diverse order of insects containing 144,695 described, extant species (Huber, 2009), fewer than the Coleoptera (beetles) and Lepidoptera (moths and butterflies), however if undescribed species are included then the Hymenoptera may be the most specious of all insect orders and there could be as many as a million species (Sharkey, 2007). There are two main groups of the Hymenoptera; the more primitive Symphyta (sawflies, horntails) and the Apocrita, which contain 93% of the species (Huber, 2009.) The Apocrita is subdivided into the Parasitica (parasitoids) and the Aculeata, the stinging Hymenoptera which includes the familiar ants, bees and wasps. There are many evolutionary and taxonomic questions concerning the Hymenoptera which can be answered using applications of morphometrics.

Morphometrics can be broadly defined as the quantitative study of the size and shapes of organisms. Often only parts (e.g. limbs) or organs of an organism are measured, and more general conclusions are drawn about evolutionary relationships, for example, from these measurements. What is now called *traditional morphometrics* or multivariate morphometics, is the application of multivariate statistical techniques (e.g. discriminate function analysis) to morphological data sets (Adams et al., 2004). One problem, in addition to others, with using standard multivariate methods for the analysis of shape is that linear distances are usually highly correlated and so much effort was expended correcting for size (Adams et al., 2004). The "Geometric Morphometric Revolution" overcame these problems by developing methods which allowed the shape of parts, or of the whole organism to be analysed (Rohlf & Marcus, 1993; Adams et al,. 2004). This is *geometric morphometrics.*

Morphological measurements of insects, including Hymenoptera and especially the eusocial species, have had a long history of use (e.g. Huxley 1972) and have often been termed *morphometrics*. This is not true multivariate morphometrics as currently defined above and often only involves plots of two variables, such as head width and antennal scape length to describe allometric growth and caste differences in ants (Huxley, 1972; Wilson, 1971), although a combination of univariate and multivariate statistics has sometimes been employed to determine caste differences (e.g. Gelin et al., 2008). In other studies, such as those on bees, multiple characters will be measured and used descriptively but multivariate statistical analysis is not employed. I will refer to this approach as *classical morphometrics.*

2. Morphometric studies of hymenoptera

2.1 Wings and wing venation characters

Classical morphometric studies have primarily used various mouthpart measurements in addition to a measure of overall size usually radial cell length or total length of the wing (Medler, 1962; Pekkarinen, 1979; Harder, 1985), however wing measurements alone have been used in the majority of traditional and geometric morphometric studies. In holometabolous insects the longitudinal veins develop first, followed by the crossveins. Wing veins contain trachea, blood lacunae and nervous tissue, and are sensitive to developmental disturbances, as shown by studies of *Drosophila* (Marcus, 2001). The primary function of the wing veins is to provide structural support and the pattern of venation is a crucial determinant of flight mechanics. During flight insects constantly adjust wing camber for optimal air flow, and this adjustment results from the flexural stiffness of the wing, which in turn depends on the position of the crossveins (Marcus, 2001). The pattern of venation can be quantified by measuring the coordinates of the junctions (which I will call *points*) of the longitudinal and the crossveins, which presumably reflect phylogenetic and developmental information. Wing morphometrics has been successfully used in taxonomic studies of Hymenoptera to differentiate between closely related taxa, and has also shown significant differences in wing shape, size and mechanical properties between species (Aytekin et al., 2007), however there are only a relatively few studies using wing morphometrics to estimate fluctuating asymmetry.

Essentially the same set or a slightly reduced set of coordinates have been employed in most studies of Hymenoptera. Forewings have been used in all studies but some have also used data from the hindwings (Aytekin et al., 2003, 2007; Klingenberg et al. 2001). Representative examples of hymenopteran forewings are shown in figure 1 (bumble bee, *Bombus*), figure 2 (solitary wasp, *Sphex*), figure 3 (social wasp *Dolicovespula*) and figure 4 (parasitoid wasp, Braconidae) with the points used for measurement. The wing venation in figure 1 is essential homologous among bees (Table 1) and a maximum of 20 points in any particular study have been used on the forewing (Table 1) and six on the hindwing (Aytekin et al., 2003, 2007; Klingenberg et al., 2001). There is a slight difference in the venation between bumble bees and honeybees which means that point 23 is not homologous. How the measurements are then analysed depends on the approach, e.g. traditional or geometric morphometrics, etc. The wing venation in figure 2 is homologous among some of the aculeate wasps (Table 1).

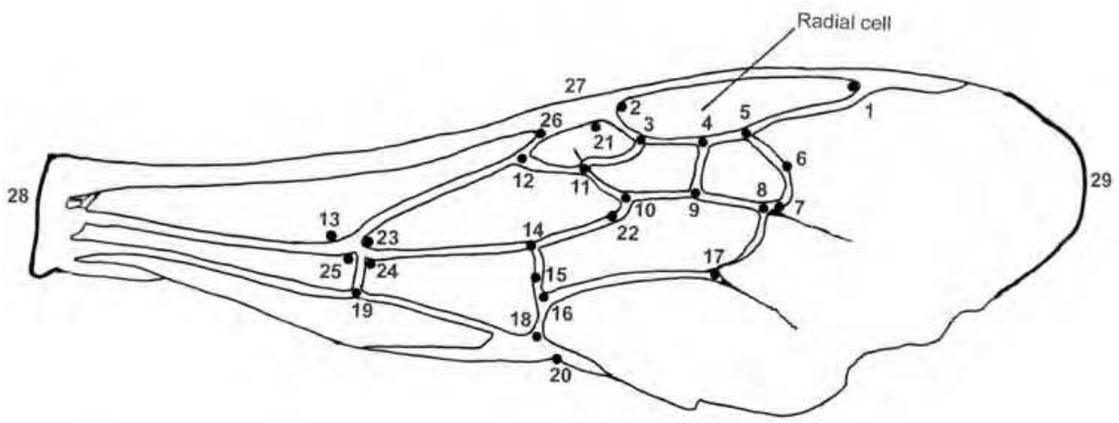

Fig. 1. Right forewing of a *Bombus rufocinctus* queen. This shows the total of 29 points which have been used in various combinations for multivariate morphometric studies of bees (see Table 1). The wing venation and the points are homologous among taxa of bees. The numbering of the first 20 points follows Aytekin et al. (2007). The length (distance 1-2) of the radial (= marginal cell) cell is also indicated as this has been used as one measure of size in some studies. The distance from the tegula (point 28) to either the distal end of the radial cell 1) or to the wingtip (29) have also been used a measures of bee size.

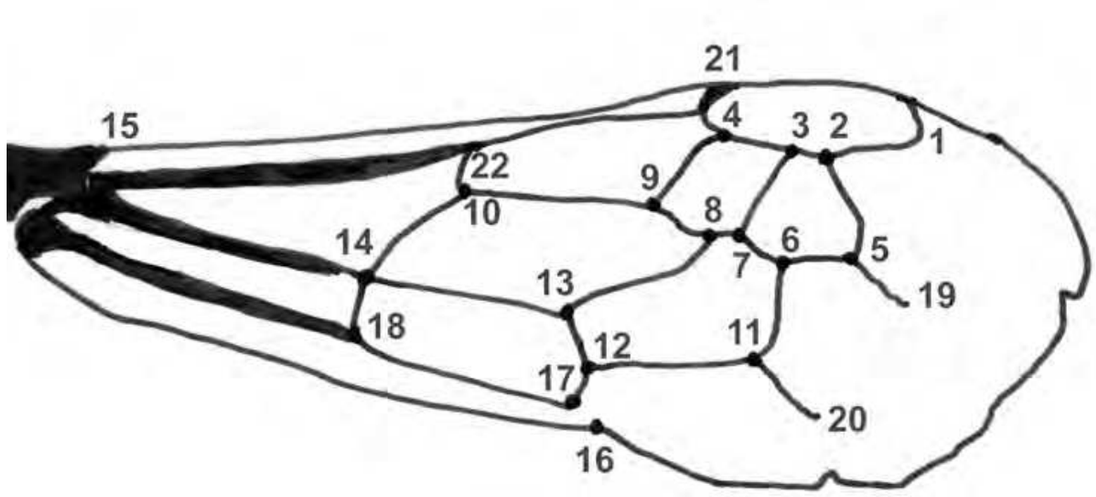

Fig. 2. Forewing of *Sphex maxillosus* redrawn from the photograph (Figure 1) of Tüzün (2009). This shows the total of 22 points which have been used in various combinations for multivariate morphometric studies of wasps (see Table 1). The wing venation and the points are homologous among taxa of aculeate wasps. The numbering of the first 20 points follows Tüzün (2009).

Family, Tribe or subfamily	Genus	Number of points	Points used (forewing)	Caste/ Sex[1]	Type of study[2]	Reference
Apidae, Bombini	*Bombus*	20	Fig. 1 : 1-20	M	G, C	Aytekin et al. 2007
Apidae, Bombini	*Bombus*	20	Fig. 1: distances (28-29),(20-27), (1-27),(1-5), (3-16),(10-13),(9-10),(3-12)	Q ,W	T, C	Aytekin et al. 2003
Apidae, Bombini	*Bombus*	19	Fig. 1: 1,2,3,4,5,8,9,10,11,12,13,14,16,18,19,21, 23,24,25	Q	T, NT	Plowright & Stephen, 1973
Apidae, Bombini	*Bombus*	19	Fig. 1: 1,2,3,4,5,8,9,10,11,12,13,14,16,18,19,21, 23,24,25	Q	T, C	Plowright & Pallett, 1978
Apidae, Bombini	*Bombus*	19	Fig. 1: 1,2,3,4,5,8,9,10,11,12,13,14,16,18,19,21, 23,24,25	Q	T, C	Plowright & Stephen, 1980
Apidae, Bombini	*Bombus*	19	Fig. 1: 1,2,3,4,5,7,8,9,10,11,12,13,14,16,17,18, 19,23,26	Q, W	T, C	Kozmus et al., 2011
Apidae, Bombini	*Bombus*	14	Fig. 1: 1,3,4,5,8,9,10,11,12,16,17,18,19,24	Q	T, C	Owen et al., 2010
Apidae, Bombini	*Bombus*	13	Fig. 1: 3,4,5,7,8,9,10,11,12,14,17,18,19,24	W	G, FA	Klingenberg et al., 2001
Apidae, Apini	*Apis*	19	Fig. 1: 1,2,3,4,5,7,8,9,10,11,12,13,14,16,17,18, 19,23,26	W	G, FA	Smith et al., 2007
Apidae, Apini	*Apis*	19	Fig. 1: 1,2,3,4,5,7,8,9,10,11,12,13,14,16,17,18, 19,23,26	W	G, ABIS, C	Francoy et al., 2009
Apidae, Euglossini	*Euglossa, Eulaema*		Fig. 1: distances M1 (1-17), M2 (1-12), M3 (12-18), M4 (17-18)	M	FA	Silva et al., 2009
Sphecidae, Sphedini	*Sphex*	20 - 24	Fig. 2: 1-20	?	G, C	Tüzün, 2009
Sphecidae, Larini	*Tachysphex*	15	Fig. 2: 1,2,3,4,6,7,8,9,10,13,14,17,18,21,22	M, F	G, C	Pretorius, 2005
Vespidae, Polistini	*Polistes*		Fig. 2: distances (1-4),(7-11), (10-12), (13-14)	Q, M	T, V	Eickwort, 1969
Vespidae, Vespinae	*Dolichovespula*	17	Fig. 3: 1-17	M	T,C	Tofilski, 2004
Braconidae, Agathidinae	*Bassus*	15	Fig. 4: 1-15	F	G, C	Baylac, et al. 2003

[1] *Q = queen, W = worker (female), F = female, M = male, ? = sex not specified.*
[2] *G = geometric morphpmetics, T = traditional morphpmetics, ABIS = automated bee identification system, FA = fluctuating asymmetry, C = classification/taxonomy, NT = numerical taxonomy, V = quantitative variation.*

Table 1. A representative selection of multivariate morphometric studies of the Hymenoptera. Either the distances between points, the distance of each point from an origin, the Cartesian coordinates of the points, or the angles between certain points are used as data. See the text for details of each study.

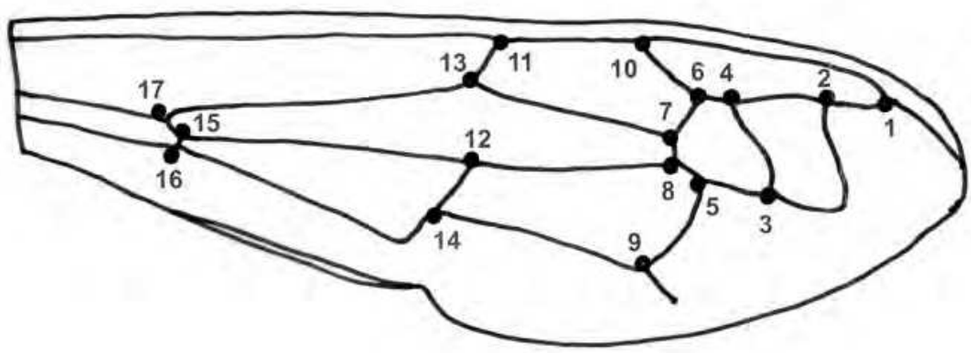

Fig. 3. Right forewing of a *Dolicovespula sylvestrimale* redrawn from figure 4 of Tofilski (2004).

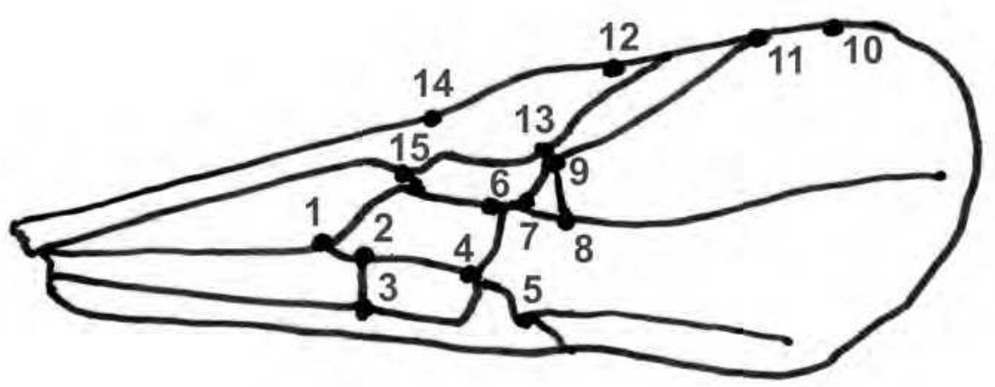

Fig. 4. Right forewing of a *Bassus tumidulus* female redrawn from figure 2 of Baylac et al. (2003).

As can be seen from the figures and Table 1, the wing venation and the wing points are not homologous among even all the Apocrita represented here, so morphometric comparisons have to be done on relatively closely related species. Wing vein characters are, however, used in cladistic analysis (Alexander, 1991; Sharkey & Roy, 2002; Shih et al., 2010) although these are generally not quantitative but instead presence/absence of veins, etc. As Sharkey & Roy (2002) point out reduction and loss characters are difficult to code and are subject to homoplasy.

2.2 Classical morphometrics

Medler (1962) measured the lengths of the radial cell of the forewing, the glossa, the prementum and the first segment of the labial palpus in 35 species of bumble bees (*Bombus* spp.). He then calculated correlation coefficients between each of these characters and calculated a wing index and a labial index (queen/worker x 100). Medler (1962) found that

these indices did vary among the recognized subgenera of *Bombus*. Univariate measures of various characters of bumble bees and correlations between characters have been reported in other studies (Pekkarinen, 1979; Harder 1982, 1985; Owen, 1988). Pekkarinen (1979) measured radial cell length and calculated mouthpart indices for 13 species of bumble bees in Denmark and Fennoscandia. He found that many closely related species, subspecies or populations could be distinguished from one another on the basis of mouthpart indices (mouthpart length/radial cell length). He also found allometric variation of wing length and some mouthpart indices with body size (Pekkarinen, 1979).

Morphometric variation in relation to foraging and resource partitioning has been extensively studied in bumble bees, but these have been limited to univariate measures or indices of characters important for the foraging behaviour of worker bees. It is well established that glossa (tongue) length is a major determinant of flower choice as there is a positive correlation between glossa length and corolla length of flowers visited (Pekkarinen, 1979; Harder, 1985; Prŷs-Jones & Corbet, 1987). However Harder (1985) found that besides glossa length other factors, such as body size, wing length flower species richness and plant abundance, also influence flower choice.

Similar morphometic studies have also been done with other bees, for example stingless bees, the Meliponinae (Danaraddi & Viraktamath, 2009), and univariate measures of size variation, usually in relationship to sex ratios and sex allocation, is well known in leafcutter bees, particularly *Megachile* (e.g. Rothschild, 1979; O'Neill et al. 2010). I am not including here studies of quantitative genetic variation and heritability as these will be discussed later.

2.3 Traditional morphometrics

Discriminant function analysis, introduced by Fisher (1936), has been widely used in traditional morphometrics. Discriminant analysis is used to classify individuals into groups, i.e. to define group boundaries (Sneath & Sokal, 1973; Hintze 1996). It derives linear functions of the measurements which best discriminate populations (Fisher, 1936). These maximize discrimination between groups, the goal being to be as certain as possible that individuals are assigned to the "correct" group according to a qualitative predictor variable. Mathematically the technique is similar to multiple regression analysis, the difference being that in discriminant analysis the dependent variable is discrete instead of continuous (Hintze 1996). The predictor variable in taxonomic studies is species name, and the null hypothesis is that the original classification of the species is correct. Since discriminant analysis derives equations that maximize distinction between groups it is an inherently conservative technique as this will correspondingly minimize the likelihood of making a Type I error. Where real differences do exist the technique does correctly discriminate between species. Canonical variates analysis is very similar to discriminant function analysis except that the discriminate scores, *D*, are plotted in a system of orthogonal axes, which are the canonical variates (Sneath & Sokal, 1973). Discriminant functions are relatively insensitive to overall size differences, but an individual of the same shape but of much different size may be classified incorrectly (Sneath & Sokal, 1973).

Traditional morphometric approaches have been applied to problems of taxonomy, classification and geographic variation in the honeybee *Apis mellifera* and the other three commonly defined species; *A. florea*, *A. cerana*, and *A. dorsata* (Ruttner, 1986). A combination

of discriminant function analysis, principal component analysis and cluster analysis allows the 23 geographic races of *A. mellifera* to be distinguished (Ruttner, 1986). Forty morphological characters were used for this analysis including angles of wing venation (Ruttner, 1986). I shall not attempt to review the large literature on honeybee morphological variation, instead I will concentrate mainly on some examples from bumble bees.

An early application of traditional morphometrics and numerical taxonomy to bumble bees was the study of Plowright & Stephen (1973) on the evolutionary relationship of *Bombus* and their social parasites, *Psithyrus*. They measured the coordinates of 19 points using point 19 as the origin and the line from 19-4 as the horizontal axis (Fig. 1, Table 1). The measurements were standardized by dividing them by the length 19-4 to give variables independent of size (Plowright & Stephen, 1973). The generalized Mahalanobis distance, D^2, was calculated for each species pair (Sneath & Sokal, 1973) and each distance was subtracted from the largest distance to give a measure of similarity (Plowright & Stephen, 1973). Plowright & Stephen (1973) then used weighted-pair-group cluster analysis (Sneath & Sokal, 1973) to produce a phenogram. The 13 species of *Psithyrus* were clearly separated from the 60 *Bombus* species. They also used multiple discriminant analysis (canonical variates analysis) to visualize the groupings (Hintze, 1996). Again *Psithyrus* was clearly separated from the *Bombus* subgenera on the plot of the first two canonical variates (Plowright & Stephen, 1973).

Traditional morphometrics has also been successful for lower level species discrimination. As will be described later, there are numerous taxonomic problems in the genus *Bombus* concerning the exact relationship of closely related species. Plowright & Pallett (1978) applied the same measurement techniques as used by Plowright & Stephen (1973) and discriminant analysis to re-investigate the taxonomic status of *B. sandersoni* Fkln. They measured previously identified museum specimens, and found a non-overlapping separation between *B. sandersoni*, and *B. frigidus* F. Sm., and *B. vagans* F. Sm. Therefore Plowright & Pallett (1978) suggested retaining *sandersoni* as the valid name for the species. However they did also point out that their results did not preclude this taxon from being a clinal variant of *frigidus*. Similarly Plowright & Stephen (1980) re-examined the taxonomic status of *Bombus franklini* (Frison) and multivariate analysis gave a clear separation of *franklini* from other species within the subgenus.

Tofilski (2004) was able to correctly classify all 22 individuals of the two wasp species *Dolicovespula sylvestrimale* and *D. saxonica* using stepwise discriminate function analysis of the coordinates of 17 wing vein points (Fig. 3, Table 1).

Not only have the distances between points been used for traditional and geometric morphometrics, but the angles described by wing veins, and some indices based on the points have also been calculated and used as characters for species discrimination (Tüzün, 2009; Kozmus et al., 2011). Also Alexander (1991) used two wing vein angles in his cladistic analysis of the genus *Apis*. Tüzün (2009) used wing vein angles to discriminate between and 30 species of wasps from different families. He used a combination of traditional and geometric morphometric techinques. He used 20 points (see Fig. 2, Table 1) and an additional four points (not shown here) on some species, measured the distance between *all* combinations of points and calculated vein length ratios (Tüzün, 2009). All possible combinations and ratios were calculated and also all angles between points were calculated, yielding a table of 77 different angle and ratio values for all species (Tüzün, 2009). One focus

of his study was to differentiate between three *Sphex* species *S. maxillosus*, *S. flavipennis* and *S. pruniosus*. He used stepwise discriminant fuction analysis and found that the three species were unambiguously separated by this method (Tüzün, 2009). He measured 27 more wasp species and entered the data into a database. He wrote a computer program to compare an unknown specimen with those in the database by calculating:

Total Angle Variation = |Angle 1 [unknown species]-Angle 1[species found in the database] |+|Angle 2 [unknown species]-Angle 2[species found in the database] |+…+ etc., and

Total length Variation = |1-Length 1[unknown species]/ -Length 1[species found in the database] |+ |1-Length 2[unknown species]/ -Length 2[species found in the database] |+…+ etc. (Tüzün, 2009).

The lower the value the higher the probability of a correct identification. Some examples are given in Table 2 which is extracted from Table 6 of Tüzün (2009). He also calculated a Similarity coefficient = (1/A x R) x K, where A = sum of the differences in wing angles, and R = sum of differences among the ratios of wing veins, and K = a constant (Tüzün, 2009.)

Pre diagnosed species	**Species estimated by the program**	**Sum of differences in wing angles (A)**	**Sum of differences among the ratios of wing veins (R)**	**Result: Similarity coefficient**
Vespa orientalis	*Vespa orientalis*	**19.873**	**2.111**	**23.8**
	Vespa crabro	51.714	2.856	6.8
	Vespa bicolor	55.962	3.368	5.3
Sphex rufocinctus	*Sphex rufocinctus*	**60.036**	**0.495**	**33.6**
	Sphex maxillatus	87.548	2.459	4.6
	Myzina tripunctata	78.030	2.001	6.4
Eumenes dubius cyranaius	*Eumenes dubius cyranaius*	**16.048**	**0.171**	**364.4**
	Eumenes coronatus detensus	33.398	3.193	9.4
	Eumenes pomiformis	60.840	4.483	3.7

Table 2. Some examples of the identification of wasp species according to wing morphometric values. (Modified from Tüzün (2009)).

His methods are clearly very successful at discriminating between wasp species, at least those represented in his data base. Kozmus et al. (2011) used eight lengths, 17 wing angles and five indices calculated from 19 points (Fig. 1, Table 1) for a total of 37 characters, and measured 530 queens and workers from 18 European species of bumble bees. They did

discriminant analysis based on Mahalanobis distance and from this assigned each specimen to a group. Canonical variates analysis was also performed and used to calculated three variables to separate the species into groups (Kozmus et al, 2011). They were able to correctly assign 97% of the bumble bees to the correct species, an in 13 species all the bees were correctly assigned (Kozmus et al, 2011). They found that three characters were particularly informative, based on high R^2 (explained variability) from an ANOVA. These were angle J16, A4 and discoidal shift (Dis D). Angle A4 is the angle described by the points (Fig. 1) 9, 7, 5 (where the vertex is denoted by the second number in the series and then first and last are the end points of line segments), J16 is the angle described by the points 3, 11, 26 and Dis D that between 1, 2, 7 (Kozmus et al, 2011). The R^2 were 63.82%, 61.91% and 60.30% respectively. This particular technique obviously holds great promise for identification and discrimination of *Bombus* species and groups.

The discussion of combined traditional morphometrics and genetic studies (e.g. Aytekin et al., 2003; Owen et al., 2010) will be left until section 4, below.

2.4 Geometric morphometrics

As mentioned earlier, the development of geometric morphometrics has led to the analysis of shape by removing the confounding effects of size. It encompasses a variety of multivariate statistical techniques for the analysis of Cartesian coordinates. These coordinates are usually (but do not have to be) based on point locations called *landmarks* (Slice et al., 2009). The studies which I will discuss here are based on landmarks so the specific suite of techniques used is referred to as *landmark based geometric morphometrics* (Adams et al., 2004). Since it is crucial to understand exactly how landmarks are defined, I have taken the definition directly from Slice et al. (2009):

"landmark - A specific point on a biological form or image of a form located according to some rule. Landmarks with the same name, homologues in the purely semantic sense, are presumed to correspond in some sensible way over the forms of a data set.

Type I landmark - A mathematical point whose claimed homology from case to case is supported by the strongest evidence, such as a local pattern of juxtaposition of tissue types or a small patch of some unusual histology.

Type II landmark - A mathematical point whose claimed homology from case to case is supported only by geometric, not histological, evidence: for instance, the sharpest curvature of a tooth."

(There are also Type III landmarks which do not concern us here). It is obvious that vein intersection points on insect wings are ideal Type I landmarks (Figs. 1, 2, 3,4), although they will not be homologous between relatively distantly related taxa (e.g. wasps and bees). It is better to use Type I landmarks and not Type II landmarks (e.g. wingtips) for evolutionary and developmental studies (Aytekin et al., 2007). Therefore differences between wings, either right and left ones of an individual, or differences between species can be analyzed using the Cartesian coordinates of landmarks as the data. The analysis proceeds by removing non-shape variation. This is variation in orientation, position and scale (Adams et al., 2004). There are a number of superimposition methods developed to remove the non-

shape variation, but the *Generalized Procrustes analysis* (or just Procrustes analysis) has become widely used. Procrustes analysis is an optimization technique which superimposes landmark configurations using least-squares estimates for translation and rotation parameters (Adams et al., 2004). After superimposition the deformation or "warping" in shape of each individual from a consensus form is given by partial warp scores (Adams et al., 2004; Aytekin et al., 2007). The partial warp scores can be analysed statistically to compare variation in shape within and between populations. Relative warp analysis is a principal component analysis of the partial warps (Adams et al., 2004). The thin-plate spline is used to plot the deformations them on a grid.

Landmark based geometric morphometrics and Procrustes methods have been used in a wide variety of studies over a wide range of taxa (Adams et al., 2004). Three applications of relevance here are (1) allometry of shape, (2) fluctuating asymmetry, and (3) taxonomy and classification.

Allometry of shape was detected by Klingenberg et al. (2001) in their study of development and fluctuating asymmetry in bumble bees, although it was not the main focus of their investigation. In another arthropod, the Fiddler crab, Rosenberg (1997) analysed shape allometry of the major and minor chilipeds. Studies of fluctuating asymmetry will be discussed in section 3, below. Here I will review a few selected studies of Hymenoptera using landmark based geometric morphometrics.

a. Bumble bees: As will be discussed in section 4, there are many taxonomic problems involving the exact status of species in some subgenera of bumble bees (*Bombus*). Aytekin et al. (2007) used landmark based geometric morphometrics to resolve some taxonomic problems in the subgenus *Sibiricobombus*. In particular the specific of *B. vorticus* and *B. niveatus* has been questioned (Williams, 1998). They collected 52 males from six species representing three subgenera (see Table 3).

Species		**Bending energy (10^{-5})**	
	n	Front-wing	Hind-wing
B. (Sibricobombus) niveatus	26	3369	1121
B. (Sibricobombus) vorticosus	6	3850	1117
B. (Sibricobombus) sulfureus	3	4004	1299
B. (Mendacibombus) handlirchianus	6	5073	1816
B. (Melanobombus) erzurumensis	3	2087	289
B. (Melanobombus) incertus	8	294	61

Table 3. The six species of bumble bees collected by Aytekin et al. (2007). Also given are the sample sizes (*n*) and the bending energies for the front- and hind-wings, calculated from the thin-plate-spline. Modified from Aytekin et al. (2007).

Principal component analysis clearly separated all species except *B. vorticus* and *B. niveatus*, also there was no significant difference in size between these two species although all others could be separated by size Aytekin et al. (2007). The bending energies, calculated from the thin-plate-spline showed some difference in the front-wing between *B. vorticus* and *B. niveatus*, but were remarkably similar for the hind-wings (Table 3). They concluded that there were no significant morphological differences between these two taxa and they should

be considered conspecific. Aytekin et al. (2007) also concluded that landmark based geometric morphometrics was a powerful method for resolving taxonomic problems in bumble bees and that venation shape may be an important factor for the mechanics of bumble bee flight. Although it must be realised that the bending energies from the thin-plate spline (Table 3) do not represent actual bending energies of a real bumble bee wing, but that they may nevertheless reflect some real mechanical differences between species. Shih et al. (2010) analysed the patterns of wing venation in extinct (fossil) and living pelecinid wasps and identified an "X" pattern of venation in the forewing which evolved and was maintained in some lineages. They suggest that this pattern could have provided a stronger wing structure and led to better flight performance for the larger species (Shih et al., 2010).

b. *Apis mellifera*: Francoy et al. (2009) used geometric morphometrics and an Automated Bee Identification System (ABIS) to examine changes in morphology of an Africanized honeybee population in Brazil 34 years after the African bee swarms escaped. This is interesting because it compare bees collected from 1965 – 1968 with those collected in 2002 at the same location (Francoy et al., 2009). In 1957 swarms of 26 colonies of the African honeybee *Apis mellifera scutellata* escaped in Brazil and hybrized with the previously introduced European honeybee races (Francoy et al., 2009). These Africanized bees have since spread throughout South America, Central America and into the USA by 1990 and are now found as far north as Nevada (Francoy et al., 2009). In 2002 Francoy et al. (2009) collected samples of five workers from 10 colonies from Ribeirão Preto, about 150 km from the original place of introduction of *A. mellifera scutellata*. They measured the right front wing of these specimens, the bees collected from 1965-1968 in the same location and also specimens of *A. mellifera scutellata*, *A. mellifera carnica, A. mellifera mellifera,* and *A. mellifera lingustica*. They used the standard 19 landmarks on the honeybee wing (Fig. 1, Table 1) and carried out two analyses: (1) geometric morphometrics was done using a Procrustes superimposition followed by the calculation of the relative warps and then a discriminant analysis. Mahalanobis distances, D^2, were also calculated (Table 4) and a dendrogram was plotted using these values (Francoy et al., 2009); (2) ABIS performs an automated analysis of images of honeybee forewings. It analyses the venation pattern and then uses various statistical techniques either linear discriminant analysis or a more powerful Kernal discriminant analysis which allows species and subspecies identification (Francoy et al., 2009). The system has to be "trained" with at least 20 specimens of each group (Francoy et al., 2009).

	RP - 1968	RP - 2002	*A. mellifera scutellata*	*A. mellifera mellifera*	*A. mellifera carnica*	*A. mellifera lingustica*
RP - 1968	-	12.43	12.40	21.60	32.98	29.83
RP - 2002		-	15.14	24.18	37.68	34.04
A. mellifera scutellata			-	22.47	27.08	23.65
A. mellifera mellifera				-	34.54	29.68
A. mellifera carnica					-	9.32
A. mellifera lingustica						-

Table 4. Mahalanobis distances, D^2, between the centroids of the Apis mellifera groups calculated through relative warp analysis (modified from Francoy et al., 2009). RP = Ribeirão Preto populations.

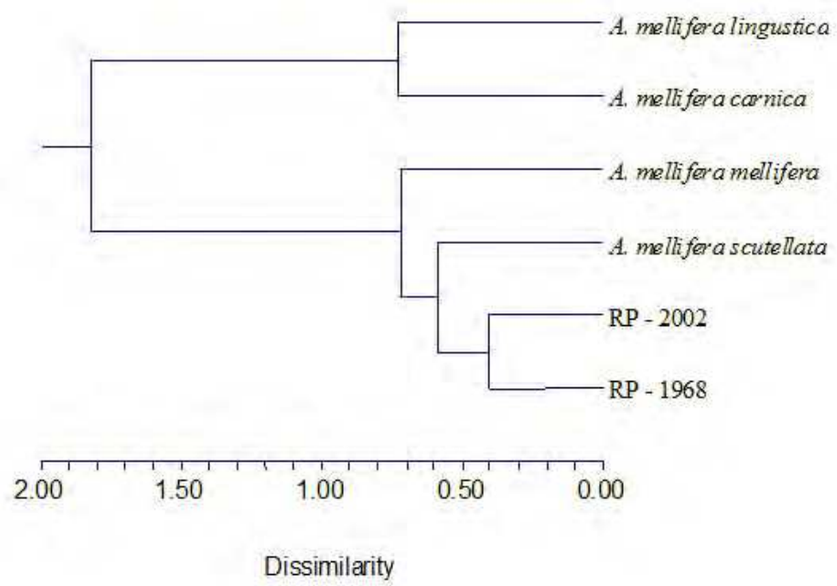

Fig. 5. A dendrogram produced by the Unweighted Pair-Group method (UPGMA) using the Mahalanobis distances in Table 4. Francoy et al. (2009) used the neighbour-joining tree method to produce a very similar dendrogram. Note that RP = Ribeirão Preto populations.

From Table 4, from which a dendrogram is constructed (Fig. 5) it is clear that Africanized bees resemble the African race more than they do the European races. Also it is interesting to see that there have been some morphological changes in the Africanized bees in Brazil over the 34 years since the hybridization event. The 1968 and 2002 Ribeirão Preto populations are clearly distinct with a Mahalanobis distance of 12.43 (Table 4, Fig. 5). The ABIS gave essentially the same results.

As mentioned earlier Tüzün (2009) used both traditional and geometric morphometric techniques. Clustering of the relative warps also separated the three *Sphex* species very well.

Two more studies are of interest as they show slightly different applications of the techniques. Pretorius (2005) used standard geometric morphometrics to examine wing shape dimorphism between male and female wasps in the genus *Tachysphex*. He used 24 species in this genus and measured 15 landmarks (Fig. 2, Table 1). He did find small but definite differences in the shapes of the wings between the sexes and cautioned that in an analysis of a genus only one of the sexes should be used as small-scale differences, may in some cases influence the results (Pretorius, 2005).

Baylac et al. (2003) studied two closely related species of Bracoinid parasitoids. The two species *Bassus tumidulus* and *B. tegularis* had been synonymised and then subsequently split. Baylac et al. (2003) used geometric morphometrics of wing venation to study this problem but they also used some aspects of pattern analysis. Pattern analysis involves statistical techniques such as kernel density estimates and Gaussian mixture analysis (Baylac et al., 2003). Baylac et al. (2003) measured 15 landmarks on the wing (Fig. 4, Table 1) and found that both methods did separate the species into two definite morphological groups.

It is often useful and informative to combine traditional and geometric morphometric techniques and other methods (Fruciano, et al., 2011; Tüzün, 2009; Baylac et al., 2003). For instance Fruciano et al. (2011) point out that it may be necessary to use traditional techniques to allow a comparison with earlier results in the literature.

3. Fluctuating asymmetry

Most animals are bilaterally symmetrical, with paired internal organs and paired appendages. However the symmetry is often not exact or "perfect". There are two general classes of asymmetry; conspicuous and subtle; conspicuous asymmetries are very obvious, for example the extreme difference in size between right and left claws in some crabs, e.g. Fiddler Crabs. However many animals exhibit less obvious types of asymmetry which can only be quantified in a *sample* of individuals, and thus statistical methods must be used to analyze it (Palmer, 1994). Measurements are made on a structure on the right (R) and left (L) sides of each individual in the sample and an index of asymmetry is then calculated. Three types of asymmetry can occur: (1) fluctuating asymmetry (FA), with a normal distribution of R- L values around a mean of zero, (2) directional asymmetry (DA) where the mean of one side is almost always greater than that of the other, and (3) antisymmetry where there is a difference between the two sides but it cannot be predicted which will show the greater value, so giving a broad-peaked or bimodal distribution of R-L values about a mean of zero (Palmer & Strobeck 1986).

Developmental stability (DS) is defined as "the ability of an organism to buffer development against genetic or environmental perturbation" (Clarke, 1997). For instance, populations undergoing decline are likely exposed to environmental and genetic stresses which may cause developmental instability (DI) of individuals (Parsons 1990, Milankov et al. 2010). This DI is often manifest by deviations from bilateral symmetry (Palmer 1994). Insect wing venation characters are ideal for assessing FA and environmental stress. Fluctuating asymmetry (FA), where the differences between right and left sides follow a normal distribution, should reflect perturbations from perfect bilaterally symmetrical development and thus serve as a measure of the stresses experienced by an individual during its development (Palmer & Strobeck 1986). In turn it can be used as an epigenetic measure of stress in natural populations (Parsons 1990). Therefore the estimation of FA could be an important indicator of the "health" of species and help guide decisions regarding conservation. Recently much attention has been paid to the significant contraction of the distributions, and the decline in the abundance, of some bumble bee species in North America and Europe (Evans et al. 2008; Goulson et al. 2008). In North America *Bombus affinis* Cresson, *B. terricola* Kirby and *B. occidentalis* Greene have all disappeared from significant parts of their historic ranges (Colla and Packer 2008; Evans et al. 2008, Grixti et al. 2009; Cameron et al. 2011). If FA is a good predictor of stress then we would predict higher levels of FA in species undergoing decline than stable species.

3.1 Developmental stability, fluctuating asymmetry and quantitative genetic variation

Here I clarify the relationship between developmental stability, fluctuating asymmetry and quantitative genetic variation in the Hymenoptera. Hymenoptera (ants, bees and wasps), have what is known as a *haplodiploid* genetic system. This means that females (queens and

workers in eusocial species), like most animals, are derived from fertilized eggs while males arise from *unfertilized* eggs. Thus males are *haploid* (*n*) inheriting only one member of each pair of chromosomes, those from their mother, whereas females are *diploid* (*2n*) having both members of each pair of chromosomes. Formally the system of inheritance follows the pattern of X-linked inheritance in organism in which both sexes are diploid. Many aspects of the genetics of X-linked or haplodiploid genes are different from that of autosomal genes, including the expression of quantitative or polygenic characters. One consequence is that for a genetically determined quantitative trait males are likely to be more variable than females (Eickwort, 1969; Owen, 1989). The variances are derived on the assumption of dosage compensation of genotypic values in males (Fig. 6), and we must distinguish between mean within-family (or within-colony) variances and population variances. Consider a single gene locus with alleles A_1 and A_2 at frequencies p and q respectively, and let the genotypes take the genotypic values shown in figure 6.

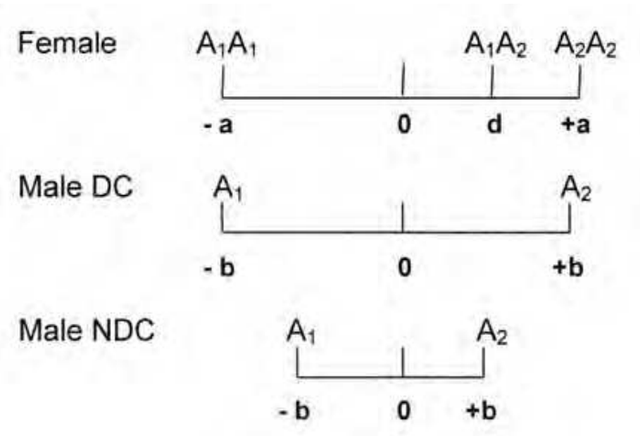

Fig. 6. Arbitrarily assigned genotypic values of a quantitative trait at an X-inked or haplodiploid locus, showing the difference between dosage compensation (Male DC) and no dosage compensation (Male NDC) of male genotypic values.

The well-known population variances (Owen, 1989) are,

Females: $V_f = 2pqa^2 + (2pqd)^2$

$= V_{Af} + V_{Df}$

Males: $V_m = V_{Am} = 4pqb^2$

Where the average effect $a = a+d(q-p)$, and V_{Af} and V_{Df} are the female additive and dominance variance components, respectively. Note that the male genotypic variance, V_m consists solely of an additive component, V_m. The corresponding mean within-colony variances are,

Females: $\bar{V}_f = \frac{1}{2}pq[\alpha^2 + 2ad(q-p) + d^2]$

Males: $\bar{V}_m = 2pqb^2$

It is assumed that the male and female offspring are full-siblings. Thus we can see that with (i) no dominance in females (d = 0), and (ii) dosage compensation in males (a = b), then for the population variances,

$$V_m = 2V_f$$

and for the mean within-colony variances,

$$\bar{V}_m = 4\bar{V}_f$$

Thus males are predicted to more variable than females, and this will generally be the case unless there is complete dominance in females and only then when the allele frequency q>0.62 (Owen, 1989). However if there is no dosage compensation then females will be more variable, i.e. $V_f > V_m$, except with intermediate dominance when q<0.16 and with complete dominance when q<0.21 (Owen, 1989). This differential variability has no relationship *per se* with developmental stability and FA, it is just the result of the different ploidy levels in males and females. However if genome wide heterozygosity promotes DS then we would expect haploid males to show more DI and FA than their diploid counterparts, thus haplodiploid organism are good models with which to partition the effects of heterozygosity and ploidy on DI and FA (Clarke, 1997; Smith et al. 1997). The prediction is that, due to the absence of heterozgosity, the haploid males will show higher FA than the diploid females.

3.2 Differential morphological variation between the sexes in the hymenoptera

Males in many species of Hymenoptera, in accordance with quantitative genetic theory are indeed more variable in morphological characters than females, although this is not always the case. For comparisons of eusocial species it is important to compare reproductives, i.e. males with queens and not workers. In the Hymenoptera worker size variation is great and due to many different factors (Wilson, 1971). Eickwort (1969) in her multivariate morphometric study of *Polistes exclamans* found that males were more variables than queens in the characters used. In addition to wing measurements (see Fig. 2 and Table 1) she also used six other morphological characters (number of hamuli, distance between the most distal and proximal hamular sockets, mesoscutal width and length, distance between compound eyes, head width). She sampled 19 nests and calculated generalized variances (D) to compare mean within-colony (nest) variances of males and females. Variance-covariance matrices were calculated across all the characters for males and females in each nest and the determinants of these matrices were defined as the generalized variances. Males were more variable than queens (P<0.01), and she noted that even the largest generalized variance for queens (0.000006020) was smaller than the smallest generalized variance of any group of males (0.000075343) (Eickwort, 1969).

Univariate studies of differential variability in the Hymenoptera are relatively common. I examined variation of radial cell length (distance between points 1-2, Fig. 1) in the bumble bee *Bombus rufocinctus.* A sample of 787 young queens from 38 laboratory reared colonies and a sample of 680 males from 38 colonies were measured. The males, with a coefficient of variation (CV) of 5.75% were significantly more variable (P<0.01) than the females (CV=3.98%). There were also significant intraclass correlations between male (t_m = 0.553) and

young queen (t_f = 0.435) offspring, indicating a considerable degree of phenotypic resemblance between bees of the same caste within each colony (Owen, 1989). Heritability as estimated from offspring-parent regression (0.20 ± 0.19 for queens and 0.47 ± 0.38 for males) was significantly lower that than estimated from the intraclass correlations, suggesting that environmental variation is of the same order of magnitude as additive genetic variation (Owen, 1989).

In other Hymenoptera environmental variation clearly is the most important determinant of phenotypic variation. Owen & McCorquodale (1994) examined variation and heritability of body size and postdiapause development time in the leafcutter bee, *Megachile rotundata*. The bees were from a domesticated population and the nests were in pine blocks (12 cm long) with about 500 standard nest tubes 5 mm in diameter (Richards, 1984). Head widths of offspring from total of 200 nests was measured and the frequency distribution is shown in figure 7.

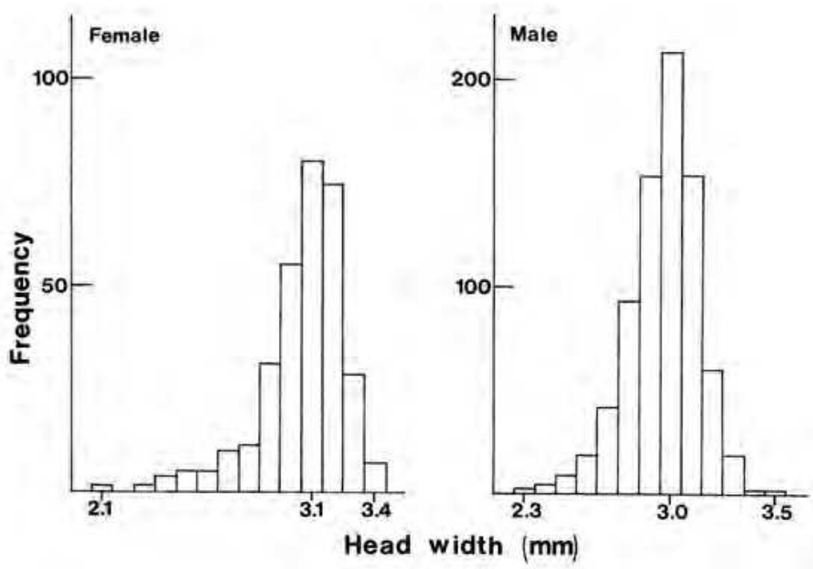

Fig. 7. Frequency distributions of head width in a sample of female and male offspring from 200 nests of Megachile rotundata (modified from Owen & McCorquodale, 1994).

Female offspring (n=312) from 151 of these nests had a mean head width (±SEM) = 3.5 ± 0.011 mm, and male offspring (n=769) from 172 of the nests were significantly smaller (P<0.00001) with mean head width = 2.96 ±0.006 mm. Interestingly the males also were *less* variable with a CV=5.65% as compared to females with a CV of 6.58%. Heritability of head width, estimated from offspring-parent regression, was not significantly different from zero and was considerably lower than that obtained from the intraclass correlation coefficient. Because intraclass correlations can be inflated by environmental variation, the difference between these two estimates implies that in this case maternal effects are the most important determinant of head width (Owen & McCorquodale, 1994). O'Neill et al. (2010) found that in feral populations of *M. rotundata* offspring size (head width) was generally positively related to tunnel diameter. Again, as Owen & McCorquodale (1994) did, they found offspring within families were more similar to each other than to bees in other families.

O'Neill et al. (2010) concluded that the most important maternal effect which probably accounted for this was the amount of provision provided by the mother. In *M. rotundata* and other leafcutter bees there is low if any, genetic variation for body size, and environmental factors are the major causes of variation in males and females (Owen & McCorquodale, 1994).

In mass provisioning wasps the size of the offspring is determined to a great extent by the mass of provision they receive (Hastings et al., 2008), and so the variation in offspring size would also reflect the variation in mass provision size. Therefore we would expect genetic sources of variance to be quite weak in comparison to environmental causes. This is illustrated well by the cicada killer wasp, *Specius speciosus* (Hastings et al., 2008; 2010). Hastings et al. (2010) studied two populations of Eastern cicada killer wasp in northern Florida, and they measured wet mass (mg) and right wing length (mm) of males and females. Both males and females were significantly larger and heavier at St. Johns than at Newberry, but in both populations males were less variable than females. The CV's calculated from Table 1 in Hastings et al. (2010) are: St. Johns, males CV=0.08%, females CV=0.10%; Newberry, males CV=0.05%, females CV=0.07%. There is a very interesting relationship between body size of the wasps and their prey. *S. speciosus* females provide male offspring with usually a single cicada, and each female offspring with usually two cicadas irrespective of prey size (Hastings et al., 2008). Hastings et al. (2008) sampled the wasps and cicadas from 12 different locations in 10 states in the USA, and they found a significant correlation between wasp size and the mean local cicada mass. However they did find the two locations in Florida (St. Johns and Newberry) where the pattern did not hold. Hastings et al. (2010) found that in these populations female wasps exhibited prey selection by size. Small wasps only collected small cicadas and large wasps only collected large cicadas. The small wasps probably cannot carry the large cicadas but the large wasps, which could carry the small ones, select only the larger sizes (Hastings et al., 2010).

3.3 Fluctuating asymmetry in haplodiploids

There are relatively few studies of FA and DI in Hymenoptera and haplodiploid organisms. Some studies have used traditional morphometric methods while others have employed geometric morphometric techniques. Clarke's (1997) study was to test the hypothesis that haploid males should show greater DI than the diploid females, as manifest by larger FA in males. He used a combination of morphometric (wing vein lengths; the details were unspecified) and meristic (number of humuli) in six taxa of Hymenoptera; two races of *Apis mellifera* (*capensis* and *scutellata*), *A. cerana*, *Trichocolletes affenutus* (Colletidae) , *Vespula germanica* (Vespidae) and *Solenopsis invicta* (Formicidae). He also assessed two haplodiploid thrip (Tysanoptera) species, *Haplothrips angustus* and *H. froggatti*, the measure used was the number of duplicated cilia along the posterior margin of the forewing. Clarke (1997) calculated mean asymmetry values for each character in each sex, and tested the difference between sexes using single classification and multivariate analysis of variance. Clarke's (1997) did not find any consistent pattern and his conclusion was that, as a whole, haploid males are no more asymmetric than diploid females. Of the 60 direct comparisons made using univariate ANOVA only 8% showed the haploid males to be more variable than the females and only 3% showed the reverse, and the other comparisons showed no significant

difference. Clarke (1997) found no significant difference in asymmetry between males and females in the two *Haplothrips* species. Crespi & Vanderkist (1997) measured FA in the thrip, *Oncothrips tepperi* also to test the hypothesis of higher FA in males, and also to compare FA in functional and vestigial traits. The latter should exhibit higher FA than the former due to relaxion of selection for functionality (Crespi & Vanderkist, 1997). They measured fore femora lengths of soldier and disperser morphs, and wing lengths of dispersers (functional traits), and wing length of soldiers (vestigial trait). Analysis was done following the methods of Palmer (1994). They found complex interactions between sex, caste and FA, namely that for wings FA was higher in female soldiers that in male soldiers, but in dispersers males had the higher FA. For the femora males and females did not differ in FA in either morph. Crespi & Vanderkist (1997) concluded that there was no consistently higher FA in males than females, but that vestigial traits did show higher FA than functional traits.

Silva et al. (2009) estimated FA in two species of Euglossine bees in Brazil to assess the effects of climatic and anthropogenic stresses on these bee populations. They collected 60 males of each species, 30 from the forest border and 30 from the interior of the forest, and half were collected during the hot, wet season and the other half during the cold, dry season (Silva et al., 2009). Four measurements (M1, M2, M3, M4, see Fig. 1, Table 1) were made on both wings of each individual, and for each measurement FA was calculated. A general body size index was obtained from a principal component analysis of measurements M 1-3, and then the transformed FA and size index data were analysed using ANOVA (Silva et al., 2009). There were no differences in FA for the four characters between areas and seasons in *Eulaema nigrita*, however in *Euglossa pleostica*, they found significant greater FA of M3 in bees collected in the hot and wet season than those collected in the cold and dry season. Silva et al. (2009) concluded that this species was responding to increased environmental stress in the hot, wet season.

The last two studies of FA that I will discuss used geometric morphometric methods. Smith et al. (1997) were interested in partitioning out the effects of ploidy and hybridization on levels of FA in *A. mellifera*. They used the coordinates of 19 points (see Fig. 1 and Table 1) on the forewings of ten workers and five males (drones) from each of 27 hives. The coordinates were digitized and subject to a Procrustes analysis of asymmetry (Smith et al., 1997). The specialized analysis described by Smith et al. (1997) produces a measure of asymmetry, A^2, for each specimen, then the mean A^2 of a series of specimens is decomposed into one term for FA and another term for directional asymmetry (DA). Smith et al. (1997) found that across all populations total asymmetry was significantly greater (one-way ANOVA, $P<0.001$)for haploid males than for diploid females, however they were surprised to find that most of the asymmetry was not due to FA but was directional asymmetry (Table 5).

	All bees	Females	Males
n=	377	261	116
Total squared asymmetry	7.29	6.34	9.42
Directional squared asymmetry	3.61	2.89	6.31
Fluctuating squared asymmetry	3.68	3.45	3.11

Table 5. Partitioning of total squared asymmetry in *Apis mellifera* into directional and fluctuating components. Note all entries are x 10^4. Modified from Smith et al. (1997).

Smith et al. (1997) concluded that perhaps DA was more common than previously thought. Klingenberg et al. (2001) examined FA and variation among individuals in the forewings and hindwings of bumble bees as part of an investigation of developmental modularity. The fore- and the hindwings develop from separate imaginal discs and so are expected to be independent developmental modules (Klingenberg et al., 2001). Klingenberg et al. (2001) predicted that patterns of variation among individuals should be similar to the patterns of FA within each wing, and that individual variation between fore-and hindwings will co-vary (depending on how much they really are independent modules), but that FA will be independent between them. They measured 13 points on the forewings (see Fig. 1 and Table 1) and six on the hindwings of worker bees. They used laboratory reared bumble bee colonies and subject sets of colonies to three treatments which consisted of providing a flow of air through the colonies, two with different concentrations of CO_2, 10% and 5%, and one a control treatment with just air (ultimately they only used the control and 5% treatments). Klingenberg et al. (2001) used geometric morphometric and Procrustes methods to characterize size and shape variation in fore- and hindwings separately. They found that the major pattern of variation within each wing was the coordinated shifts in sets of landmarks over the entire wing. This suggests that each wing is a developmental module which is not further subdivided into smaller domains (Klingenberg et al., 2001). As a consequence they also concluded that any small perturbations causing FA are transmitted throughout the entire wing, affecting all landmarks. Since shape asymmetry co-varied only between fore- and hindwings in the CO_2 treatment Klingenberg et al. (2001) concluded that the developmental interactions between wings are probably related to gas exchange.

4. Taxonomic and systematic problems: Concordance between genetic and morphometric approaches in bumble bees

Here I will discuss the use of combined genetic and morphometric approaches to resolve taxonomic problems, with examples from bumble bees. Bumble bees (tribe Bombini) form a well-defined monophyletic group containing a relatively small number of species (239 according to Williams 1998), thus it may seem surprising that bumble bees pose many taxonomic and systematic problems. At the specific level the taxonomic status of closely related taxa is often unclear and subject to contradictory interpretations. Bumble bees are relatively quite invariant or 'monotonous' morphologically compared to other bees (Michener 2000), but many species show considerable pile colour variation. Some of this has a simple (Owen & Plowright 1980) or relatively simple (Owen & Plowright 1988) genetic basis, but most variation is continuous and probably polygenic in nature (Stephen 1957), and to complicate matters further, considerable convergence in colour pattern, often between distantly related species also occurs (Plowright & Owen 1980). The root of the problem is that traditional taxonomic approaches are limited when applied to bumble bees. Genetic and statistical methods must be used to understand processes of speciation in *Bombus*. For example, Scholl *et al*. (1990) found that *B. moderatus* differed from *B. lucorum* at 3 out of 26 enzyme-gene loci, with the electromorphs exhibiting fixed differences in each species. Again, Scholl *et al.* (1992) found fixed electrophoretic differences between *B. auricomus* and *B. nevadensis* at 5 out of 18 enzyme loci. In both cases the authors suggested the return to the original specific designations. A powerful approach, which has been very successful in resolving some of these problems, is to combine genetics and morphometrics.

Aytekin et al. (2003) combined these approaches to elucidate the relationship between two subspecies of *Bombus terrestris*. In the eastern Mediterranean region two subspecies have been recognized, *B. terrestris dalmatinus* from the Balkans and surrounding areas; and *B. t. lucoformis* from Anatolia (Aytekin et al., 2003). Aytekin et al. (2003) sampled 157 specimens of queens and workers from Bulgaria, Greece and Turkey. They assessed allozyme variation by using six enzyme systems and morphometric variation by using 28 morphological characters. Of the morphological characters employed 13 were distances measured between points, eight on the front wing (Fig. 1, Table 1) and five on the hindwing. They found that the allozymes exhibited very little variation and the electromorphs appeared to be fixed in all populations, and both taxa were monomorphic in all loci scored (Aytekin et al., 2003.). They found no heterozygotes or different electromorphs, except *B. t. lucoformis* found in the Ankara region had two alleles for malic enzyme (*Me*) with electrophoretic mobilities of 100 and 102. The morphological characters were analysed by multigroup discriminant function analysis (canonical variates CANOVAR) and principal component analysis (PCA), and also failed to separate the two groups, so (Aytekin et al., 2003) concluded that there was not enough of a difference between *lucoformis* and *dalmatinus* to warrant separate sub-species status. I will now discuss two examples of some of my own work in more detail.

4.1 *B. melanopygus*/ *B. edwardsii*

Owen et al. (2010) examined the relationship between the two nominate taxa *B. melanopygus* Nylander, and *B. edwardsii* Cresson, using a combination of genetic and morphometric analyses. Traditionally there was absolutely no question that these taxa represented two distinct species (Stephen 1957; Milliron 1971) since the bees differ dramatically in the colour of the abdominal terga two and three, these being ferruginous (or red) in *B. melanopygus* and black in *B. edwardsii*, although other morphological differences between the two are minor (Stephen 1957; Owen et al. 2010). Moreover, the distributions have relatively little overlap. *B. edwardsii* occurs throughout California and just into neighbouring Nevada, while *B. melanopygus* extends north through Oregon, Washington, British Columbia, Alaska, east into Alberta, Saskatchewan, and across northern Canada possibly to Labrador (Stephen 1957; Laverty and Harder 1988). They are sympatric only in southern Oregon and northern California (Stephen 1957). However, the taxonomic status of these bees was called into question when Owen & Plowright (1980) reared colonies from queens collected in the area of sympatry. They discovered that pile coloration was due a single, biallelic Mendelian gene, with the red (*R*) allele dominant to the black (*r*). Also, the observed numbers of queen genotypes and colony types at each collection location conformed to those expected under Hardy-Weinberg equilibrium. This suggested that the two taxa are in fact conspecific, in which case there is a gene frequency cline running from north to south where the red allele is completely replaced by the black allele over a distance of about 600 km (Owen & Plowright 1980; Owen 1986). Although this genetic evidence is compelling, because the bees were only collected from the region where both alleles are present, it still leaves open the logical possibility that *B. edwardsii* is the dimorphic species and *B. melanopygus* exists as a separate, northern species.

Owen et al. (2010) showed that both enzyme electrophoresis and wing morphometrics do unambiguously distinguish between these two species. Allozyme electrophoresis can be useful for distinguishing closely related species. If there are fixed differences, or large gene

frequency difference between two taxa then this would strongly suggest either complete, or a very high degree of, reproductive isolation. Conversely, if two taxa have identical allozyme profiles, then this would strongly suggest conspecificity (see above Aytekin et al., 2003); however it cannot of course *prove* it. Similarly, morphometric analysis of wing venation patterns has also proved to be very successful for differentiating between bumble bee species as discussed earlier. Owen et al. (2010) included in their analysis a closely related species, *B. sylvicola* with which *B. melanopygus* is sympatric in Alberta. This was to verify that the techniques they used were sensitive enough to correctly discriminate closely related species if real differences do exist. Specimens were collected from Alberta and locations in Oregon and California (Fig. 8) and 113 bees were scored at 16 enzyme-gene loci using horizontal starch gel electrophoresis. For details see Owen et al. (2010). Traditional morphometrics was used and the points measured were a subset of those used by Plowright & Stephen (1973) The distance from 18 to the 13 points shown (Table 1, Fig. 1) was measured (for more details see Owen et al. (2010). Discriminant analysis was done using the statistical software package NCSS (Hintze 1996).Owen et al. (2010) did not standardize the measurements as done by Plowright & Stephen (1973), for two reasons: one was to ensure that any differences between taxa would be *maximized* by the discriminant analysis and the other reason was because size of bumble bee queens is important ecologically (Owen 1988), which might reflect real differences between the species if they exist.

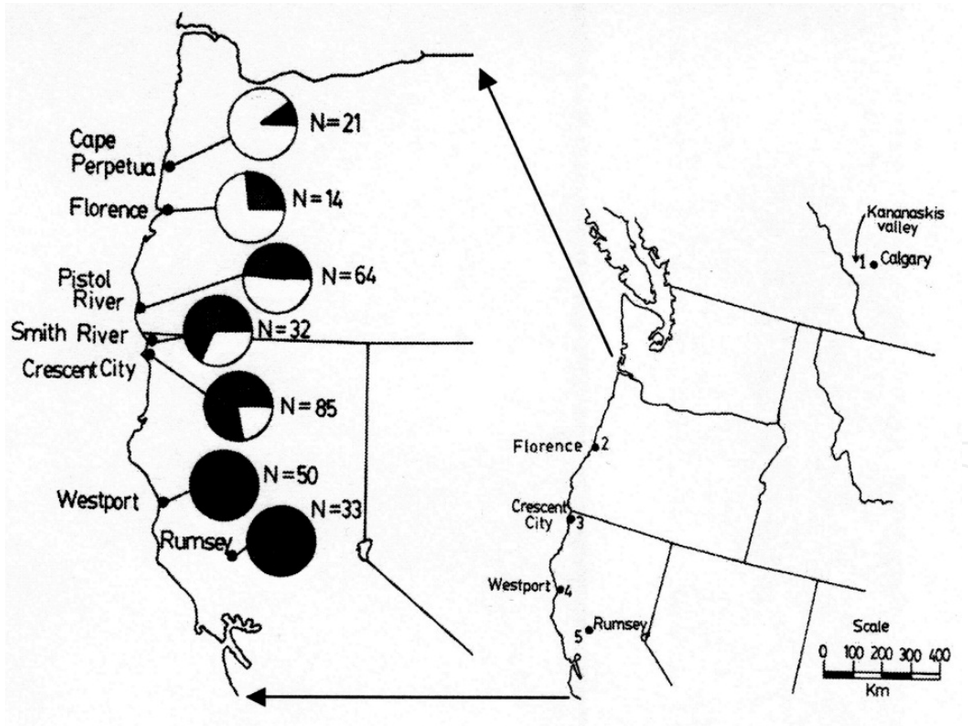

Fig. 8. Collection locations for bees examined electrophoretically and morphometrically. The enlarged section shows the gene frequency cline in *Bombus melanopygus* in Oregon and California. Pie diagrams give the relative frequency of the *R* (red) allele (clear portions) and the *r* (black) allele (shaded portions). The sample size (N) at each location represents the combined total of queen bees collected in1978, 1979, 1980, 1981 and 1988.

All bees had identical electrophoretic mobilities, and were invariant at 11 of the 16 enzyme loci examined. Five loci exhibited either differences between taxa and/or variation within taxa (Table 6). The nominate forms of *sylvicola* and *melanopygus* from Alberta clearly have different electrophoretic profiles (Table 6). The electrophoretic profiles of *melanopygus* and *edwardsii* from all locations were entirely consistent with each other. There was a very small amount of variation present, with heterozygotes being detected at a few locations (Table 6).

What was really interesting was the six bees ("MEL X"), collected in Alberta, that were assigned to *melanopygus* by eye when they were collected but turned out to have an electrophoretic profile inconsistent with that of *melanopygus* but consistent with that of *sylvicola* (Table 6). Going back to the collection records it was found that these bees (plus another three that were not electrophoresed) came from high elevations in the Kananaskis Valley (Fortress Mountain and Highwood Pass) where typical *sylvicola* had been collected. These were later reassigned to *sylvicola* on the basis of the wing morphometric analysis (see below).

	Enzyme electromorph													
	Pgm				*Gpi*		*Idh* (NAD)			*Hk*			*Sdh*	
Taxon*	72/82	82	93	93/100	92/96	96	95	100	102	100	100/105	105	100	105
***B. sylvicola* (*n*=18)**	1	17				18	16	2				18	1	17
"X" (*n*=6)		6				6		6			1	5	6	
***"B. melanopygus"* AB (*n* = 16)**			16			16		13	3	16			16	
***"B. melanopygus"* OR/CA (*n*=25)**			23	2	1	24		15	10	25			25	
***"B. edwardsii"* OR/CA (*n*=48)**			24	2	2	46		35	13	48			48	

* Taxon: *B. sylvicola*; "X" = the bees from Alberta resembling melanopygus, but with an electrophoretic profile inconsistent with the other *melanopygus*; *B. melanopygus* AB = from Alberta; *B. melanopygus* OR/CA = from Oregon and California; *B. edwardsii* OR/CA = from Oregon and California

Table 6. Electrophoresis results for the five enzymes exhibiting either differences between taxa and/or variation within taxa. The other 11 loci were invariant within, and showed no differences between, all taxa. The body of the table gives the number of individual bees of each electromorph. Electromorph mobilities (mm) are standardized relative to those of B. occidentalis (= index 100, Scholl et al. 1990). Modified from Owen et al. (2010).

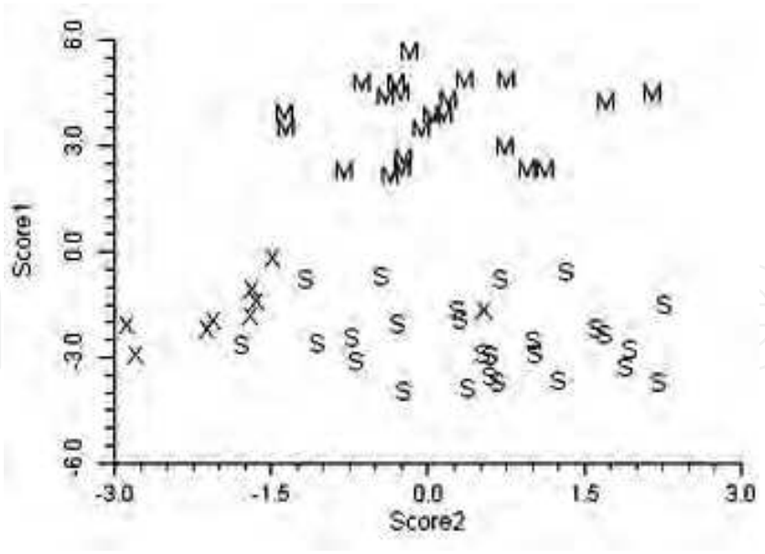

Fig. 9. Plot of the first two Canonical scores for *B. sylvicola* (S), the Alberta *B. melanopygus* (M) and the anomalous Alberta *B. melanopygus* ("Mel X"). Modified from Owen et al. (2010).

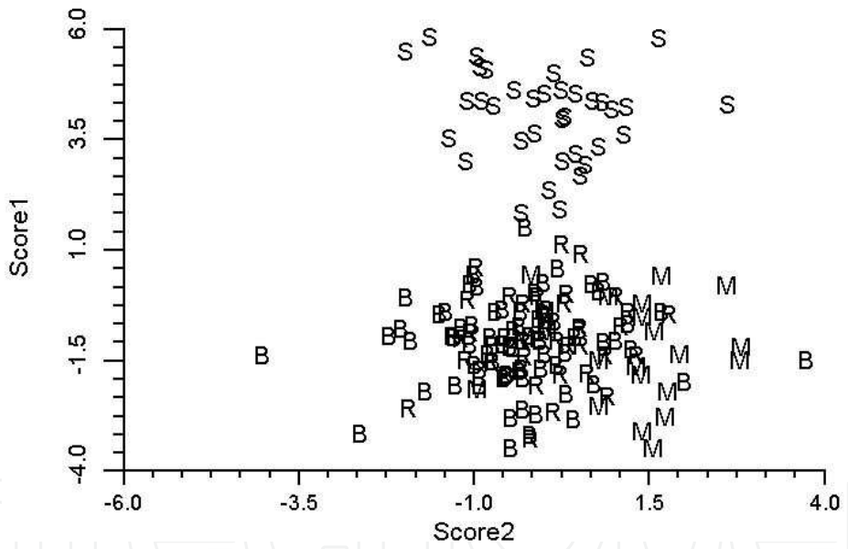

Fig. 10. Plot of the first two Canonical scores for the total data set. S = *B. sylvicola*, R = red "*melanopygus*" from Oregon and California, B = black "*edwardsii*" from Oregon and California, M = *B. melanopygus* from Alberta. Modified from Owen et al. (2010).

The discriminant functions analysis was run three times. Initially only specimens from Alberta were included. This was to verify that the technique could separate closely related species (*melanopygus* and *sylvicola*) in sympatry, and to determine the status of the aberrant *melanopygus* ("MEL X"). In addition to the six "MEL X" bees that were electrophoresed (Table 6) three other queens that were collected on the same dates and at the same locations were reassigned from *melanopygus* and included in the "MEL X" category. The plot of the first two canonical scores is shown in Figure 9. *B. melanopygus* is clearly separated from *B. sylvicola* by

the first canonical score. Similarly the "MEL X" bees are obviously distinct from *melanopygus* and are grouped with *sylvicola*. Next, the analysis was run using the complete data set (Figure 10) with the "MEL X" bees now being reclassified as *sylvicola*. Again, *B. sylvicola* is clearly separated by canonical score one, but *melanopygus* and *edwardsii* are not obviously resolved.

Enzyme electrophoresis and wing morphometrics failed to distinguish the nominate species *B. edwardsii* and *B. melanopygus*, yet clearly separated *B. sylvicola* from the latter. This, together with the colour dimorphism genetic data (Owen and Plowright 1980), and the lack of other morphological differences led Owen et al. (2010) to conclude that *melanopygus* and *edwardsii* are conspecific. If *B. melanopygus* is a "good" species, then there is a gene frequency cline for the color dimorphism (Fig. 8).

4.2 *B. occidentalis/B. terricola*

Two other taxa, where the evolutionary status and taxonomic classification are also unclear, are *B. terricola* Kirby and *B. occidentalis* Greene. The basis of this confusion originates with their classification being based primarily on pile colour pattern. Greene's original description of *B. occidentalis* reads "…first four abdominal segments black… "(Franklin 1913). Given that this is the type specimen description, specimens with the first four abdominal segments being black should be considered 'typical' *B. occidentalis.* In contrast, typical *B. terricola* have TIII and TIV that are consistently and clearly defined by complete yellow bands, and lack the large amount of white to cream-coloured pile typical of *B. occidentalis* on TV and TVI. However, in some parts of its distribution including areas of overlap with *B. terricola*, *B. occidentalis* exhibits considerable pile colour variation with some specimens closely resembling *B. terricola* (Stephen, 1957; Milliron, 1971). The primary ambiguous components of these bees are the complete to incomplete yellow bands on gastral terga III and IV. Nevertheless Stephen (1957) noted that *B. terricola* was "one of the most color stable species in western America" (p. 82) showing little or no variation throughout its range, and that it could be distinguished from *B. occidentalis* in having TII always yellow and TIV black. On this basis many authors have regarded *B. occidentalis* and *B. terricola* to be separate species (Stephen 1957; Thorp et al. 1983). However, Milliron (1971) reduced *B. occidentalis* to subspecific status under *B. terricola*, citing a lack of evident reliable or constant morphological features by which to differentiate specimens in areas of overlap. Milliron (1971) also suggested that these two subspecies most probably interbreed, producing numerous perplexing subspecific hybrids. This is certainly one possible explanation for the rare occurrence of colonies headed by definite *B. occidentalis* queens which produce *B. terricola*-like offspring.

Recently Bertsch et al. (2010) sequenced part (1005 bp) of the mitochondrial cytochrome oxidase subunit I (COI) gene and found a difference of 30 nucleotides between *B. occidentalis* and *B. terricola*, which is significantly larger than that found within a species. On this basis Bertsch et al. (2010) concluded that *B. occidentalis* and *B. terricola* do represent good biological species. They also suggested that to clarify the situation these taxa should be studied in greater detail in their area of contact in British Columbia and southern Alberta.

Whidden (2002) studied sympatric populations of *B. occidentalis* and *B. terricola* in Alberta using randomly amplified polymorphic DNA (RAPD) analysis. For comparison he also analyzed one consubgeneric species, *B. moderatus*, and one non-consubgeneric species *B.*

(*Pyrobombus*) *perplexus*. Ninety two bands using four different PCR primers were generated. Fixed differences occurred between all groups, and individual haplotypes did not occur in more than one taxonomic group, although there was overlap in haplotype components. The corrected average number of pairwise differences of between *B. moderatus* and *B. terricola* and *B. moderatus* and *B. occidentalis* was 6.98 and 5.92 respectively, and that between *B. occidentalis* and *B. terricola* was 5.07 (Table 7).

Species (n)	***B. terricola***	***B. occidentalis***	***B. moderatus***	***B. perplexus***
B. terricola (87)	1.28	6.27	7.91	54.76
B. occidentalis (79)	5.07	1.11	6.77	53.87
B. moderatus (104)	6.98	5.92	0.59	53.55
B. perplexus (54)	53.21	52.41	52.34	1.81

Table 7. Average pairwise differences between and within bumble-bee species.Above diagonal: Average number of pairwise differences between groups (P_iXY).Diagonal elements: Average number of pairwise differences within groups (P_iX). Below diagonal: Corrected average number of pairwise differences ($P_iXY-(P_iY)/2$). Sample sizes are given in parentheses.

Traditional morphometric analysis was done on some specimens of *B. occidentalis* and *B. terricola* queens collected in 1985 and 1986. The left forewing was removed and measured using the methods of Owen et al. (2010) as described above, and discriminant analysis performed. The classification counts are given in Table 8, and the first and third canonical scores are plotted in Fig. 11.

Species		**Predicted**				
Actual[1]	*occidentalis* 1985	*occidentalis* 1986	*terricola* 1985	*terricola* 1986	Total (n)	% correctly classified
occidentalis 1985	16	15	4	5	40	77.5%
occidentalis 1986	17	39	2	5	63	88.9%
*terricola*1985	2	0	17	4	23	91.3%
terricola 1986	1	3	6	15	25	84.0%
Total	36	57	29	29	151	85.4%

[1] Reduction in classification error due to variables measured = 43.5%.

Table 8**.** Classification count (actual and predicted) of the *B. occidentalis* and *B. terricola* from 1985 and 1986 using discriminant analysis of wing venation.

The taxa are clearly separated by both the genetic and morphological evidence. The corrected average number of pairwise differences of between *B. moderatus* and *B. terricola* and *B. moderatus* and *B. occidentalis* was 6.98 and 5.92 respectively, and that between *B. occidentalis* and *B. terricola* was 5.07. Therefore since *B. terricola* and *B. occidentalis* are

differentiated from each other to the same extent as they are from *B. moderatus*, they should regarded as distinct taxa. Discriminant function analysis of wing morphometric data correctly classified over 85% of the specimens of *B. occidentalis* and *B. terricola*, indicating significant morphological divergence.

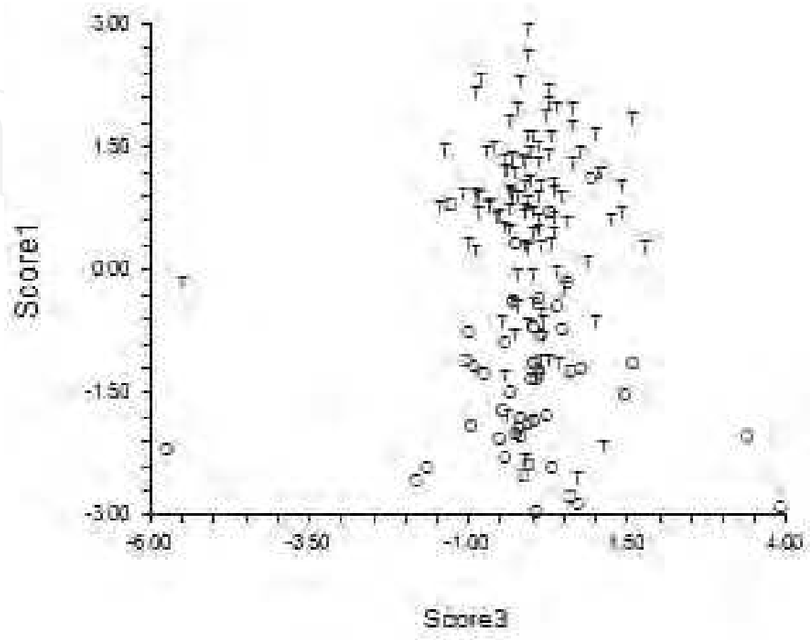

Fig. 11. Plot of the first and third Canonical scores for the 1985 and 1986 specimens of *B. occidentalis* (O) and *B. terricola* (T).

5. Conclusions

Morphometric analysis has been applied in a number of different ways to problems in the Hymenoptera and has proved to have an important and useful set of techniques for answering interesting questions. It is particularly useful for species identification and classification. The more traditional approaches appear to be as sensitive as geometric morphometrics for many problems. A powerful approach is to combine morphometric genetic methods, particularly to help answer questions of systematic and taxonomy.

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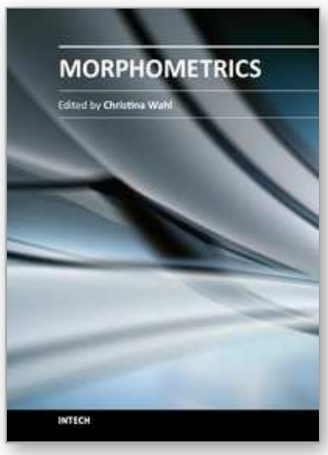

Morphometrics

Edited by Prof. Christina Wahl

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It is human nature to measure things, and this holds true for science as well as everyday life. The five papers in this book demonstrate the usefulness of a morphometric approach to a variety of subjects in natural history, including systematics, phenotypic plasticity in response to environmental variation, and ontogenetic adaptation. As our understanding of genetic control mechanisms and epigenetics has matured over the last several decades, it has become clear that morphometric assessment continues to be important to our overall understanding of natural variability in growth and form. The tremendous growth of our knowledge base during the last century has necessitated that we find new ways to measure and track greater detail as well as greater numbers of parameters among populations and individuals.

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Phone: +385 (51) 770 447
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Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
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Phone: +86-21-62489820
Fax: +86-21-62489821

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