# GEOMETRIC MORPHOMETRIC ANALYSIS OF WING SHAPE VARIATION IN TEN EUROPEAN POPULATIONS OF *CALOPTERYX SPLENDENS* (HARRIS, 1782) (ZYGOPTERA: CALOPTERYGIDAE)

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The wings of 10 C. splendens populations were examined by landmark-based geometric morphometric analysis. Subspecific taxa in this group are currently based on wing spot size in  $\delta \delta$ . Here, the variation in wing shape and size is evaluated, to test whether shape is different at a population level, and whether this has implications at a taxonomic level. It was found that Geometric Morphometrics successfully discriminates populations; overall wing shape significantly differed between populations but the results were only partly compatible with taxonomic studies based on wing spot size. Irrespective of wing spot, all populations showed differentiation in wing shape even though not in wing size; 4 groups were recognized based on wing shape: (1) Turkish1 population; (2) Spanish, Finnish, Russian and Turkish2 populations; (3) Italian, German and French populations; (4) Greek and Albanian populations. Ordination of the populations based on consensus data and cluster analysis phenogram confirmed such a pattern. The Spanish population (C. xanthostoma), did not show a strong identity, while the Turkish1 (C. s. waterstoni) was quite isolated. The Italian population (C. s. capral) showed more relation to the French (C. s. faivrei) and German populations than to Albanian and Greek populations.

# INTRODUCTION

*Calopteryx splendens* is a widespread damselfly, found in most of Europe, large parts of Siberia and much of western and central Asia (MERTENS et al., 1992). There is great variation among males in wing coloration (see SIVA-JOTHY, 1999). At sexual maturity, the amount of pigmentation becomes fixed (HOOPER et al.,

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1999; CORDOBA-AGUILAR, 1993). Traditionally subspecific taxa have been distinguished by the size and position of the pigmented wing spot, and by (mating) behavior (MERTENS et al., 1992; DUMONT et al., 2005). About a dozen of subspecies have been recognized. C. s. splendens occurs from Britain and southern Scandinavia over most of northern and western Europe; C. s. xanthostoma (often considered a good species) lives in southern France, northern Italy, western and southern Switzerland, the Iberian Peninsula and North Africa; C. s. caprai is in central Italy and possibly Mediterranean France; C. s. balcanica inhabits eastern Mediterranean Europe (ASKEW, 2004). Various other names, such as C. s. intermedia, C. s. faivrei, C. s. taurica, C. s. mingrelica refer to putative subspecies, all of which are more or less geographically confined, but often with overlapping ranges and strong variation in wing spot size.

Few authors deny a correlation between degree of wing pigmentation and male mating or territorial success (HOPEMAN & ABRAMSON, 2005); most results suggest that wing pigmentation is a reliable signal of male quality and plays a role in mate recognition by females (GRETHER, 1996; TYNKKYNEN, 2004; HOPEMAN, 2005). Thus, visual discrimination based on wing pigmentation is a major component of reproductive isolation in *Calopteryx* species (WAAGE, 1975). Size and density of wing pigmentation is also correlated with resistance against disease and immunological condition (RANTALA et al., 2000; SIVA-JOTHY, 2000; CORDOBA-AGUILAR, 2002; KOSKIMAKI et al., 2004).

In spite of these indications, the question arises whether variation in wing spot is really a taxonomically valid discriminator. The delimitation of many subspecies is indeed fuzzy, and clines are common (DUMONT et al., 1993). Moreover, even if valuable, it would still be meaningful to try and reinforce it by additional markers. Here, we attempt translating morphological traits in unambiguous numerical data, and confront the results with traditional taxonomy.

Geometric morphometrics is a relatively new technique that has generated valuable results in many fields of classic morphometry. A major advantage of the geometric framework is a comprehensive use of information about shape, available from a set of landmarks (BOOKSTEIN, 1996). Variations in body shape (and wings as part of the body) have important fitness consequences because they can affect the ability to occupy habitats successfully (GATZ, 1979; LOSOS & SINERVO, 1989), to prevail in predator-prey interactions (WALKER, 1997; NAGEL & SCHLUTER, 1998), and to reproduce successfully. So, we can expect selection to act upon wing phenotype.

Insect wings have been the subject of geometric morphometric analysis in the past (ROHLF & SLICE, 1990; BAYLAC & DAUFRESNE, 1996); they are especially attractive because they can be treated with biological realism in only two dimensions. Wing morphometrics can help to characterize populations within a species, as shown by the analysis of geographic variation in populations

of Drosophila lummei (HAAS & TOLLEY, 1998), Drosophila serrata (HOFF-MAN & SHIRRIFS, 2002) and Scythris obscurella (Lepidoptera) (ROGGERO & d'ENTREVES, 2005). Wings also proved useful to study complexes of species, for example in Diptera (DE LA RIVA et al., 2001), or examine the effects of hybridization, such as in Apis melifera subspecies (SMITH et al., 1997). The wing venation pattern of Odonata (like that of most other flying insects) has long provided students of Odonata with a rich source of diagnostic characters at all taxonomic levels (REHN, 2003).

In this study we use landmark-based geometric morphometrics method to quantify and analyze wing morphological features in ten European and Anatolian *C. splendens* populations. The central aim of our study was to evaluate wing shape variation, testing the possible use of wing shape patterns for intraspecific taxonomy, and attempting to distinguish effects of local adaptation from taxonlinked morphological differences.

#### MATERIAL AND METHODS

Specimens were dried or preserved in 70% ethyl alcohol. Since the number of specimens from one location was not enough for a strong statistical result, in some cases we used two geographically very close adjacent populations with no significant difference between the means (F & T test, p>0.1) as a single population (Tab. I, Fig. 2). In all, we used 344 specimens from 10 European localities. For simplicity we named the populations after their country of origin.

Population (country)	No.	Localities
Albania	29	- Mesopotam near to Delvine, 5-VI-1993, (39:59 N, 20:04 E)
	27	- Tirana, Albania, 6-VI-1993, (41:20 N, 19:49 E)
France	16	- Sissonne, 9-V-1993, (49:34 N, 03:53 E)
	20	- Canal au bord du Rhone, Gard, 12-VII-2004
Greece	25	- Saulopoulo, Ioaninna, 13-VI-1993, (39:44 N, 20:53 E)
	13	- Eleftheri, Thesprotia, Greece, 25-V-1998, (39:18 N, 20:25 E)
Spain	15	- Cuenca, 14-VII-1988, (40:04 N, 02:08 W)
-	9	- Alcaniz, 20-VIII-1991, (41:03 N, 00:09 W)
Turkey 1	22	- Derecik, near Trabzon, Turkey, 19-VIII-1988, (41:00 N, 39:43 E)
-	7	- Besikduzu, W Trabzon, Turkey, 20-VIII-1988, (41:02 N, 39:13 E)
Finland	27	- Kitee, SE Finland, 13-VIII-2004, (62:10 N, 30:08 E)
Germany	32	- Gerlenhofen, Ulm, Germany, 31-VIII-1993,(48:20 N, 10:04 E)
Italy	16	- Castel di Sangero, Italy, 30-V-1993,(41:47 N, 14:07 E)
•	16	- Gildone-Campobasso, Italy, 01-VI-1993, (41:33 N, 14:39 E)
Russia	31	- Ismaylowsky Park, Moscow, 12-VII-1989, (57:44 N, 37:37 E)
Turkey 2	19	- Golderesi-kemer, Fethiye, Mugla, 21-VII-87, (36:39 N, 29:22 E)
2	20	- 9 km. West of Seki, Mugla, 21-VII-87, (36:24 N, 29:13 E)

Table I							
Number of specimens and sampling localitie	s						

The anterior left wing of each male specimen was scanned on a flatbed table scanner (Agfa SNAP-SCAN 1236) as a digital RGB color image with 400 dpi resolution. Before scanning, dried specimens were soaked in 70% ethyl alcohol for 20 minutes and were left to dry at room temperature to gain flexibility for handling. Damaged wings were excluded from analysis.

WING STRUCTURE. – Wing venation in calopterygids is dense, with numerous antenodal and postnodal cross-veins and an anal field that is composed of variable number of cells. A dense and variable venation, along with dark and shiny metallic color on wings in *Calopteryx splendens* (male), restricts our choices for landmarking and makes it difficult to find identical landmark places on wing pictures.

We collected 19 homologous landmarks on the nodes of wing venation (Fig. 1) using tpsDig2 (ROHLF, 2006). The landmarks represented wing shape, and included all those that could reliably be identified. They were chosen at the intersection of wing veins in the hyaline part of the wing and/ or at the wing edge. Hence, 18 landmarks can be considered as type I landmarks and one (LM 4) as a type II (BOOKSTEIN, 1991). The following landmarks were used (see Fig. 1): (1) costa - subcosta connection, (2) nodus, (3) radius2 - wing margin connection, (4) distal tip of the wing, (5) medius - wing margin connection, (6) cubitus1 - wing margin connection, (7) cubitus2 - wing margin connection, (8) ventral tip of anal triangle, (9) proximal apex of anal triangle, (10, 11 & 12) distal angles of arculus, (13) origin of medius, (14) origin of cubitus1, (15) distal angle of anal triangle, (16) origin of cubitus2, (17) Ru+Rs and R+M connection, (18) origin of IR3 (third interradial), (19) origin of radius1. The nomenclature of the wing venation used follows DUMONT (1991).

To estimate the digitization error and defining those landmarks that can be digitized with the highest accuracy, we used protocol by Adriaens, http://www.fun-morph.ugent.be/Research/Methodology/ Morphometrics.pdf). This protocol allows quantifying the extent of digitization (and orientation) error with respect to the variation observed in the populations (based on a subsample). After testing, approximately 11.6 % of the observed variation was due to digitization (and orientation) error (due to nature of the wings and the approach followed here, orientation error can be considered to be ab-



Fig. 1. C. splendens waterstoni: (a) nomenclature of wing veins; - (b) landmarks position.

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sent). We also compared different combinations of 24 and 20 landmarks in two separate pilot studies to select the combination with the highest number of landmarks that showed the lowest amount of noise. As such, these 19 landmarks were the best combination.

A Generalized Procrustes analysis (GPA) was performed to superimpose landmark configurations using least-squares estimates for translation and rotation parameters (ADAMS et al., 2004). GPA is an important procedure because it removes variation due to differences in translation, orientation, and size, and superimposes the objects in a common coordinate system. Shape distances between GPA aligned specimens in Kendall shape space are subsequently projected into a Euclidean space that is tangent to this Kendall's shape space (ROHLF, 1999; SLICE, 2001). To visualize wing shape differences we generated thin-plate spline deformation grids (ROHLF, 2004).

STATISTICAL ANALYSIS. – Correlations between the Procrustes and tangent shape distances were calculated using tpsSmall software (ROHLF, 1998), to ensure that the amount of shape variation in the original data set was adequately represented after projection in the tangent space. The sample showed perfectly correlated distances ( $R^2 = 1.000$ ), allowing further statistical testing using the projected dataset (see ROHLF, 1998).

As a measure of overall size variation of the wings, the centroid size (the square root of the sum of the squared interlandmark distances) was calculated for each population (Fig. 3) (BOOKSTEIN, 1991; 1996, SLICE et al., 2007; ZELDITCH, 2004). Centroid size was calculated using tpsRelw (ROHLF, 2007) and tested for normality using the Shapiro-Wilk test. All populations were normally distributed (p>0.05). Leven's test was used to test for homogeneity of the variance (MILLIKEN & JOHNSON, 1984). A one-way ANOVA was conducted on whole data set to test significant centroid size differences between and within populations and a *post hoc* test (Tukey's test) defined pairwise differences in centroid size of populations (SOKAL & ROHLF, 1995).

For analyzing wing shape variation within and among populations, principal component analysis



Fig. 2. Distribution map of the populations studied and thumbnail pictures of their anterior left wing. Map source: http://z.about.com/d/geography/1/0/2/L/eurasia.jpg

(PCA) and canonical variates analysis (CVA) were conducted on the landmark coordinates data set, PCA as a tool for exploring patterns of variation within population using variance-covariance matrix and CVA for analyzing and testing differences between populations.

A MANOVA and two-group permutation tests (2000 permutations) were performed, with squared Mahalanobis distance calculated on the landmark coordinates data set using PAST (HAMMER & HARPER, 2007) to determine whether geographically separated populations from different countries differ in wing shape. In this analysis the criteria *Wilk's lambda* is used and when the MANOVA showed significant overall difference between groups, the analysis proceeded by pair-wise comparisons (post-hoc) by pairwise Hotelling's tests. We also performed two-group permutation test (2000 permutations) and extracted squared Mahalanobis distance for every two populations.

We also measured consensus shape data (mean shape) of the separate populations to illustrate ordination of the shapes' consensus by a relative warp ordination plot using tpsSmal and tpsRelw (ROHLF, 2003, 2007). In all figures, the consensus landmark configurations (i.e. the configuration that is obtained by averaging specimen landmark coordinates in the generalized procrustes analysis) are used as a reference, with the subtle differences between populations shown as a three times exaggerated transformation grid.

Phenetic relationships between the ten populations were also investigated through a cluster analysis using the matrix of procrustes distances between pair-wise population consensus configurations. A UPGMA (Unweighted pair-group method with arithmetic means) was chosen as they led to a phenogram with the largest cophenetic correlations to the original procrustes distance matrix (ROHLF, 2002).

All statistical analyses were performed in PAST (Paleontological Statistics) version 1.57 (HAM-MER, 2007) and SPSS (version 15.0.1, 2006). Graphical depictions of wing-shape transformations in tpsSplin (ROHLF, 2004) and IMP (SHEETS, 2000) and of the phenograms were generated in NTSYSpc (Version 2.1, ROHLF, 2000).



Fig. 3. Mean centroid size of ten European populations. Abbreviation of populations: Al: Albanian; Fi: Finnish; Fr: French; Ge: German; Gr: Greek; It: Italian; Ru: Russian; Sp: Spanish; T1: Turkish1; T2: Turkish2 populations.

## RESULTS

### WING SIZE VARIATION

The mean centroid size of the populations singled out the Greek and Albanian populations as those with the largest, and the Spanish population as that with smallest wing size (Fig. 3).

A Shapiro-Wilk test revealed a normal distribution of all populations (p>0.05) and Levene's test showed significant homogeneity of variances (p<0.05) based on means. A one-way ANOVA of mean centroid sizes showed significant differences between inter- and intera-population variations (F = 63.47, p = 0.000). The results of Tukey HSD as a *post hoc* test on centroid sizes are summarized in Table II as pair-wise differences. The result of homogeneous subsets of centroid sizes extracted from Tukey HSD identified four groups with significantly different centroid size: the Greek and Albanian populations (first group) had the biggest size, the Spanish, Turkish1 and Turkish2 populations were the smallest (second group). The Italian, French and German populations (fourth group) were situated between the largest and medium size groups (Fig. 3).

No significant difference in centroid size was found among members of each group. We found that group one (Albania and Greece) was highly significant in difference with other populations in mean centroid size.

#### WING SHAPE VARIATION

PCA or relative warp analysis of all specimens explained 65.7% of shape variation within samples by the two first PCA axes extracted from the variance-cov-

Results of Tukey HSD (post-noc) test on wing centroid size, insigning ant values bolded $- * < 0.05$ ; - ** < 0.01										
Country	Al	Fr	Gr	Sp	TI	Fi	Ge	It	Ru	T2
Al	-			-				<u> </u>		
Fr	48.54**	-								
Gr	3.26	51.80**	-							
Sp	71.06**	22.52**	74.33**	-						
TI	62.40**	13.86	65.67**	8.66	•					
Fi	40.07**	8.46	43.34**	30.98**	22.32**	-				
Ge	52.82**	4.28	56.09**	18.24**	9.58	12.74	-			
It	51.04**	2.50	54.31**	20.02*	11.36	10.96	1.78	-		
Ru	36.98**	11.56	40.24**	34.08**	25.42**	3.10	15.84	14.07	-	
T2	65.6**	17.10*	68.91**	5.42	3.24	25.56**	12.82	14.60	28.67**	•

 Table II

 Passults of Tukey HSD (nost hos) test on using contraid size insignificant values holded = \$<0.05.</td>

ariance matrix (PC1 explains 50.5% and PC2, 15.2%). A total of up to seven axes were required to cover more than 90% of the shape variation. At first glance PC1 vs PC2 scatter plot showed an overlap of most populations and their distribution on this plot (Fig. 4) did not correspond with geographical differences between populations. However, PCA weakly showed some population shape variation (Turkish1 population from Albanian, Spanish, Russian, and Turkish2 populations). Furthermore, through PC1 the vectors on landmarks shows inclination of landmarks 3 and 4 to posterior and landmarks 5, 6, 7 to anterior part of the wing which leads to increase distance between landmarks 2 and 3 in one side and between 4 and 5 in other side. Through PC2 the landmarks 3 to 8 incline to the central part of the wing which leads to a decline in length and width of the wing (see Appendix 2). These landmarks refer to connecting point of Radius2 with posterior wing margin, distal tip of the wing, and connection of Medius, Cubitusl and Cubitus2 and anal triangle tip with ventral wing margin respectively (Fig. 1).

MANOVA found a significant overall difference between populations (*Wilk's lambda*: 0.0076, F: 6.47, p = 0.0000). Hotelling's pair-wise comparisons (*posthoc*) showed highly significant differences between all populations except between French and German populations which were at a low significance level (p<0.05). Squared Mahalanobis distance of populations confirmed these results (Tab. III).



Fig. 4. PC1 vs PC2 screen plot. For abbreviations see Figure 3.

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Country	Al	Fr	Gr	Sp	ті	Fi	Ge	It	Ru	T2
Al	0	11.4**	3.0**	14.8**	30.6**	8.5**	10.6**	9.7**	8.3**	13.1**
Fr	0.408**	0	13.8**	6.8**	14.7**	2.6**	1.8*	3.5**	3.5**	5.9**
Gr	0.302**	0.586**	0	12.8**	38.1**	14.7**	11.7**	16.7**	10.1**	14.3**
Sp	0.661**	0.828**	0.821**	0	15.7**	9.4**	7.1**	9.2**	8.8**	6.6**
Tl	0.952**	1.129**	1.397**	1.548**	0	10.3**	8.5**	13.7**	15.6**	15.1**
Fi	0.696**	0.539**	1.01**	1.237**	1.271**	0	3.1**	6.0**	3.7**	6.5**
Ge	0.519**	0.415*	0.703**	0.763**	0.973**	0.583*	0	2.9**	8.0**	4.8**
It	0.537**	0.466**	0.860**	1.313**	1.19**	0.804**	0.498**	0	7.2**	6.9**
Ru	0.455**	0.552**	0.598**	1.014**	1.28**	0.680*	0.815**	0.801**	0	5.8**
T2	0.519**	0.562**	0.467**	0.718**	0.971**	0.707**	0.547**	0.617**	0.558**	0

 
 Table III

 Hotelling's pair-wise comparisons (F-scores and p values) and Mahalanobis distances and p values for 10 populations on upper and lower diagonal respectively - \* <0.05; - \*\* <0.01</td>

Every couple of populations significantly differed (p < 0.05) in squared Mahalanobis distances.

Differences between populations were not well illustrated by a CVA plot. A scatter plot of CV1 (eigenvalue 3.206) vs. CV2 (eigenvalue 1.455) showed a general pattern similar to PCA but with less overlap. CV1 revealed Turkish1 prominently, Albanian and Greek (together), and Italian populations more or less differed in wing shape from others (Fig. 5).

Moreover, considering CV2, Italian and German populations separated in shape from Russian, Spanish and Turkish2 (together) populations. Other populations more or less overlapped each other (Fig. 5). There were seven distinct CVs (Appendix 1) and other CVA plots also showed good separation from other populations. Cv1 vs CV4 well defined shape differences between Spanish and Turkish2 and CV2 vs. CV3 (eigenvalue 0.8595) and CV4 (eigenvalue 0.6239) plots (not shown here) showed good differences between Finnish and Russian populations, and between Turkish2 and Finnish, Spanish and French, Italian and Russian populations.

There was a high correlation between CV1 and PC2 (Spearman's rho correlation = 0.65). This correlation refers to landmarks situated in the middle part of the wing, that show an expansion between landmarks 2, 7, 8, and 16 and a contraction between landmarks 10-18 in the anterior part, a part of the wing informative in taxonomic studies.

UPGMA cluster analyses and also relative warp ordination of shape consensus showed the same population relationships (Figs 6, 7). In a relative warp ordination plot, Greek and Albanian (together), Italian, Turkish1 and Turkish2 populations showed the largest differences, while Spanish, Russian and, Finnish populations on the one hand and, French and German populations on the other hand were close to each other. The Albanian population was closer to Greek population, and the French population fell between the Spanish and German populations. Russian, Finnish, Turkish2 and Spanish populations and French, German and Italian populations are closely related. Albanian and Greek populations as third and Turkish1 population as fourth group settled at two extremes of the X- axis. These relationships are quite compatible with the UPGMA dendrogram, estimated from Procrustes distances.

The wing shape transformation grids adjacent to the dendrogram depicted shape similarities and differences between populations. These illustrated transformations of the wing landmarks which led to such a clustering.

# DISCUSSION

Geographic variation in wide-ranging species is ubiquitous (MAYR, 1963) and often reflects adaptations of a population to local environments and biotic factors (RICKLEFS & MILES, 1994; McPEEK, 1990); such adaptations are expected to end up in (sub)speciation, but it is not always easy to determine when this process has taken place. One difficulty is the choice of characters upon which to base such a decision. Many aspects of flight performance correlated to fitness directly or indirectly, are determined by wing form. A proper comprehensive analysis of wing shape may thus provide an insight in phenotypic variation related to flight



Fig. 5. CVA plot, CV1 (eigenvalue 3.434) vs CV2 (eigenvalue 1.925). For abbreviations see Figure 3.

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performance, a character that should be under selection.

Wing variation among ten populations of *Calopteryx splendens* irrespective of their wing spot, measured by wing centroid size, reveals a relationship between some populations in the west (French, German and Italian) and east (Finnish, Russian) of Europe and differences between southern (Greece, Albania) populations with others. Non-significant differences of centroid size between Turkey1 and France, and between Turkey1, Turkey2, Italy and Spain populations as well as the occurrence of the smallest and largest wings' mean in Spain and Greece rejected the existence of a simple Bergman cline in wing size.

Wing shape differences between paired populations in this study show no simple pattern. There are significant differences in shape, squared Mahalanobis distance and even wing spot area and ratio of spot area to wing area (S/W ratio) (not worked out in this study)

Turkish1 and Turkish2 are clearly differentiated. Turkish1 shows much relation to West European populations (France and Germany) whereas Turkish2 is related to East European (Russia and Finland) and Spanish populations. Turkish populations emerge from almost every analysis as highly heterogeneous.

Turkish1 males again have a wing phenotype significantly different from all others; this population has hyaline wings (without spot) and only occurs along the north-eastern coast of Turkey (south-eastern Black sea coast). Taxonomically, it is known as sub-species, *waterstoni*. Its wing shape, squared Mahalanobis distance, and all other analyses except wing centroid size (and evaluation of wing area in another study) are unique. This form was originally described as a good species (SCHNEIDER, 1984) but DUMONT et al. (1987) and HEIDARI



Fig. 6. Relative warp ordination plot of consensus data of 10 European C. splendens populations.

& DUMONT (2002) showed that it hybridizes with forms with any type of wing spot. They hypothesize that it may have played a role in the genesis of *C. splendens* populations with a small to medium wing spot, and that its current range is just a fraction of a much wider range during the late Pleistocene.

Thin-plate spline deformation grids second our results for shape and cluster analysis and also confirm population differences based on wing shape. It appears that the posterior half of the wing plays a more important role than the anterior part in population differentiation. Regardless of Turkish1, which is restricted at north east coast of Turkey and shows a unique wing deformation, populations of *C. splendens* in south and east of Europe show other deformations than western populations. The wings of European populations lead to three main types of wing deformations (based on position of landmarks of deformation grids), a narrow and rectangular form in the west, and two types in the east and south, the first wide and narrowing in the middle, and the second wide with or without a narrowing of its posterior part.

Our data also confirm that the Spanish population (*C. s. xanthostoma*) is morphologically distinct (see DUMONT, 1972). Their wing differences especially appeared in thin-plate deformation grid. However, the results of cluster analy-



Fig. 7. UPGMA phenogram of ten European C. splendens population and related thin-plate deformation grids.

sis showed its closer relation to eastern European populations including Turkish2 than western ones. The nature of this relationship cannot be decided by this analysis only, since it could represent as well the result of an old connection between Spanish (*C. s. xanthostoma*) and Turkish2 (*C. s. amasina*) populations, as a homoplastic effect. Sadeghi et al. (in review) have, however, shown, using AFLP, that there is indeed a genetic relationship between these two that is closer than between both of them and the (geographically) much nearer *waterstoni*. According to BODENHEIMER (1938) and DUMONT (1975) at the end of the Cenozoic, a fauna of Irano-Turanian origin extended from the Near-East to North Africa and southern Spain, It seems that at this time, a passage for ancestral population of Turkish2 (*C. s. amasina*) as far west as Iberia took place.

On the other hand Turkish2 (*C. s. amasina*) seems more related to North European populations than Greek and Albanian populations. This suggests a common origin of these populations which more southeasterly situated *Calopteryx* populations (*C. s. intermedia* of Hatay province, southeastern Turkey).

The relationship between the French and Italian populations is more than with the Spanish population, which suggests a greater taxonomic distance. Geographic distance is clearly not the major separating factor in this group. Populations may take much time to cross a mountain barrier like the Alps or the Pyrenees (DUMONT, 1975), but can rapidly expand across a newly invaded river basin. It seems that, through this mechanism, genetic mixing can easily and quickly take place over large distances. A river that seems to have played a major role in the re-invasion of Europe following glacial epochs is the Danube. Several consecutive waves of *Calopteryx* with different wing shapes seem to have used this pathway from East to West at different times. At the same time, with sea levels lower than today, the coastal zone on the Balkans was much wider than today and permitted easy dispersal between Greece and the Dalmatian coast, and at times even Italy, explaining the similarity of the Greek and Albanian, and Italian and southern French populations as well. This "wave" is clearly distinct from the waves that traveled up the Danube (separated from the Mediterranean by mountains), and perhaps further North, the Dniepr, Ural and Volga. Since these movements may have happened shortly after the last pleniglacial, some amount of local adaptation may have become superimposed on each population since. Testing this idea further will, however, require the examination of many more populations situated at critical sites of the species' range.

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#### **APPENDIX 1**

Results from CVA/MANOVA extracted from IMP

Axis I	Lambda= 0.0093	chisq=1503.2575	df=306	p<2.22045e-016
Axis 2	Lambda= 0.0389	chisq=1042.1590	df=264	p<2.22045e-016
Axis 3	Lambda= 0.0955	chisq=753.8607	df=224	p<2.22045e-016
Axis 4	Lambda= 0.1776	chisq=554.7413	df=186	p<2.22045e-016
Axis 5	Lambda= 0.2884	chisq=399.1084	df=150	p<2.22045e-016
Axis 6	Lambda= 0.4254	chisq=274.3850	df=116	p=7.32747e-015
Axis 7	Lambda= 0.5863	chisq=171.3907	df=84	p=6.08586e-008
Axis 8	Lambda= 0.7617	chisq=87.3702	df=54	p=0.00272366

# **APPENDIX 2**

Principal deformation from mean shape, note on variation on posterioventral landmarks

