A comparison of the intraspecific variability of *Phlebotomus sergenti* Parrot, 1917 (Diptera: Psychodidae)

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ABSTRACT: *Phlebotomus sergenti* populations from different areas of the Mediterranean basin are known to exhibit high intraspecific variability. Previous studies of ITS2 revealed the presence of two branches that may represent sibling species. To corroborate this finding by other tools, two colonies of *P. sergenti* originating from Turkey and Israel, each belonging to a different ITS2 branch, were compared by three different methods: geometric morphometric analysis of wing shape, RAPD (random amplified polymorphic DNA), and cross-mating study. For geometric morphometric analysis, two-dimensional Cartesian coordinates of 16 landmarks from the wings were digitized and analyzed. Significant shape differences were found between colonies but not between sexes within each colony. RAPD results formed two distinctive clades corresponding to the origin of the colony but also showed heterogenity among members of both colonies. In cross-mating studies, viable hybrid F1 and F2 progeny were obtained when both Turkish males/Israeli females and Israeli males/Turkish females were crossed. F1 progeny was included in RAPD analysis and these hybrids formed a distinctive clade with an intermediate position between the two parental clades. No significant differences were found in egg production of crossed sand flies. The cross-mating study showed that there is no reproductive barrier between *P. sergenti* from different geographical areas. On the other hand, RAPD and geometric morphometric analysis revealed a significant difference between colonies and confirmed the suitability of previous ITS2 analysis for discrimination among sand fly populations. Further development of molecular markers should resolve a possible existence of sibling species within *Phlebotomus sergenti*. ***Journal of Vector Ecology*** **31 (2): 229-238. 2006.**

Keyword Index: Phlebotomus sergenti, RAPD, intraspecific variabilty, sibling species, geometric morphometrics.

INTRODUCTION

As the main vector of *Leishmania tropica*, *Phlebotomus sergenti* Parrot, 1917 is a sand fly of great medical importance. Originally described from Algeria in 1917, this species has a broad range of distribution which covers areas of the southern Mediterranean (Morocco, Algeria, Tunisia), the northern Mediterranean (Portugal, Spain, Sicily), Middle East, Arabia, Afghanistan, Pakistan, and northern parts of India. Because of the broad distribution, we can expect some degree of intraspecific variability.

Intraspecific variability of the internal transcribed spacer 2 (ITS2) was studied by Depaquit et al. (2002) with 12 populations of *Phlebotomus sergenti* from ten different countries. According to this study, two branches can be identified: One is related to the north-eastern Mediterranean area (Cyprus, Pakistan, Syria, and Turkey), while the other is south and west of the first one (Egypt, Morocco, and Israel). These branches are in accordance with postulated migration routes of *P. sergenti* along the Thetys Sea during the Miocene era. According to Depaquit et al. (2002), groups belonging to these two different branches seem to differ in ecology, host preferences, and possibly also in vectorial capacity. It is judicious to consider the potential existence of sibling species as was demonstrated by means of molecular biology in the case of *Lutzomyia longipalpis* (Diptera: Psychodidae), where different cryptic species were established (Arrivillaga et al. 2002, Maingon et al. 2003). If sibling species within *P. sergenti* were proven, it would have important implications in epidemiology as well as in experimental studies. Because we have maintained two laboratory colonies originating from areas belonging to two different above mentioned branches, we were able to study the intraspecific variability of this taxon by three different methods: RAPD, geometric morphometrics, and a cross-mating study.

Random-amplified polymorphic DNA (RAPD) represents a powerful tool for identification of species and strains and the estimation of genetic variability between isolates (Williams et al. 1990). The technique amplifies random fragments of genomic DNA by polymerase chain reaction (PCR) using single primers of arbitrary nucleotide sequences. It is considered to be a fast, easy, inexpensive, and yet very informative method (Diakou and Dovas 2001). In the New World sand flies, RAPD was succesfully used to discriminate between two closely related species, *Lutzomyia youngi* and *L. spinacrassa* (Adamson et al. 1993). It distinguished succesfully between different laboratory populations of *L. longipalpis* complex (Dias et al. 1998) and distinct biogeographical populations of *L. whitmanni* complex (Margonari et al. 2004). Only two studies using RAPD were performed on the Old World sandflies. RAPD was succesfully used to compare genetic variation within and between five *Phlebotomus* species sympatric in southern Spain (Martin-Sanchez et al. 2000) and to develop species-specific diagnostic profiles of *Phlebotomus papatasi* and *P. duboscqi* (Mukhopadhyay et al. 2000). Here we present

RAPD as a useful method for discrimination of different laboratory colonies of *Phlebotomus sergenti* and a powerful tool for studies of intraspecific variability in this species.

In spite of the growing number of molecular methods, morphological approaches are also valid. Among these, geometric morphometrics represents an important new paradigm for the statistical study of "shape" and "size" in biology and other fields of science (Rohlf and Marcus 1993). The landmark-based geometric morphometrics has gained significant support among entomologists (Alibert et al. 2001). Unlike analytical approaches, the geometric one is aimed at a comparison of the shapes themselves (Pavlinov[3]). The method is based on capturing the two- or three-dimensional Cartesian coordinates of landmarks which are the homologous points among the structures that have been previously assigned the same names (Bookstein 1991). Differences among individual configurations of landmarks can be translated to several mathematical functions which fit the differences (Alibert et al. 2001). The Procrustes distance (the square root of the sum of squared differences) can then be used as a metric for comparing shapes (Rohlf 1999). Together with warps, Principal Components Analysis (PCA) of Procrustes residuals, consensus shape of wing-triangulation (average positioning of the landmarks in a set of specimens, *eg*. a species with their artificial triangular connections), and UPGMA (unweighted pair-group method using arithmetic averages) phenograms can provide an excellent combination of techniques for the two purposes of such studies: first to *detect* and then to *describe* the differences among taxa (Lockwood et al. 2002). Secondly, the size measure can be visualized in terms of the centroid size, which is uncorrelated with shape in the absence of allometry (Zelditch et al. 2004).

Several cross-mating studies have been performed within both Old and New World sandflies. The main object of these studies was *Lutzomyia longipalpis*, a possible complex of sibling New World species (reviewed by Uribe 1999). Cross-mating between populations with different male sex pheromones was unsuccesful (Ward et al. 1988). This, in combination with other approaches, suggests an existence of pre-zygotic reproductive barriers within this complex (Maingon et al. 2003). In the Old World sand flies, cross-mating studies were performed between *Phlebotomus papatasi* and *P. duboscqi* (Madulo-Leblond et al. 1991, Ghosh et al. 1999) and between *P. bergeroti* and *P. papatasi* (Fryauff and Hanafi 1991). The aim of our study was to hybridize males and females of *P. sergenti* from colonies derived from two different geographical regions (Turkey and Israel) to examine a possible reproductive barrier among suspected sibling species and compare the results with those of morphometry and RAPD analysis.

[3]Pavlinov, I.Y. 2001. Geometric morphometrics, a new analytical approach to comparision of digitized images. Information Technology in Biodiversity Resarch. Abstracts of the 2nd International Symposium. St. Petersburg. 41–90.

MATERIALS AND METHODS

Sand fly colonies

Two colonies of *P. sergenti* were maintained at Charles University, Czech Republic. The colony originating from Turkey (TK) was established from gravid females collected in 1998 in Urfa, southeast Anatolia. The colony originating from Israel (IS) was derived from females collected in Amnun, northern Israel in 2001.

Both colonies were maintained under the same conditions at 26±1° C, 90% RH and 14/10 L:D photoperiod. Adults had constant access to cotton wool soaked with 50% honey, sugar, and a water source. Once a week, females were fed on anaesthetized mouse (ketamin 150 mg/kg, xylazin 15 mg/kg). The blood-fed females were transfered into separate cages and after defecation they were moved to a plaster-lined breeding pot to lay eggs. Larvae were maintained on a diet of mixed mold, rabbit feces, and rabbit chow that was aged for three weeks, air dried, and finely ground.

RAPD analysis

DNA was extracted from individual males and unfed females with the High Pure PCR Template Preparation Kit (Roche, France). Only the thorax was used; the digestive tract, heads, wings, and legs of flies were dissected out prior to DNA extraction to minimalize possible contamination. Five males and five females were taken from each colony. We also included F1 progeny obtained from the cross-mating study into the RAPD analysis (five males and three females descended from a Turkish female/Israeli male crossing).

To prevent possible contamination by symbionts, both colonies were previously screened for presence of *Wolbachia* using the general *wsp* primers 81F and 691R (Braig et al. 1998, Zhou et al. 1998) as described elsewhere (Benlarbi and Ready 2003). *P. papatasi* from Urfa, Turkey, was used as a positive control. No *Wolbachia* symbionts were found in any *P. sergenti*.

For RAPD analysis, 60 decamer random primers were tested (OPA 1-20, OPD 1-20, OPF 1-20, by Operon Technologies Inc, U.S.A.). Twelve of these primers were found suitable: OPA3, OPA5, OPA9, OPA10, OPA11, OPA20, OPD5, OPD8, OPD13, OPF14, OPF19, and OPF20. The PCR reaction was optimized (number of cycles, thermic profile of reaction, Mg^{2+} concentration) in order to obtain informative and reproducible RAPD patterns. The volume of each reaction was 25 µl. The reaction mixture was prepared as follows: 12.5 µl of Master Mix (75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0,001% Tween 20, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 200 µM dTTP, 2,5 U Taq purple DNA polymerase, by Top-Bio, Czech Republic), 1.5 µl $MgCl_2$ (1.5 mM), 2 µl primer (10 pmol), and 8 µl dH_2O. RAPD reactions were performed by a PTC-200 thermocycler (MJ Research Inc, U.S.A.) and subjected to 45 amplification cycles. The temperature profile was 94° C for 1 min, 35° C for 2 min and 72° C for 3 min. An initial denaturation step of 94° C for 4 min and a final extension step of 72° C for 10 min were added.

After PCR amplification, the reaction products were

separated on 1.5% agarose (Serva) gel in TAE (40 mM Tris acetate/1 mM EDTA) at 80 V for 3 h and stained with ethidium bromide. Ten µl of each product was loaded on the gel, and ethidium bromide was added into the gel prior to the separation. The electrophoretograms were captured with a digital camera using the SkyPro program (Software Bisque). Bands were transformed into a binary matrix data where presence or absence of a band was codified as 1 or 0, respectively. All detected bands were included in the analysis. Genetic distances of samples were computed from Nei-Li´s coefficient of similarity (Nei and Li 1979). Phylogenetic trees were constructed by the unweighted pair-grouping analysis (UPGMA) (Sneath and Sokal 1973). The robustness of trees was assessed by bootstrap analysis. PC program FreeTree (Hampl et al. 2001) was used for computations of genetic distances and construction of trees.

Geometric morphometric analysis

For morphometric analysis, 100 specimens were randomly taken from each of the two colonies. All the specimens were screened for the presence of known ecto- and endo-parasites to shield the morphometric data from possible traumatic variations (Mayr and Ashlock 1991, Aytekin et al. 2002). The wings were removed from each specimen by forceps and stained for proper vision of veins using the following procedure: the wings were kept in 5% KOH for 20 min to clear hairs, washed in 95% ethanol, and then washed in distilled water. The wings were transformed to methylene blue for 20 min, re-washed by distilled water and ethanol, soaked in xylene for 5 min, and mounted in entellane on labelled slides. All slides were photographed using a Leica MZ-7.5 stereoscopic zoom dissection microscope with a DC-300 digital camera system, digitized, coded, and archived. Some of the 2,000 specimens were eliminated because of problems during their preparation and 81 were used for the morphometric analysis (15 males and 66 females). Three females of the IS population were also removed as they caused a Pinocchio effect, which is a large change concentrated only at one landmark. All specimens were scored by the same person (A.M.A.). In order to reduce the measurement error, all specimens were digitized twice. The second session of measurement was conducted after the specimens had been removed and replaced under the microscope in order to take positioning error into account (Arnqvist and Mårtensson 1998, Alibert et al. 2001).

Two-dimensional Cartesian coordinates of 16 landmarks (Figure 1) were digitized by tps-DIG1.40 software (Rohlf 2004a). The landmark configurations obtained were then scaled, translated, and rotated against the consensus configuration by GLS Procrustes superimposition method (Bookstein 1991, Rohlf and Marcus 1993, Dryden and Mardia 1998) and used in Morphologika® (O'Higgins and Jones 1999) to perform Principal Components Analysis (PCA) and to calculate centroid sizes. The principal components were later used for SAHN (Sequential, Agglomerative, Hierarchical, and Nested clustering method) clustering to obtain an UPGMA phenogram by Ntsys-Pc2.1® (Rohlf 2000). Euclid distance was preferred for the pooled interval data to obtain the similarity matrix. The size morphometry of the taxa were investigated by using the centroid sizes of the front wings as an estimator with the nonparametric Kruskal-Wallis test (Zelditch et al. 2004).

Cross-mating analysis

For the cross-mating experiments, single pupae from both colonies were placed into individual vials to secure virgin individuals. Virgin females of a given colony were grouped with virgin males from the other colony (TK male / IS female, IS male / TK female) in an approximate 1:1 ratio of sexes and allowed to feed on mice. Blood-fed females were places individually into oviposition vials and ovipositing females, eggs, and emerging adults were counted. Adult F1 hybrids were used for F2 brother-sister mating, which was performed in the same fashion.

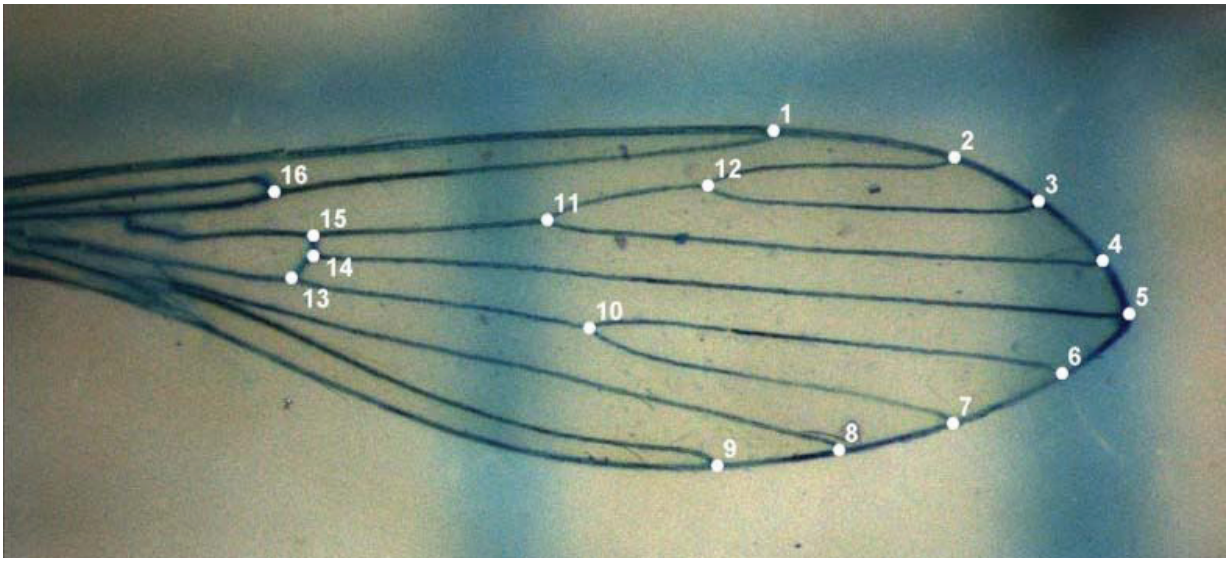

Figure 1. Location of the 16 landmarks on the wing of *Phlebotomus sergenti*.

Special attention was paid to possible differences in egg production of IS females as the results of preliminary experiments suggested that their oviposition might be affected by mating with TK males. The number of eggs in batches of IS females inseminated by TK males was compared with the number of eggs in batches of IS females inseminated by IS males (T-test, Statistica©). Care was taken to perform the mating and oviposition under the same conditions.

RESULTS

RAPD analysis

PCR conditions produced a reproducible banding pattern for each primer used for the analysis. The reproducibility was tested for several primers, and aside from minor variations in band intensity, there were no variations in banding pattern for any primer tested.

A total of 149 fragments was scored. Of these, 21 were monomorphic (shared by all individuals) and 128 (86%) polymorphic. The size of amplified products ranged from 200-1500 bp. The UPGMA analysis of the RAPD data revealed that members of each colony formed a distinct subgroup. A similar grouping pattern was also acquired by the neighbor-joining method (data not shown). There was no significant grouping pattern based on sex in any of the two groups formed. However, there was a considerably high level of variability within each subgroup with Israeli sand flies showing higher levels of such variability. When F1 progeny obtained from the cross-mating study were included in the analysis, it formed a distinct subgroup with an intermediate position between the Turkish and Israeli subgroup (Figure 2).

Geometric morphometric analysis

When the four taxa were analyzed (TK-female, TK-male, IS-female, and IS-male) in terms of relative warps, the TK and IS populations showed significant differences where there were small changes among sexes of each population. The most significant differences were determined in the shape of the basal part for TK populations and mid and apical of the IS populations when the mean shape used as reference. The intra-variation of the sexes was not determined to be as high as inter-regional differences. The results showed that the TK populations are typically different by having a thinner wing than those of IS populations when the generalized least-square superimposition for the landmark configurations are rendered to wire-frames superimposed on the reference configuration (Figure 3 A-D).

The PCA of the wing shape produced a similar grouping pattern. The SAHN clustering showed no significant difference among sexes. But when an UPGMA phenogram was conducted for each sex, distinct groups (TK and IS) in both females (Figure 4) and males (Figure 5) clustered perfectly, which indicated that there was a distinct group pattern in terms of shape morphometry among Turkish and Israel taxa. The same clustering was also observed for data obtained from the centroid sizes. The size differences among the populations were significant and in a linear gradient (Figure 6) (Kruskal-Wallis Test: $H = 38.64$ at $P < 0.001$).

Cross-mating analysis

In the first experiment, successful mating, insemination, and viable hybrid F1 and F2 offsprings were obtained from both combinations of parents (TK male/IS female and vice versa). Of 25 TK females grouped with IS males, 17 females oviposited a total number of 542 eggs. F1 progeny descended from these eggs was a total of 310 sand flies. Of these, 90 females were used for production of F2 progeny and they produced a total number of 712 F2 generation sand flies. Of 46 IS females grouped with TK males, 20 females oviposited a total number of 460 eggs. F1 progeny descended from these eggs was 25 sand flies in total. Of these, 12 females were used to produce a total number of 171 F2 generation sand flies.

In the second experiment the difference in egg production was tested between combinations of TK male/IS female and IS male/IS female. The total number of 103 IS females was mixed with TK males. Of these, 78 females fed on mice and 50 oviposited. These females produced batches with total number of 2,052 eggs, an average of 41 eggs per female. Of 97 IS females maintained under the same conditions and mixed with IS males, 71 females blood fed and 46 oviposited. Their egg production was 2,242, corresponding to an average of 48 eggs per female. Although IS females mixed with TK males produced a lower number of eggs, the difference was non-significant (T-test: $T = 1.22$, $P = 0.2$).

DISCUSSION

ITS2 sequencing by Depaquit et al. (2002) revealed that populations of *P. sergenti* from the Mediterranean basin exhibit intraspecific variability (Depaquit et al. 2002). The aim of our study was to corroborate this finding and study the differences between populations of *P. sergenti* from different branches postulated by Depaquit et al. (2002) using three different approaches: geometric morphometric analysis of wing shape, RAPD analysis, and cross-mating study.

RAPD was able to clearly distinguish between members of the Turkish and Israeli colonies. When 15 different arbitrary primers were deployed, both UPGMA analysis and neighbor-joining method analysis of RAPD-PCR revealed the existence of two distinct groups according to colony origin. All members of one colony fell into the same group. Moreover, there was no significant grouping pattern based on sex in any of these two groups. RAPD analysis also revealed a considerable variability within each of the two colonies. The patterns of bands obtained from RAPD-PCR were very complex and showed intracolonial differences, with the Israeli colony showing a higher degree of this variability. As we succesfully obtained F1 progeny from the cross-mating study, these progeny were included in the RAPD analysis. Interestingly, these samples formed a distinct group with position intermediate between the Turkish and Israeli subgroups.

As the RAPD patterns obtained were stable and reproducible, we believe that RAPD-PCR reflects a prevailing

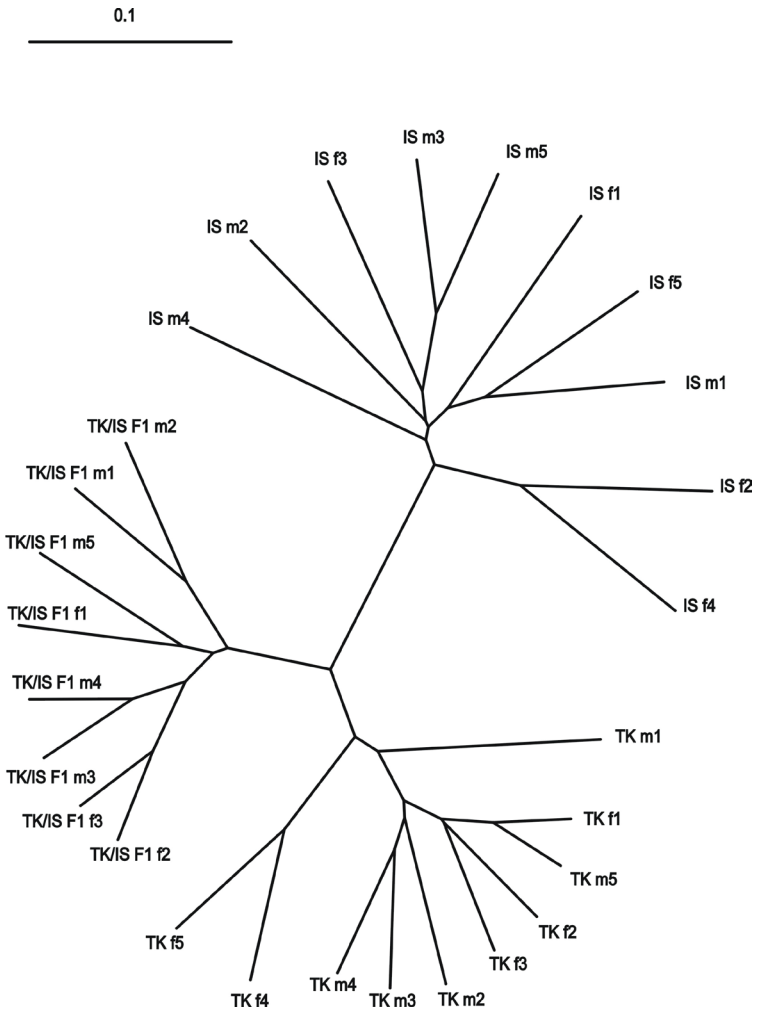

Figure 2. Unrooted UPGMA tree based on RAPD analysis of 28 individuals: 10 Turkey (5 males, 5 females), 10 Israel (5 males, 5 females), 8 F1 progeny (T♀/I♂, 5 males, 3 females). (TK-Turkey, IS–Israel, m-male, f-female).

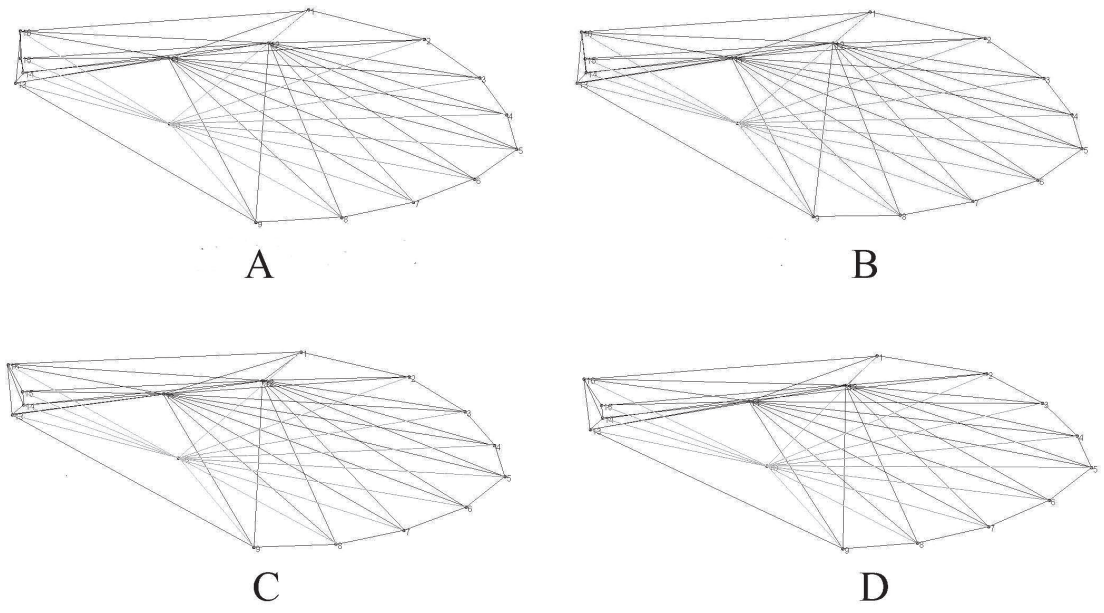

Figure 3. Results of the generalized least-square superimposition for the landmark configurations of *Phlebotomus sergenti* rendered to wire-frames of different populations superimposed on the reference configuration using affine generalized resistant fit analysis. **A**: IS-Female, **B**: IS-Male, **C**: TK-Female and **D:** TK-Male.

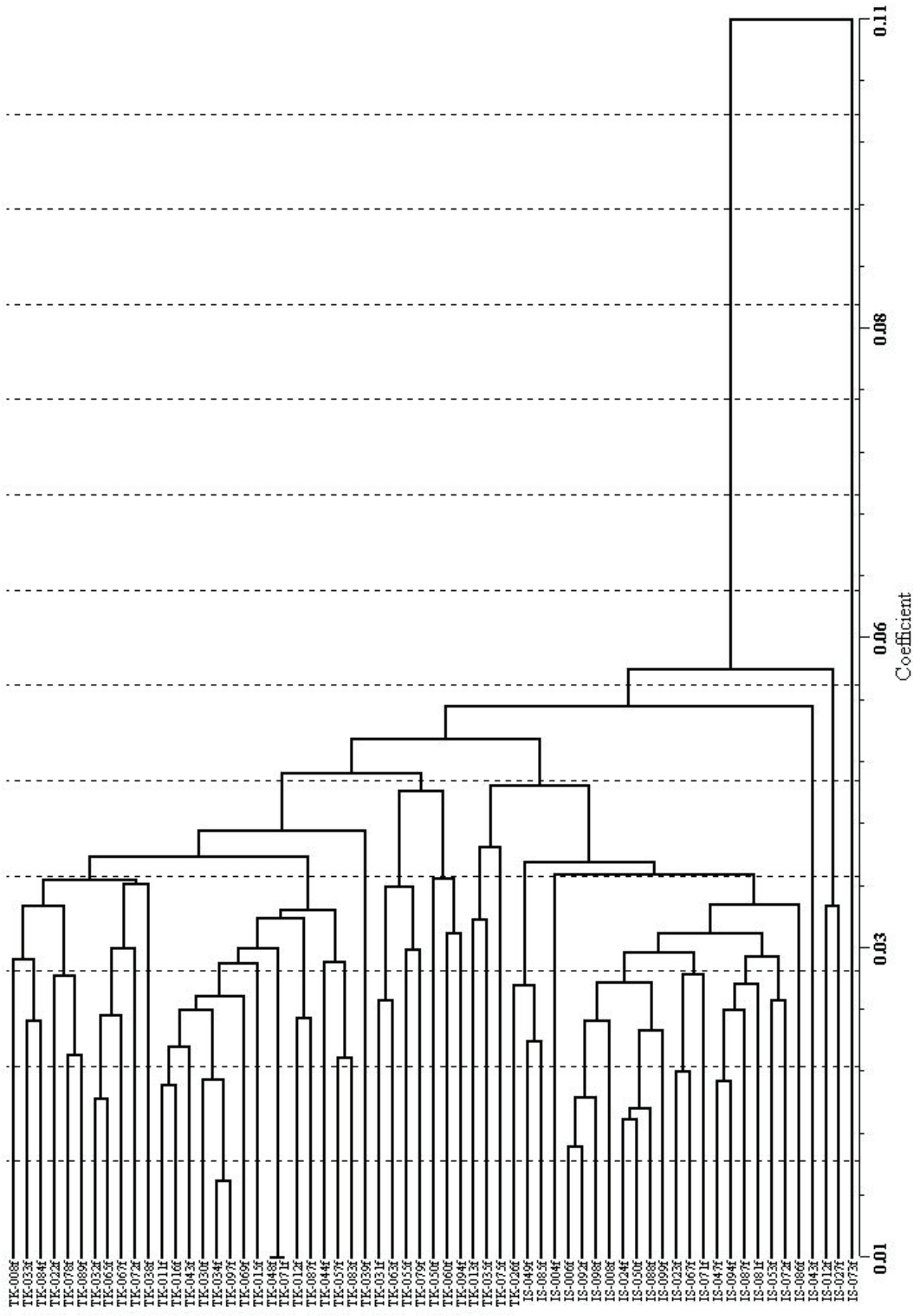

Figure 4. UPGMA phenogram showing the difference between Turkish and Israeli females (Euclid distance SAHN clustering).

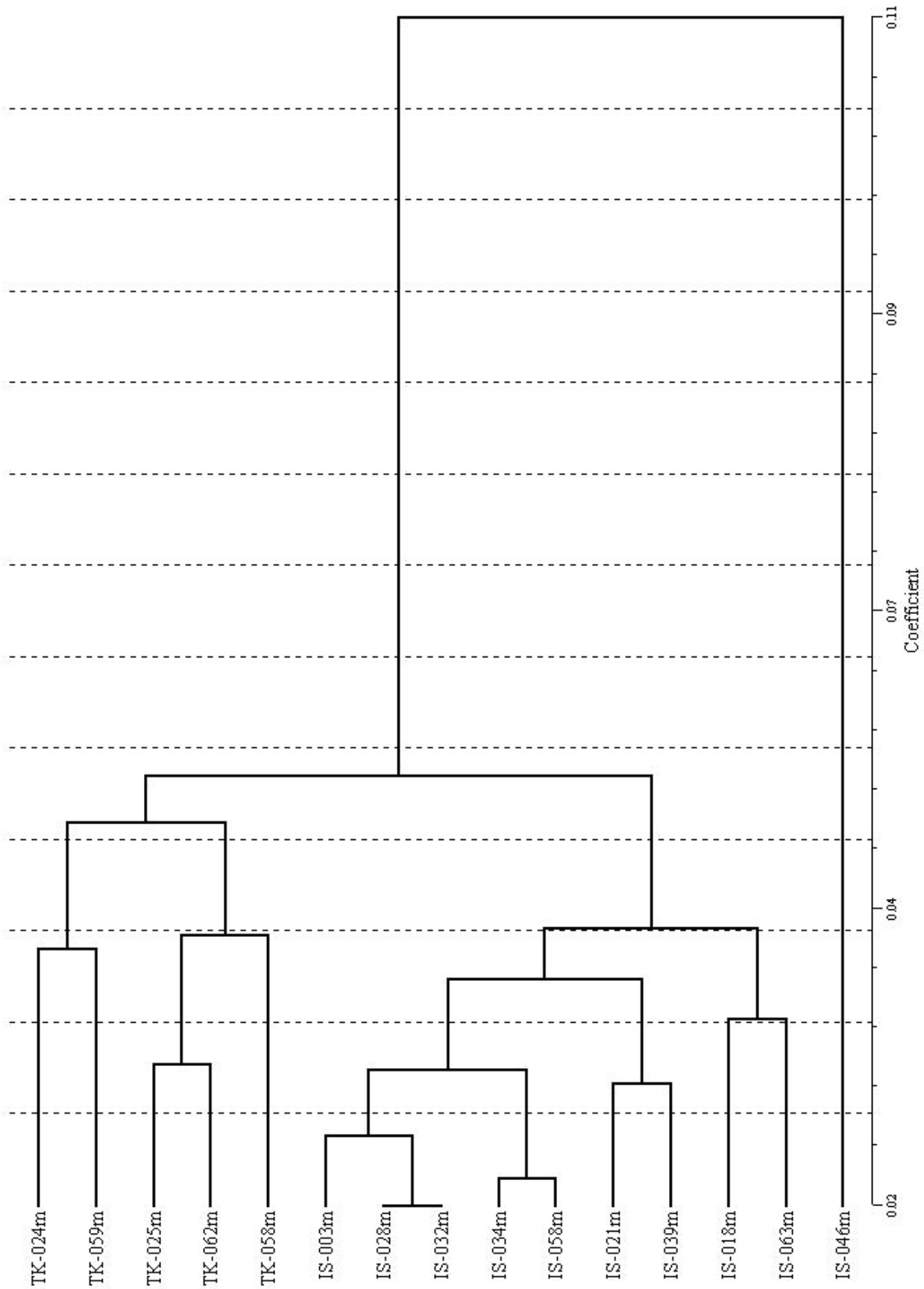

Figure 5. UPGMA phenogram showing the difference between Turkish and Israeli males (Euclid distance SAHN clustering).

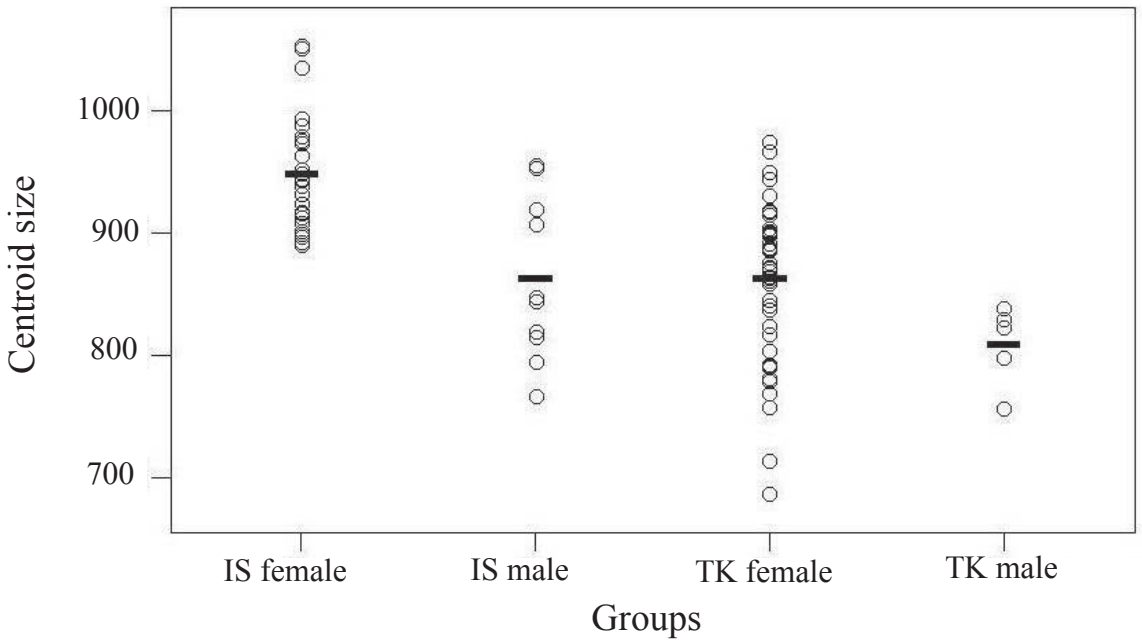

Figure 6. Plot of the individuals of the *Phlebotomus sergenti* populations examined from the data of centroid sizes from the wings. Group means are indicated by lines and dotplots by open circles.

variability in both colonies. Neither of these two colonies underwent any bottle-neck event and it is reasonable to assume that this intracolonial variability reflects a variability of original wild populations. The next step would be a comparison of field samples of *P. sergenti* from different regions of the Mediterranean basin.

Despite its limitations, RAPD analysis has been repeatedly used to evaluate intraspecific variability in sand flies. It proved to be useful in revealing heterogenity among different laboratory populations of *L. longipalpis* (Dias et al. 1998) and also different geographical populations of *L. whitmani* (Margonari et al. 2004). Both taxa are considered to be complexes of sibling species that are difficult to distinguish morphologically. The results of RAPD analysis of *L. whitmanni* sand flies originating in different regions of the distribution of this complex were in partial accordance with a previous morphometric survey from the same regions and provided additional evidence to support the existence of distinct biogeographical populations (Margonari et al. 2004). Here we present a similar kind of results for an Old World sand fly species which may also represent a complex of sibling species.

The insect wings with the least degrees of freedom are the most appropriate structures for geometric morphometric studies (Pavlinov[3]). The results obtained from the wing shape and size supported those obtained from the RAPD. Both PCA and UPGMA results showed no shape difference between sexes, but the deformation of the wing shape among the Israel and Turkish populations is significant. The means of the centroid sizes are also supported by the same scheme obtained from the configuration of the landmarks. The sizes are also significantly different in both colonies. There is a larger wing in IS females than in TK females, while in male TK populations, wing size is more typically thinner in general. The main shape deformation is generally concentrated on the tip of the wings and the basal part. These are kinematically more reflected by the different physical conditions that had the same effects on both sexes.

Cross-mating studies within the Old World sand flies of subgenus *Phlebotomus* yielded various results. Attempted hybridization between *Phlebotomus papatasi* and *P. duboscqi* was first reported to be unsuccesful (Madulo-Leblond et al. 1991). Later, however, female *P. duboscqi* inseminated by male *P. papatasi* did produce viable interspecific hybrid adults (Ghosh et al. 1999). Interspecific hybrid offsprings were also obtained when *P. bergeroti* females were inseminated by *P. papatasi* males, described as intermediate between the parent species in morphology and behavior. Male hybrids of F1 progeny were fertile (Fryauff and Hanafi 1991).

Our cross-mating study demonstrates that crossing is possible between *P. sergenti* specimens from Turkey and Israel. We observed successful mating and insemination and obtained viable hybrid F1 and F2 offspring from both Turkish male/Israeli female and Israeli male/Turkish female combinations. We tested possible differences in egg production and batches descended from Turkish male/Israeli female were compared with egg production from Israeli male/Israeli female mating. Although preliminary results showed a reduction of egg production in females from Israel inseminated by males from Turkish colony, this effect was not statistically significant. According to our results, there is no reproductive barrier among sand flies from Turkish and Israeli colonies. The examples of *P. duboscqi*/*P. papatasi* (Madulo-Leblond et al. 1991, Ghosh et al. 1999) and *P. bergeroti*/*P. papatasi* (Fryauff and Hanafi 1991) crossings show, however, that reproductive isolation may be

incomplete even among closely related, but well established species. If we consider *P. sergenti* populations from two branches postulated by Depaquit et al. (2002) being in the state of speciation or representing sibling species within *P. sergenti* complex, we may expect an incomplete reproductive barrier.

The results of RAPD analysis and geometric morphometric analysis of wing shape of *P. sergenti* from Turkey and Israel corroborated the ITS2 results of by Depaquit et al. (2002). In light of these findings, it seems judicious to extend the application of different approaches to studies of intraspecific variability of different geographic populations of the Mediterranean basin to elucidate the taxonomic status of these populations and reveal a possible existence of sibling species among *P. sergenti.* A panel of microsatellite markers is currently being developed for this purpose and the application of these markers should resolve relationships and taxonomic status of these populations.

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