Phenotypic variation among local populations of phlebotomine sand flies (Diptera: Psychodidae) in southern Turkey

A. Murat Aytekin[1], Bulent Alten[1✉], Selim S. Caglar[1], Yusuf Ozbel[2], Sinan Kaynas[1], Fatih M. Simsek[3], Ozge Erisoz Kasap[1], and Asli Belen[1]

[1]*Hacettepe University, Department of Biology, Faculty of Science, ESRL, 06800 Beytepe, Ankara, Turkey*
[2]*Ege University, Faculty of Medicine, Department of Parasitology, 35100, Izmir, Turkey*
[3]*Adnan Menderes University, Art and Science Faculty, Department of Biology, 09010, Aydin, Turkey*

ABSTRACT: The wing-shape morphology of local populations of the medically important phlebotomine sand flies, *Phlebotomus sergenti, P. papatasi, P. tobbi,* and *P. similis,* were examined in both sexes by using geometric morphometrics. There are three major mountain ranges that may serve as geographical barriers for species distribution in the study area and four main gaps were recognized among these barriers. **We found no statistically important differences in wing morphology** in all examined species in both sexes for all local populations. These results show that the barriers are not sufficient to stop gene flow among local populations of sand flies. The graphical depiction of PCA, CVA, and F-test confirmed our morphometric study suggesting that the difference in wing morphology between *P. similis* and *P. sergenti* indicates that these are clearly different species. These two show sympatric distribution in the Konya Plain of Anatolia. ***Journal of Vector Ecology*** **32 (2): 226-234. 2007.**

Keyword Index: Sand flies, *Phlebotomus*, geometric morphometrics, thin plate spline, geographical distribution, south Anatolia.

INTRODUCTION

Two clinical types of leishmaniasis exist in Turkey. Human cutaneous leishmaniasis (HCL), caused by *Leishmania tropica* and *L. infantum* (Serin et al. 2005), is highly endemic in southern and southeastern Anatolia. Human visceral leishmaniasis (HVL), caused by *L. infantum*, is endemic along the Aegean and Mediterranean coasts and occurs sporadically in other regions (Ozbel et al. 1995, Ok et al. 2002, Volf et al. 2002, Yaman and Ozbel 2004). In some provinces, including Adana and Osmaniye on the Mediterranean coast of Cukurova Plain (Pazarbasi et al. 2006) and Hatay on the Mediterranean coast in the southeast (Yaman and Ozbel 2004), HCL and HVL have posed an important public health problem for many years.

Previous studies of sand flies in Turkey identified 19 *Phlebotomus* species (or subspecies recently raised to species level) belonging to the subgenera *Phlebotomus* Rondani, 1840, *Adlerius* Nitzulescu, 1931, *Larroussius* Nitzulescu, 1931, and *Paraphlebotomus* Theodor, 1948. It is also known that there are five *Sergentomyia* species of the subgenus *Sergentomyia* Franca and Parrot, 1920 (Alptekin et al. 1999, Volf et al. 2002, Alten et al. 2003, Yaman and Ozbel 2004, Toprak and Ozer 2005). Nine of these species are proven or probable vectors of the parasites causing human leishmaniases, such as *P. sergenti* Parrot, 1917, *P. tobbi* Adler & Theodor, 1930, and *P. papatasi* Scopoli, 1786 in the Old World (Killick-Kendrick 1990).

Geometric morphometrics plays an important role in many kind of biological studies, especially in systematics and ecology (Zelditch et al. 2004). It is more advantageous compared to traditional morphometrics in several ways (Adams et al. 2004). Instead of measurements of distances between landmarks which are homologous anatomical loci matching between and within populations, the geometric method uses Cartesian coordinates of these landmarks (Alibert et al. 2001, Gumiel et al. 2003, Adams 2004, Zelditch et al. 2004). The landmark coordinate data then superimposes these landmarks by the Procrustes method which has been shown to have the highest statistical power among its alternatives (Rohlf 2000). The thin plate splines algorithm of Bookstein (1991) can also be used to compute the deformation grid in between different landmark configurations. For mathematical details, see Bookstein (1991), Zelditch et al. (2004) and Adams et al. (2004).

Southern Anatolia is located between the Taurus, Amanos, and Anti-Taurus mountains and represents a geographical crossroad for sand fly dispersion between the western and eastern parts of Anatolia, showing various ecological, geographical, and climatic differences that are important in the epidemiology of leishmaniasis. This study explores whether there are statistically important changes in the morphology of the wings among different local populations of some medically important phlebotomine sand fly species, *P. sergenti, P. papatasi, P. tobbi,* and *P. similis* collected from the southern and middle Anatolia regions by using geometric morphometric methods.

MATERIALS AND METHODS

Field studies were performed in an area of approximately 170,000 km^2, containing plains, rivers, streams, and high mountains, in the southern and middle Anatolia regions between April and October of 2004 and 2005. The study area is outlined by the Mediterranean Sea to the south, West Taurus Mountains to the west, the mountain ranges of Taurus and Anti-Taurus to the north, and the Amanos Mountains to the east in south Anatolia, and Konya province from middle Anatolia. There are three important mountain ranges that can serve as geographical barriers for species distribution, and four main gaps were recognized among these barriers. The study area was separated into six sub-regions (Region I: Hatay-east 35°47'N-36°07'E; Region II: Hatay-west and Osmaniye 36°12'N-35°50'E; Region III: Kahramanmaras 37°51'N-37°39'E; Region IV: Adana 36°53'-36°52'; Region V: Nigde and Kayseri 28°22'N-31°16'E, 38°22'N-34°39'E; Region VI: Konya 36°43'N-32°28'E), including nine provinces according to these barriers: Konya, Nigde, Kayseri, Adana, Osmaniye, Hatay, Kahramanmaras, Malatya, and Adiyaman (Table 1). Because they had a single sampling site in each, the last two provinces were not included in the evaluations.

Sampling by CDC light traps, sticky traps, and mouth aspirators was conducted in different biotopes at 90 sites and 360 sampling points, distributed around the circumferences of the provinces, with an altitude ranging between 0-1,600 m above sea level. The specimens collected by light traps and aspirators were directly transferred to 96% ethanol in the field. Specimens caught by sticky paper traps were immersed first in 96% ethanol to remove the oil, transferred to 90% ethanol, cleared in lactophenol, and mounted, together with the specimens collected by other methods, in Berlese medium on labeled slides for identification. Before mounting, the heads and genitalia of the sand flies were removed from each specimen with forceps, and the wings were stained to better view the veins using the method of Belen et al. (2004). The number of specimens used in the geometric morphometrics analysis is shown in Table 2.

All slides were photographed and analyzed to depict the results graphically (Zelditch et al. 2004). Photographs were first entered into tps-UTIL (Rohlf 2006a) and the data were collected in the form of 2-D coordinates of the 16 landmarks and four sliders from the right wing (Figure 1) using tps-DIG (Rohlf 2006b). The landmark configurations were then scaled, translated, and rotated against the consensus configuration by Generalized Procrustes Analysis (GPA, formerly termed GLS) (Bookstein 1991, Dryden and Mardia 1998) by using tps-RELW (Rohlf 2006c) and Morpheus (D. Slice, Department of Ecology and Evolution, SUNY, Stony Brook). MANOVA and permutation tests were performed on the landmark Procrustes distances. The wing-shape deformations from the reference were shown along the first two axes on a PCA graph performed by tps-RELW (Rohlf 2006$_c$). A canonical variates analyses (CANOVAR) was also conducted on landmark data by IMP CVAGEN6n (Zelditch et al. 2004) to compare the local populations by using group membership information. Barlett's test was used to determine if there were differences among groups (Zelditch et al. 2004). Goodall's F test was also used to compare the reliability of different species using the software IMP TwoMorphGen6 (Zelditch et al. 2004). The size morphometry of the examined species was investigated by using the centroid sizes of the wings as an estimator with one-way ANOVA (Sokal and Rohlf 1995). Centroid size is the square root of the sum of squared distances of a set of landmarks from their centroid or, in other words, it is the square root of the sum of the variances of the landmarks about that centroid in x- and y- directions (Bookstein 1991).
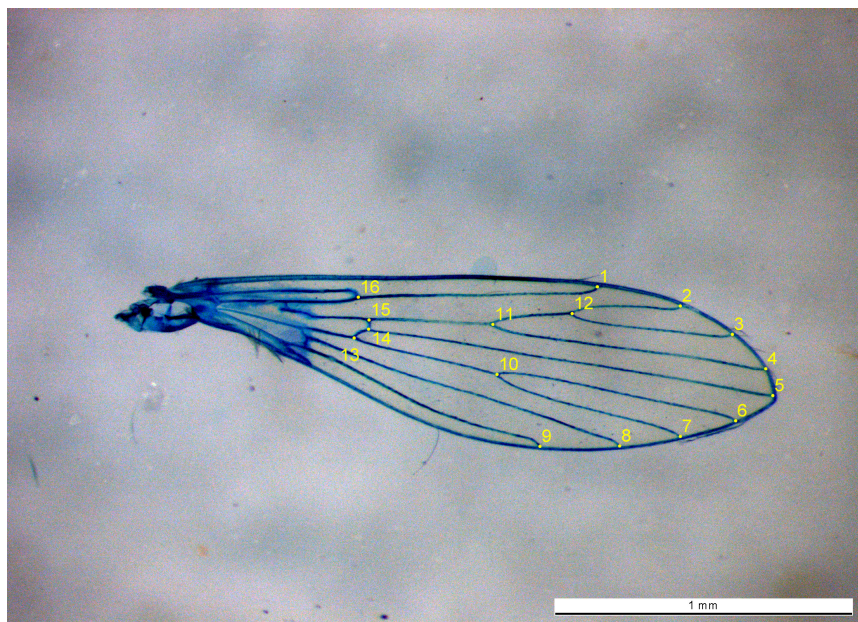

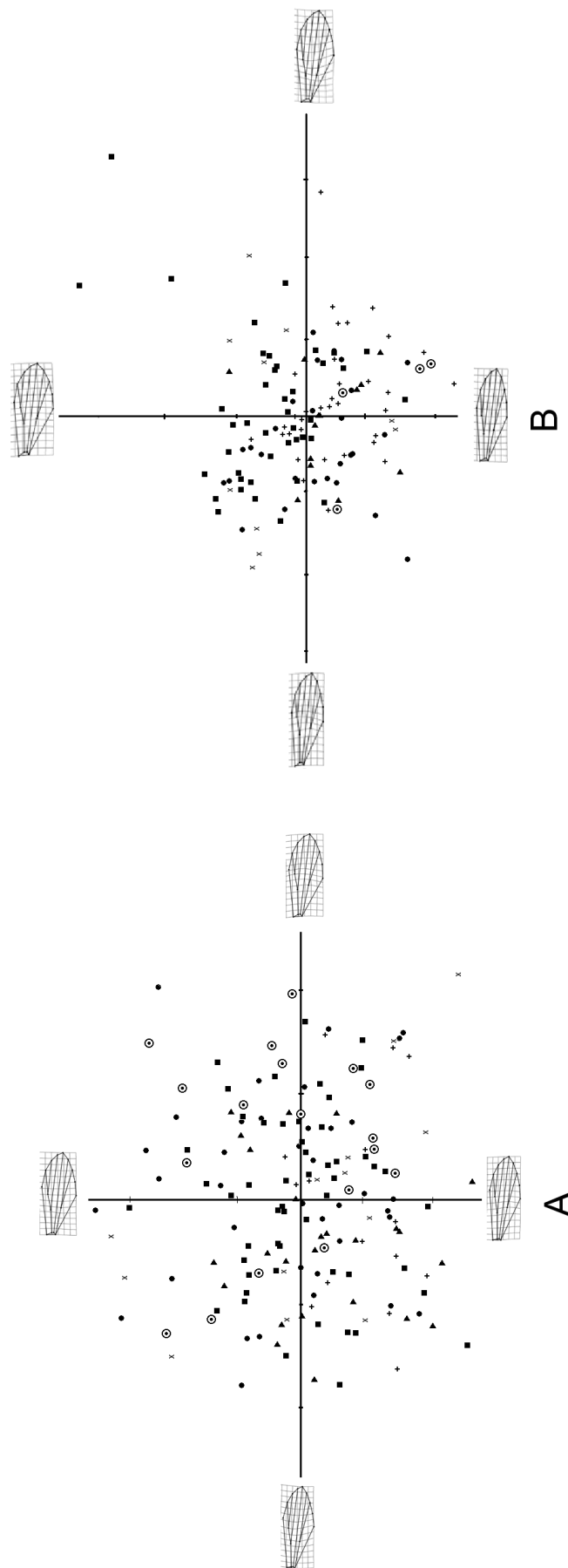Figure 1. Location of the 16 landmarks on the wing of a female *P. papatasi*.

Figure 2. A- Distribution of the females of P. papatasi along the first two PC's. Principal component analysis of tangent space coordinates derived from GPA of the original coordinates that was conducted for the 16 landmarks digitized from the wings. Horizontal axis, PC1; vertical axis PC2. Region I: ●; Region II: x; Region III: ☉; Region IV: ■; Region V: +; Region VI: ▲. B- Distribution of the males of P. papatasi along the first two PC's. Principal component analysis of tangent space coordinates derived from GPA of the original coordinates that was conducted for the 16 landmarks digitized from the wings. Horizontal axis, PC1; vertical axis PC2. Region I: ●; Region II: x; Region III: ☉; Region IV: ■; Region V: +; Region VI: ▲.
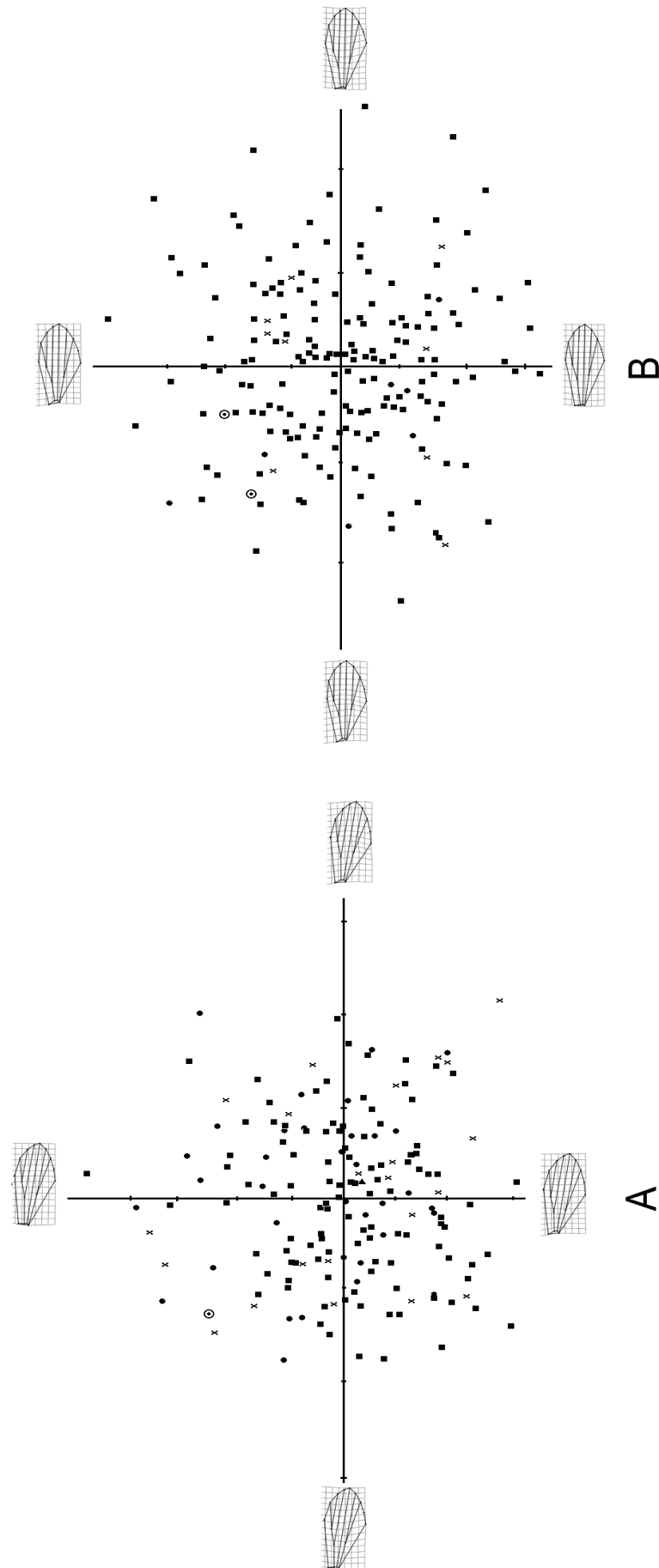
Figure 3. A- Distribution of the females of P. tobbi along the first two PC's. Principal component analysis of tangent space coordinates derived from GPA of the original coordinates that was conducted for the 16 landmarks digitized from the wings. Horizontal axis, PC1; vertical axis PC2. Region I: ●; Region II: x ; Region III: ⊙ Region IV: ■ ; Region VI:▲ . B- Distribution of the males of P. tobbi along the first two PC's. Principal component analysis of tangent space coordinates derived from GPA of the original coordinates that was conducted for the 16 landmarks digitized from the wings. Horizontal axis, PC1; vertical axis PC2. Region I: ●; Region II: x ; Region III: ⊙; Region IV: ■ .
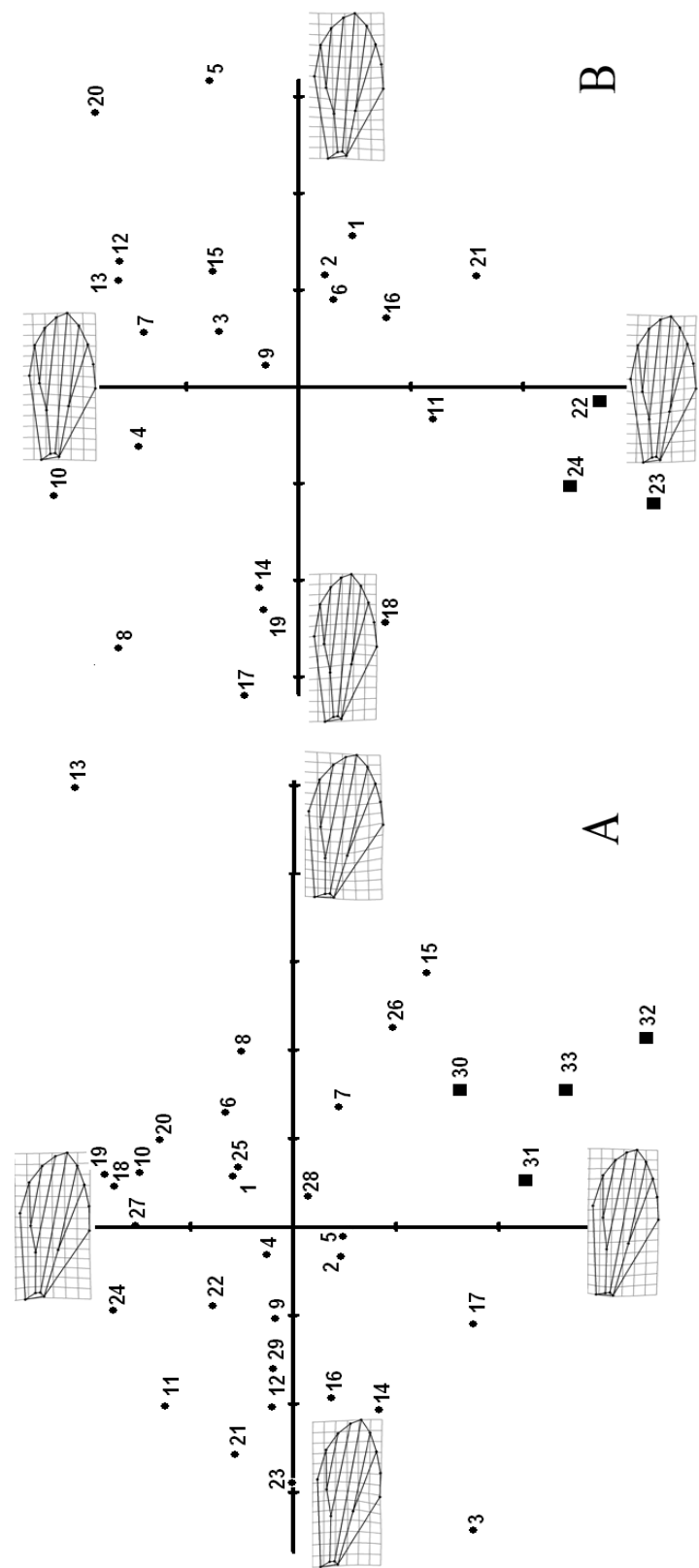
Figure 4. A- Distribution of the females of P. sergenti and P. similis along the first two PC's. Principal component analysis of tangent space coordinates derived from GPA of the original coordinates that was conducted for the 16 landmarks digitized from the wings. Horizontal axis, PC1; vertical axis PC2. Region I: 1-6; Region II: 7-16; Region III: 17-18; Region IV: 19-20; Region V: 21; P. similis only in Region VI: 22-24 (Filled Rectangle). Principal component analysis of tangent space coordinates derived from GPA of the original coordinates that was conducted for the 16 landmarks digitized from the wings. Horizontal axis, PC1; vertical axis PC2. Region I: 1-8; Region II: 9-15; Region III: 16-17; Region IV: 18-24; Region V: 19-26; Region VI: 27-29 P. similis only in Region VI: 30-33 (Filled Rectangle) . B- Distribution of the males of P. sergenti and P. similis along the first two PC's. Numbers indicates each individual.

Table 1. Physical parameters and site description of the localities in the study area.

| Localities | Physical parameters | | | | Site description | |
	Altitude (m)	MSRH (%)	MST (°C)	MSP (mm)	Rural	Domestic
K.MARAS	568	62	16.7	723	Bushes, *Quercus* and *Pinus* forests, entisols, semi-humid, caves, pelagic limestones	Briquette, cement, and adobe houses, animal barns, food stores
NIGDE	1300	55	11.1	349	Steppe, *Pinus nigra* forests, aridisol, mollisol, semi-arid, highland, caves, tuffs	Briquette, cement, stone and adobe houses, animal barns, food stores, apple orchards
KA YSERI	1054	64	10.8	366	*Astragalus, Quercus, Pinus* forests, rodent burrows, alluvial soil, volcanic facies, semi-arid, highland	Briquette, cement, and adobe houses, animal barns, chicken houses
ADANA	23	66	18.7	637	Fertile, agricultural area, *Pinus, Abies* forests, alluvial soil, mollisol, humid, lowland	Briquette, cement, stone and adobe houses, animal barns, food stores, citrus farms, cotton fields, chicken farms
OSMANIYE	118	67	19.6	761	Agricultural area, bushes, *Pinus* forests, inceptisols, vertisols, serpantinite, humid, lowland	Briquette, cement and stone houses, animal barns, food stores, citrus farms, cotton fields, chicken farms
HATAY	85	69	18.2	1173	*Laurus, Quercus, Pinus* forests, entisols, semi-humid, Amanos mountains, caves, peridodite	Briquette, cement and stone houses, animal barns, food stores, citrus farms, cotton fields, banana farms
KONYA	1016	60	11.5	324	Steppe vegetation, rodent burrows, agricultural area, mollisols, aridisols, semi-arid, plato, caves, calstic and carbonate rocks	Briquette, cement, stone and adobe houses, animal barns, food stores, corn and wheat fields

MSHR: Mean seasonal relative humidity; MST: Mean seasonal temperature; MSP: Mean seasonal precipitation.

RESULTS

The wing shape of female *P. papatasi* becomes narrower through the PC1 but broader and smaller through PC2 (the first two PCs summarize 23.9% and 17.7% of the total variance, respectively). The first PC suggests some differences in the relative positions of the landmarks 8, 9, 10, and 11 (Figure 2A) regarding the base of the wing. CVA (Canonical Variates Analyses-CANOVAR) also show some overlapping in local areas (data not shown) except in regions I and VI that have statistically different shapes according to Barlett's test. There are two distinct CVs in which Axis 1 Lambda= 0.1113, χ^2=316.1455 df=140, $p <$ 0.001; Axis 2 Lambda= 0.2788 χ^2=183.9043 df=108, $p <$ 0.001. In males of the same species, the first two PCs give the same impression as in females, and it becomes smaller through the positive extremes. The first two PCs summarize 25.1% and 18.9% of the total variance, respectively (Figure 2B). There are only small deformations at the base of the wing. CVA show less overlapping in local areas (data not shown) when compared to the females. These lead to some groupings which have statistically different shapes according to Barlett's test (there are three distinct CVs in which Axis 1 Lambda= 0.0893 χ^2=275.3325 df=140, $p <$ 0.001; Axis 2 Lambda= 0.1979 χ^2=184.6885 df=108, $p <$ 0.001; Axis 3 Lambda= 0.3766 χ^2=111.3176 df=78, $p <$ 0.05). However, the grouping of region III could possibly be due to the few specimens examined.

In female *P. tobbi,* the landmarks 8, 9, and 10 show some differences among individuals that are not along a local population gradient through PC1. The first two PCs summarize 27.12% and 19.62% of the total variance, respectively, and the wing gets somewhat broader and longer through PC2, but this is not due to the localization

		I	II	III	IV	V	VI	Total
P. papatasi	♀	38	13	18	53	15	25	162
	♂	28	10	4	42	35	13	132
P. tobbi	♀	35	22	1	109	0	1	168
	♂	7	9	2	162	0	0	180
P. sergenti	♀	6	10	2	2	1	0	21
	♂	8	7	2	7	2	3	29
P. similis	♀	0	0	0	0	0	4	4
	♂	0	0	0	0	0	3	3

Table 2. Detailed number of specimens used in the geometric morphometrics analysis.

and the differences are randomly distributed (Figure 3A). CVA also overlaps in all local areas (data not shown) except in regions III and VI in which there is only one specimen each. Barlett's test shows no significant difference among groups ($p > 0.001$) and there were no distinct CVs. In males of the same species, the specimens overlap through the first two PCs like in those of females (Figure 3B); the first two PCs summarize 25.7% and 18.4% of the total variance, respectively. In the PC1, the landmarks 10, 11 and 12 show some deformation, whereas through PC2 all the wings get broader. CVA showed the same overlapping in local areas (data not shown) when compared with the females. According to the Barlett's test, there is only one distinct CV in which Axis 1 Lambda= 0.4775 (χ^2=120.5002 df=84, $p <$ 0.05).

Because we found *P. similis* in only one region, we decided to compare this species to *P. sergenti* to determine whether or not they are conspecific. In females, the wing gets narrower along the first PC which is not along a local population gradient, but PC2 discriminates *P. similis* from others on the negative extreme (Figure 4A). The differences are mainly on landmarks 1, 2, 3, 7, 8, and 9. The first two PCs summarize 30.9% and 19.9% of the total variance, respectively. We also compared two groups on species level. A bootstrapped F-test with 100 bootstraps showed that these are different species (F_{score}= 2.51, $p < 0.05$). In males, the PCA provides the same determination although the number of specimens are relatively low (Figure 4B). The first two PCs summarize 27.76% and 22.46% of the total variance, respectively. The bootstrapped F-test for the males also indicated that these are different species (F_{score}= 2.32, $p < 0.05$). As the CVA requires more specimens, we used a more specific model, where instead of using landmark coordinates we used the PCA axis scores of our specimens. Here we chose six axes for females and according to the Barlett's test, there is only one distinct CV in which Axis 1 Lambda= 0.0672 χ^2=68.8690 df=36 p=0.000792171. With ten axes for males, 95% of the original variance in the data were preserved. According to Barlett's test, there is only one distinct CV in which Axis 1 Lambda= 0.0099 χ^2=69.2269 df=50 p=0.0371409.

Centroid sizes were used as measures of overall wing size differences among different regions in each individual of the examined species. One way ANOVA was conducted

to test the significance. In both females and males of *P. papatasi* individuals from the regions 1, 2, and 4 have relatively smaller wings ($F_{(5,156)}$ = 18.48, $p < 0.001$ in females and $F_{(5,126)}$ = 22.84, $p < 0.001$ in males). In *P. tobbi*, there is no size difference among the regions for both male and females ($p > 0.05$). We found the centroid size to be significantly different in species level between *P. similis* and *P. sergenti* females ($F_{(6,26)}$ = 2.70, $p < 0.05$) and highly significant in males ($F_{(5,18)}$ = 11.43, $p < 0.001$).

DISCUSSION

Among sand fly populations, phenotypic variation is influenced by an assortment of environmental factors that include, but are not limited to, altitude, host population, and temperature (Ghosh et al. 1999, Belen et al. 2004). The relatively low dispersal ability of sand flies (Munstermann et al. 1998, Killick-Kendrick 1999) is an important factor that determines the fixation of morphological characters in the local populations. Several hypotheses can be tested in order to examine the possible gene flow among these populations. We tested this here with geometric morphometrics.

The current study is one component of a large scale multi-level work we have completed. The main study demonstrated that phlebotomine sand flies are abundant and widespread in southern Anatolia. More importantly, all proven or suspected vectors of leishmaniasis in Turkey are also present throughout the area under study. Because it was the first large-scale study including nine provinces, rivers, mountains, and plains, we obtained information about vertical and horizontal geographical distribution of sand fly species and about direct or indirect effects of geographical barriers to this distribution in terms of allopatry.

Although altitude per se is not a selective factor, biotic and abiotic properties of the environment are highly correlated with altitudinal gradients, most obvious of which is climate (Karan et al. 2000). Mountains in the study area represented interesting sites in terms of the discrete division observed in the distribution of its sand fly species in domestic and rural habitats. There, most of the *Phlebotomus* species were restricted to domestic and slightly rural habitats. This patchy adult distribution is typical for most sand fly species because of their poor dispersal ability (Munstermann et al. 1999). On the other hand, the presence of transitional gaps

between the western and eastern parts of Hatay Province on the south (GAP 1), between Adana Province and K.Maras Province north of the Amanos Mountains (GAP 2), and between Adana Province and Nigde-Konya Provinces (GAP 3) helped large-scale horizontal distribution of some species such as *P. sergenti, P. papatasi,* and *P. tobbi* throughout the study area.

We also found no statistically important differences in means of wing morphology in all examined species for both sexes of all local populations. The morphological change that is examined is in terms of their shape. We also examined the sizes of these wings among the same local populations. Both females and males of *P. papatasi* individuals from the regions 1, 2, and 4 were found to have relatively smaller wings. In these regions, climate reflects the typical characteristic of much of the Mediterranean coast, with a mean temperature of 20-25° C, high relative humidity, and occasional summer showers that may cause rapid development of pre-adult stages. Bates (1947) and Detinova (1955) indicated both in the laboratory and in nature that when larvae are provided with abundant food and are not overcrowded, the size of the adults is largely determined by temperature, with large adults being produced at low temperatures and smaller adults at high temperatures.

Contrary to Depaquit et al. (2002), we found that two of the most important vector species, *P. sergenti* and *P. similis* were both present in the sampling sites in Konya Province at an altitude of 1,132-1,385 m (unpublished data). Their study hypothesized that these two species were allopatric at the present time in different countries of the Old World, including Turkey. *P. similis* is probably present only in the western part of Turkey and *P. sergenti* only in the eastern part of Turkey. Molecular studies confirmed that the specimens were separated into two groups and represent two different species, *P. sergenti* and *P. similis*. This suggests that *P. sergenti* is a widely distributed species throughout the study area and that these two closely related species are sympatric in Konya Province (unpublished data). In our morphometric study, the graphical depiction of PCA, CVA, and F-test indicates that the difference in wing morphology between *P. similis* and *P. sergenti* suggests that these species are not conspecific. There is also a statistically significant difference between them when their wing sizes are observed. Although we keep in mind the possibility of phenotypic plasticity, we can clearly say that our results and field experiences support the conclusion that *P. similis* and *P. sergenti* are separate species and are not allopatric, but sympatric in the Konya Plain of Anatolia.

In conclusion, the results presented here indicate that because of the presence of transitional gaps between the regions, geographical variation associated with large-scale vertical and/or horizontal distribution in wing morphology does not exist among local populations of presented species. It seems likely that Region I and Region IV are transitional areas between the west and east of Anatolia. The mountains in the study area are not important geographical barriers for sand fly distribution.

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