

Altitudinal variation in morphometric and molecular characteristics of *Phlebotomus papatasi* populations

A. BELEN, B. ALTEN and A. M. AYTEKIN

Department of Biology, Faculty of Science, Hacettepe University, Turkey

Abstract. Four populations of the phlebotomine sandfly *Phlebotomus (Phlebotomus) papatasi* (Scopoli) (Diptera: Psychodidae), in different ecoregions at altitudes between 368 and 1117 m in the Sanliurfa Province of Turkey, were compared using morphometric and isoenzyme analyses. A similarity phenogram obtained from allozyme data showed that heterozygosity was extremely low, particularly for the alleles which were found to be completely fixed in populations at Hamdun (HMD) and Alitas (ALT). Populations at Akcakale (AKL) and ALT branched as a separate group from populations at Hayatiharrani (HHR) and HMD. The ALT population at the highest altitude (1117 m), and the HHR population (488 m) were clustered distinctly when linear measurements of 46 morphological characteristics were examined. A UPGMA (unweighted pair-group method using arithmetic averages) phenogram also showed that ALT and HHR clustered separately, whereas AKL and HMD formed another group.

Key words. *Phlebotomus papatasi*, altitude, electrophoresis, geographical variation, local population, traditional morphometry, Sanliurfa, Turkey.

Introduction

Cutaneous leishmaniasis, transmitted by phlebotomine sandflies, is one of the most hyperendemic vector-borne diseases in south-eastern Turkey, particularly near the border with Syria and Iraq (Lewis, 1982; Volf *et al.*, 2002). However, there is little information available on the annual incidence, distribution of cases, seasonal abundance patterns, and control methods of proven phlebotomine vector species in Turkey. It is known that the Sanliurfa province, in south-eastern Anatolia, is the largest focus of typical cutaneous leishmaniasis. The geographical, ecological and socio-economical characteristics play an important role in the distribution and epidemics of cutaneous leishmaniasis (Alten *et al.*, 2003). This disease has become a greater threat in recent years, since economic opportunities in the provinces of the South-eastern Anatolia Irrigation Project (GAP) have attracted human populations to Sanliurfa.

Ozensoy *et al.* (1997) reported that the number of documented cases of cutaneous leishmaniasis had risen in Sanliurfa from 552 in 1990, to 1955 in 1993. In the first 9 months of 1994 alone the number of reported cases was already over 3000. At the end of 1995, the number of patients had reached to 5027. The proportion of children in this total number was 61% (Aslan *et al.*, 1997). According to the Turkish Ministry of Health (Anonymous, 2002a), cutaneous leishmaniasis has been controlled and/or suppressed in Sanliurfa in the last 3 years (2001–2003), yet based in a study of 10 468 individuals between 1999 and 2002, the rate of prevalence of cutaneous leishmaniasis in Sanliurfa was between 1.9 and 2.3% (of the total 1.3 million population of Sanliurfa) (Alten *et al.*, 2003).

Previous studies with sandflies in Turkey have shown that there are 18 *Phlebotomus* species (or subspecies) present in the subgenera *Adlerius* Nitzulescu, *Larrousius* Nitzulescu, *Paraphlebotomus* Theodor and *Phlebotomus* Rondani & Bérte (Yagci *et al.*, 1998; Alptekin *et al.*, 1999) and 12 of these species can be found in Sanliurfa Province (Toprak, 2003), nine of which are proven or candidate vectors of the Old World leishmaniasis (Killick-Kendrick, 1999). The predominant species in the province were *Phlebotomus*

Correspondence: Bulent Alten, Department of Biology Ecology Section, Faculty of Science, Hacettepe University, 06532 Beytepe-Ankara, Turkey. E-mail: kaynas@hacettepe.edu.tr

papatasi (Scopoli) and *Phlebotomus sergenti* Parrot, with *P. sergenti* being the dominant species in Sanliurfa city centre (Volf *et al.*, 2002). However, Toprak (2003) found *P. papatasi* to be relatively more abundant in different sites and altitudes. The latter species is a proven vector of *Leishmania tropica* in most endemic areas elsewhere, such as in Morocco and Saudi Arabia (Al-Zahrani *et al.*, 1988). Also, this peridomestic and anthropophilic sandfly species is highly susceptible to *L. tropica* (Killick-Kendrick *et al.*, 1995). There has been some suggestion that *P. sergenti* may also be a vector of cutaneous leishmaniasis in Sanliurfa, although many studies still claim that *P. papatasi* is the most important probable vector of cutaneous leishmaniasis and/or sandfly fever (Alptekin *et al.*, 1999; Volf *et al.*, 2002).

The main objective of the current study was to investigate the effects of altitude on population of *P. papatasi*. It was hypothesized that habitat differences associated with altitude may affect gene flow and also morphological and physiological life-history, such as the reproductive capacity, generation time, intrinsic rate of increase and reproductive value.

Materials and methods

Study area

The study was conducted between May 2001 and October 2002 in Sanliurfa province (37°09' N; 38°47' E), SE Anatolia, Turkey. The province, located along the border of Syria in the south, comprises 18 500 km² of the western part of the south-eastern Anatolia region. It is the centre of the GAP irrigation project, which includes 22 dams, 19 hydroelectric plants and 13 irrigation projects with an irrigation network covering 1.7 million ha (Anonymous, 2002b). The river Euphrates drains the west province border of the study area. The city of Sanliurfa lies 550 m above sea level (a.s.l.) and has a semi-arid climate with four distinct seasons: a very hot and dry summer (June–August; mean temperature, rainfall; 31.5°C, 3.20 mm), a warm wet autumn (September–October; mean temperature, rainfall; 20.7°C, 14.14 mm), a moderately warm and rainy winter (December–February; mean temperature, rainfall; 7.50°C, 71.33 mm) and a warm and rainy spring (March–May; mean temperature, rainfall; 17.0°C, 37.03 mm).

Based on results of a previous study (Alten *et al.*, 2003), four localities at different elevations were selected for sampling purposes. The Akcakale (AKL) region (36°42'42" N, 36°56'31" S) is the lowest region, with an altitude of 363 m. The Hayati Harrani (HHR) region (37°07'22" N, 38°48'37" S), situated in the city centre, has an altitude of 488 m. They are both dry, plain and semi-urban areas. The Hamdun (HMD) region (37°29'44" N, 39°07'80" S) has an altitude of 644 m, is mountainous, and is situated in semi-urban area. The Alitas (ALT) region (37°48'56" N, 39°40'47" S) is the highest mountainous and rural area, with an altitude of 1117 m. The incidence of cutaneous

leishmaniasis ranged from 1% to 2.3% between these locations (Alten *et al.*, 2003). The major plant species found in the region are: *Astragalus aleppicus* Boiss., *Astragalus aduncus* Willd., *Onobrychis crista-galli* (L.) Lam., *Adonis dentata* Del., *Medicago orbicularis* (L.) Bart., *Eryngium criticum* Lam., *Trifolium pillulare* Boiss., *Linum mucronotum* Bertol. and *Salvia brachyntha* (Brodz.) Pobed. The main agricultural products of the Sanliurfa region are *Pistacia vera* L., *Vitis sylvestris* Gmelin, *Triticum aestivum* L., *Oryza sativa* L. and *Gossypium* spp. Apart from agriculture, livestock farming is the major source of income in the area. Sheep are the most common livestock but cattle and chickens can also be found in some areas.

Sandfly collection

Phlebotomus papatasi adults were collected by aspirating from houses and barns and from animal-bait traps made from polyester net containing cows, placed near houses of current or past cutaneous leishmaniasis patients in each locality (Alexander, 2000). Collections were also made using CDC miniature light traps (John W. Hock Co. Florida, U.S.A.) during summer (August–September). On each trapping night, four to six light traps were placed in each of the sampling localities. Houses and barns used as sampling stations varied from two-story cement block enclosures to simple brick, stone or cement houses with basements, cellars, caves or barns for keeping poultry or livestock. There had been no periodic spatial spraying of insecticides in the study area since 1996, except in the HHR region. Field-collected live females (fed or gravid) were transported to the laboratory in standard plaster-lined containers, which were in turn placed into ice containers for the establishment of new laboratory colonies (Urbanelli *et al.*, 2000; Volf *et al.*, 2002). Taxonomic identification was made using the keys and descriptions of Perfiliew (1968), Artemiev (1980) and Killick-Kendrick *et al.* (1991). Identifications were reconfirmed using voucher specimens of *P. papatasi* from Sanliurfa, Turkey. After identification, *P. papatasi* females were separated from the other live flies and introduced into standard cages for rearing and laboratory experiments. A total of 325 females and 225 males were used.

Laboratory studies

Colonization. The rearing and feeding of adults and larvae of *P. papatasi* in the laboratory followed the methods of Endris *et al.* (1982) and Modi & Tesh (1983), with modifications by Ferro *et al.* (1998). A temperature of 27 ± 1°C, a relative humidity between 65 and 75%, and a LD 14:10 h period, with 1.5 h simulated crepuscular periods, were maintained.

Fed or gravid adults of *P. papatasi* collected from different altitudes were kept in rearing chambers (each 6.6 cm diameter and 5.5 cm high, with a 1.5 cm thick Plaster-of-Paris base).

All chambers were labelled and stored in large Styrofoam boxes. Adults were provided with sterile 30% sucrose solution as an energy source. Larvae obtained from field-collected samples were maintained on a diet of an aged and finely ground mixture of rabbit faeces, rabbit laboratory chow and mould (produced during the ageing process) (Young *et al.*, 1981). For egg development, females of uniform age were permitted to feed in cloth cages (35 cm × 35 cm) on hamster blood daily for 1 h within 12 h of emergence. Hamsters were anaesthetized with an intraperitoneal injection of 0.3 mL ketamine hydrochloride. After blood feeding, approximately equal numbers of males and females were transferred to small cloth cages (20 cm × 20 cm) for oviposition in 4–5 days. Mating was also observed in these cages. Sucrose solution (30%) was provided daily in cotton swabs. All cages were checked daily and gravid females, together with approximately equal number of males, were passed to new boxes to provide eggs and immature forms of F₁. Separate chambers of flies were maintained for morphometric and electrophoretic analysis.

Electrophoresis. The thoraxes of females were ground up and homogenates were kept at –80°C until needed for electrophoresis. Five enzyme systems, MDH (malate dehydrogenase, EC 1.1.1.37), ME (malic enzyme, EC 1.1.1.40), PGM (phosphoglucosmutase, EC 5.4.2.2 formerly EC 2.5.7.11), EST (esterase, EC 3.1.1) and HK (hexokinase, EC 2.7.1.1), were studied by horizontal starch-gel electrophoresis. Two enzyme systems (EST and HK) were studied using the Tris-citrate, pH 7.0 buffer system (Shaw & Prasad, 1970; Hillis & Moritz, 1990), two (MDH and ME) were studied using the Tris-HCl, pH 8.6 buffer system (Shaw & Prasad, 1970) and PGM was studied using the Tris-EDTA-maleate-magnesium, pH 7.4 buffer system (Shaw & Prasad, 1970). Sample and gel preparation and experimental conditions were similar to those of Kandemir & Kence (1995).

Genetic variations were analysed using a BIOSYS-2 package for desktop computer (Black, 1997). This program is a modification of BIOSYS-1 (Swofford & Selander, 1981).

Morphometric analysis. All the specimens were screened for the presence of known ecto- and endoparasites to prevent possible traumatic variations affecting the morphometric data (Mayr & Ashlock, 1991). The body parts were then removed from each specimen with forceps and mounted in entellane on labelled slides. Before mounting, the wings were stained for proper vision of veins using the following procedure: after separation from thorax, the wings were kept in 5% KOH solution for 20 min to clear hairs. The wings were then placed in 95% alcohol for < 10 s, after which they were transferred for washing to cups containing distilled water. Next, wings were transformed to methylene blue and kept for 20 min before re-washing them. As a last step, the wings were again put in 95% alcohol for < 10 s and finally, soaked in xylene for 5 min for fixation (Belen, 2003). All slides were photographed using a Leica MZ-7.5 stereoscopic zoom dissection microscope with a DC-300 digital camera system,

digitized and archived. A total of 46 characters were measured using TPSdig (Rohlf, 2003) software are as follows: (1) length of femur, (2) length of tibia, (3) length of tarsus, (4) length of basitarsus, (5) length of 5th segment of basitarsus, (6) length of 6th segment of basitarsus, (7) length of 7th segment of basitarsus (fore, mid and hindlegs 21 characters), (8) length of compound eyes, (9) width of compound eyes, (10) length of the distance between compound eyes, (11) width of head, (12) length of frons, (13) length of clypeus, (14) length of labrum, (15) length of head, (16) length of wing, (17) width of wing, (18) length of costa, (19) length of subcosta, (20) length of R₁, (21) length of R₂, (22) length of R₃, (23) length of R₄, (24) length of R₅, (25) length of m₁, (26) length of m₂, (27) length of m₃, (28) length of m₄, (29) length of cubitus, (30) length of halter, (31) width of halter, and (32) length of the apical part of halter. For the paired organs, the ones on the right side were measured (Aytekin & Çagatay, 2002). The collected data were tested for allometry by Huxley's model and transformed into natural logarithmic form (Strauss, 2002).

For statistical analysis of morphology, after the normality test, the data were transformed into natural logarithmic form, which is clearly an advanced method in traditional morphometry (Debat *et al.*, 2003). These data were discriminated using a discriminant multigroup function analysis (Canonical variate analysis CANOVAR) Syn-tax 2000 (Podani, 2001) package (Exeter-Software, U.S.A.). The arithmetic means of the 46 morphometric characters were then discriminated by UPGMA. Manhattan distances were used for the pooled interval data to obtain the similarity matrix by Ntsys-Pc. 2.1 (Rohlf, 2000). The significant differences among characters were also tested by one-way ANOVA.

Results

Morphometric results

Individual distribution and clusters obtained from the morphometric analysis are shown in Fig. 1. For morphometrics, although the personal measurement error (ζ_p) (Arnqvist & Mårtensson, 1998) as well as the standardization and transformation procedure errors were kept at a minimum, unexpected intravariation was still seen in three characters (hind leg segment, wing length and haltere length). The errors in accuracy and precision of the data were mostly due to ambiguity of the reference points of these characters, resulting in shifts during measurement or/and the difficult procedure in the preparation and processing of these body parts. Errors due to two-dimensional viewing of a three-dimensional object could possibly be another reason. Analysis conducted taking the measurement errors into consideration clearly showed that ALT (1117 m) formed a distinct group from the other three regions (AKL, 368 m; HHR, 468 m; HMD, 644 m) for the 46 characters measured along the first two principal axes (Fig. 1). The differences between the local populations of *P. papatasi* were clear when all populations were

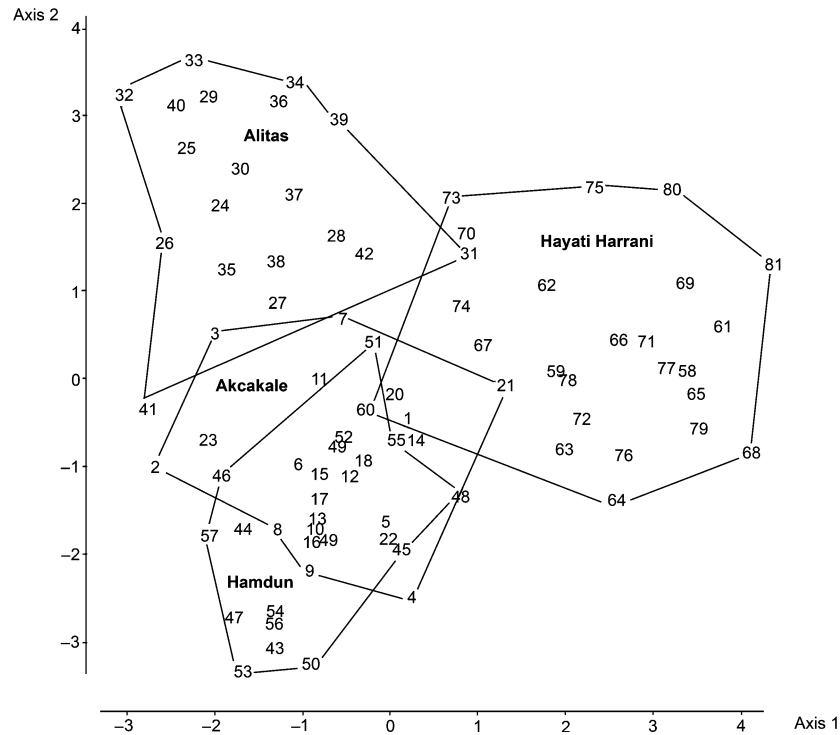


Fig. 1. Population distribution of *Phlebotomus papatasi* collected at four different altitudes (Akcakale (AKL) 368 m, Hayati Harrani (HHR) 488 m, Hamdun (HMD) 644 m, Alitas (ALT) 1117 m), Sanliurf, southeastern Turkey (May 2001–October 2002), along the first two principal axis (CANOVAR) based on 46 morphological characters. Numbers within the scatters indicate identification numbers of each individual. 1–23, AKL; 24–42, ALT; 43–57, HMD; 58–81, HHR.

considered. AKL and HMD populations were the closest ones along the first two axes, while ALT appeared as a different group. When a UPGM tree was constructed,

HMD and HHR populations showed distinct group patterns although they were nearly at the same altitude (Fig. 2). Means with standard errors were also calculated

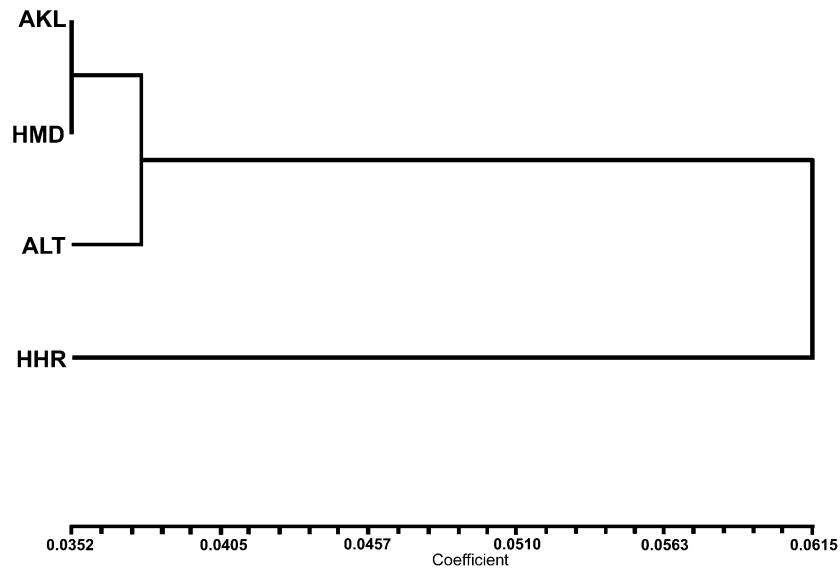


Fig. 2. The UPGMA phenogram based on the arithmetic means of 46 morphological characters measured from *Phlebotomus papatasi* collected at four different altitudes (Akcakale (AKL) 368 m, Hayati Harrani (HHR) 488 m, Hamdun (HMD) 644 m, Alitas (ALT) 1117 m), Sanliurf, southeastern Turkey (May 2001–October 2002). (SAHN clustering, Manhattan distance).

Table 1. Means (\pm standard deviation) of the 17 morphological characters which show significant differences between four populations of *Phlebotomus papatasi* collected from four altitudes (Akcakale (AKL) 368 m, Hayati Harrani (HHR) 488 m, Hamdun (HMD) 644 m, Alitas (ALT) 1117 m), Sanliurfa, southeastern Turkey (May 2001–October 2002).

Characters (mm)	AKL	HHR	HMD	ALT	P
1. Length of femur (foreleg)	0.95 \pm 0.09	1.10 \pm 0.06	1.00 \pm 0.06	0.95 \pm 0.08	0.017
2. Length of femur (midleg)	0.94 \pm 0.10	1.00 \pm 0.10	1.02 \pm 0.06	0.98 \pm 0.06	0.029
3. Length of tibia (midleg)	1.16 \pm 0.08	1.26 \pm 0.08	1.22 \pm 0.11	1.24 \pm 0.06	0.001
4. Length of femur (hindleg)	1.13 \pm 0.10	1.22 \pm 0.09	1.20 \pm 0.08	1.19 \pm 0.07	0.003
5. Length of tarsus (hindleg)	0.86 \pm 0.07	0.93 \pm 0.07	0.91 \pm 0.07	0.90 \pm 0.04	0.003
6. Length of basitarsus (hindleg)	0.43 \pm 0.03	0.46 \pm 0.04	0.43 \pm 0.03	0.45 \pm 0.03	0.029
7. Length of 6th segment of basitarsus (hindleg)	0.22 \pm 0.02	0.22 \pm 0.03	0.23 \pm 0.02	0.24 \pm 0.02	0.14
8. Length of wing	2.27 \pm 0.26	2.48 \pm 0.25	2.52 \pm 0.16	2.26 \pm 0.29	0.001
9. Length of costa	1.05 \pm 0.16	1.13 \pm 0.09	1.12 \pm 0.07	1.12 \pm 0.07	0.040
10. Length of R1	0.33 \pm 0.44	0.36 \pm 0.04	0.36 \pm 0.04	0.36 \pm 0.04	0.021
11. Length of R2	0.50 \pm 0.04	0.55 \pm 0.06	0.55 \pm 0.05	0.54 \pm 0.04	0.005
12. Length of R3	0.70 \pm 0.07	0.75 \pm 0.06	0.78 \pm 0.07	0.75 \pm 0.05	0.004
13. Length of R4	1.16 \pm 0.10	1.23 \pm 0.08	1.25 \pm 0.06	1.27 \pm 0.18	0.017
14. Length of M2	1.00 \pm 0.10	1.06 \pm 0.07	1.08 \pm 0.06	1.09 \pm 0.06	0.003
15. Length of M3	0.82 \pm 0.09	0.87 \pm 0.07	0.88 \pm 0.05	0.89 \pm 0.06	0.009
16. Length of M4	1.15 \pm 0.11	1.24 \pm 0.10	1.24 \pm 0.09	1.21 \pm 0.08	0.010
17. Length of haltere	1.05 \pm 0.19	1.25 \pm 0.13	1.11 \pm 0.14	1.09 \pm 0.25	0.004

for each character, and 17 of them showed significant differences (Table 1) among the populations.

Electrophoresis results

Data obtained from the five enzyme systems used here showed that heterozygosity was extremely low for all localities considered and it was especially noted that the HMD and ALT were fixed for the given alleles. HHR and HMD showed different clusters according to general similarities from AKL and ALT but due to low sample size it was hard to reach an exact conclusion. Allele frequencies and genetic differentiation of all populations for the five loci are given, together with their standard errors in Table 2. The similarity phenogram obtained from these data is given in Fig. 3. From Table 2 it can be determined that although the differentiation was not exact, the HHR and HMD constituted one group, whereas AKL and ALT constituted another group.

Discussion

Phlebotomus papatasi is widely distributed around the Mediterranean basin, throughout the Middle East and eastward across the entire Indian subcontinent. However, its distribution is highly disjunctive within that range, depending on locally occurring environmental factors, precipitation, temperature, physical barriers, altitude, latitude, habitat availability and the distribution and abundance of vertebrate hosts (Ghosh *et al.*, 1999). Of course, in small regions such Sanliurfa province, full isolation for any kind of flying insects should not be expected. But according to the literature, sandflies are poor fliers and the individuals rarely disperse more than 100 m (Munstermann *et al.*, 1998; Killick-Kendrick, 1999) so it may be assumed that they may exist as small local populations. Thus, genetic differences may be expected not only between geographical strains from widely sepa-

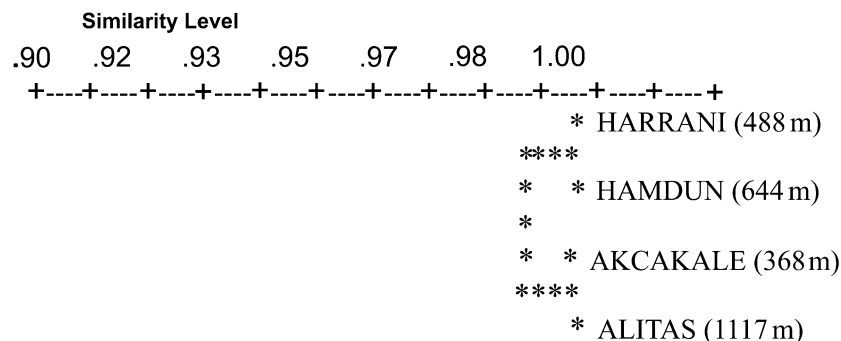
**Fig. 3.** Similarity phenogram obtained from the data of allozymes for four different populations of *Phlebotomus papatasi* collected from four altitudes (Akcakale (AKL) 368 m, Hayati Harrani (HHR) 488 m, Hamdun (HMD) 644 m, Alitas (ALT) 1117 m), Sanliurfa, southeastern Turkey (May 2001–October 2002).

Table 2. Values of genetic differentiation based on allozymes for four different populations of *Phlebotomus papatasi* collected from four altitudes (Akcaakale (AKL) 368 m, Hayati Harrani (HHR) 488 m, Hamdun (HMD) 644 m, Alitas (ALT) 1117 m), Sanliurfa, southeastern Turkey (May 2001–October 2002).

Population	Allele frequencies												Mean heterozygosity		
	HK		MDH-1		EST-3		ME		PGM		Mean sample size per locus	Mean number of alleles per locus	Percentage of loci polymorphic*	Observed	Expected (H _d -W _{bg} **)
	A	B	A	B	A	B	A	B	A	B					
AKL	0.500	0.500	0.700	0.300	0.500	0.500	1.000	1.000	0.778	0.222	6.2 (1.9)	1.8 (0.3)	66.7	0.044 (0.029)	0.301 (0.105)
HHR	0.625	0.375	0.600	0.200	0.750	0.250	1.000	1.000	0.833	0.167	7.5 (2.3)	1.7 (0.2)	66.7	0.037 (0.037)	0.310 (0.101)
HMD	0.857	0.143	0.667	0.167	0.583	0.417	1.000	0.917	0.083	0.083	7.7 (2.4)	1.8 (0.3)	66.7	0.000 (0.000)	0.244 (0.098)
ALI	0.625	0.375	0.692	0.308	0.625	0.375	1.000	0.824	0.176	0.176	8.5 (2.4)	1.7 (0.2)	66.7	0.000 (0.000)	0.290 (0.097)

*A locus is considered polymorphic if the frequency of the most common allele does not exceed 0.95

**unbiased estimate (see Nei, 1978)

rated origins but also between local populations of *P. papatasi*. Recently in Iran, however, relatively little genetic differentiation was detected between populations of *P. papatasi* sampled from different biotypes (Parvizi *et al.*, 2003). In our Turkish study area, previous studies have pointed out that the incidence of leishmaniasis decreases from the low altitudes to the high altitudes and from the city centre to the rural regions (Volf *et al.*, 2002; Alten *et al.*, 2003).

The results presented here indicate that there are some significant differences between populations. When allozyme data were examined it was recorded that the level of heterozygosity is low especially in the ALT population and no heterozygotes were observed for the alleles. Allele fixation is a general phenomenon in Diptera and Hymenoptera (Thorpe & Solé-Cava, 1994). The main reason for this might be long-term isolation, but other factors such as behaviour, mating choice and genetic drift via bottlenecks may also play a role. Long-term isolation would also indicate less human influence over the population's genetic structure. The higher level of heterozygosity in the HHR population and its morphological traits suggest that some factors other than altitude, such as effects of insecticide and/or socio-economical structure of the region may exert pressure on the genetic structure of the populations.

When analysed by one-way ANOVA the populations were found to be significantly different for 17 morphological characters (Table 1). The differences among the four populations occurred primarily in the leg (femur and tibia) and wing characters. This may be associated with factors such as climatic and ecological or socio-biological effects. This may indicate a directional selection or genetic drift especially in the AKL population. The HHR population showed especially great differences in the length of haltere and length of 6th segment of the foreleg. These differences would indicate a bottleneck effect on the HHR population as a result of some specific reason such as uncontrolled insecticide applications, which is very common in the HHR region. When the whole size differences were screened by UPGMA it showed that HHR grouped separately and AKL and HMD clustered together. The size differences also showed similar results when obtained by CANOVAR. In Fig. 1, again, HHR grouped separately from AKL and HMD.

In conclusion, these size and genetic approaches indicate that geographical variations exist among local populations of *P. papatasi* in Sanliurfa. Isoenzyme studies are expensive and time-consuming, but are less informative, suggesting that studies should be based on morphometrical characters. DNA analyses, which give more information about the populations, would possibly be an alternative. The morphometrical studies conducted here were based on only size differences. Future studies should consider size and shape differences using geometric modelling. With the increased pressure to use biological control methods, especially those that rely upon altering the genetic structure of a population, greater attention must be given to understanding the life history characteristics of sandfly populations in the field.

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