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Low-stringency single specific primer- PCR as a tool for molecular characterization of sand fly species (Diptera: Psychodidae) populations collected from Al-Madinah and Asir regions, Saudi Arabia

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Abstract

Sand flies are parasitic hematophagous insects that are vectors for several human and veterinary pathogens. Earlier studies stated that about 20 different species of sand flies have been recorded in Saudi Arabia. Surveillance of the distribution of sand fly species is important to predict the risk and expansion of *Leishmania* infection in endemic and nearby areas. In the present study, low-stringency single specific primer-PCR (LSSP-PCR) technique was established for typing seven sand fly species collected from Al-Madinah and Asir regions, Saudi Arabia. LSSP-PCR analysis was performed to detect DNA polymorphisms of 700 bp 18S rRNA fragment amplified from different species of sand flies. The obtained results revealed intra-individual genetic diversity within the species, however genetic variations among species were higher. Genetic differentiation coefficient between species (Gst) was significant (0.694), indicating that most of the genetic variability (69%) occurred between species, and therefore 31% of the total genetic variability was found within the species level. The genetic diversity was more significant among species belonging to the genus *Sergentomyia* (24%), compared to *Phlebotoumus* genus (15%). Results showed genetic distance with a low percentage (7.6%) between *P. papatasi* and *P. bergeroti*, indicating a very close relationship between these two species and possibly suggesting sister species. In conclusion, genetic variability among and within sand fly species were confirmed based on the efficient LSSP-PCR technique that used for the first time in the molecular characterization of sand fly species. The simplicity and accuracy of the LSSP-PCR technique were found to be suitable for the molecular identification and classification of sand flies. This work has provided genetic data of populations of sand flies in Saudi Arabia necessary for a better understanding of the epidemiology of leishmaniasis in Saudi Arabia.

Keywords: Molecular identification,18S rRNA, Sand fly, LSSP-PCR.

1. Introduction

Phlebotomine sand flies (Diptera: Psychodidae) are blood-sucking insects that play an important role in the transmission of bacteria (e.g., *Bartonella bacilliformis*), viruses (e.g., Phlebovirus, Vesiculovirus) and *Leishmania* spp. parasites (Birtles, 2001; Depaquit *et al.,* 2010). So far about 800 sand fly species have been recorded. However, majority of the species play no role in the transmission of leishmaniasis in nature, and less than 10% of sand flies has been incriminated as vector species of leishmaniasis (Munstermann, 2004; Bates, 2007; Kato *et al.,* 2010). There are 20 different species of sand flies found in Saudi Arabia. *Phlebotomus* and *Sergentomyia* represent the two major genera of the sand fly population (Doha and Samy, 2010). There are several reports available on the distribution of sandflies in Saudi Arabia (Ibrahim and Abdoon, 2005; El-Badry *et al.,* 2008, 2009; Doha, 2009; Doha and Samy, 2010; Alahmed *et al.,* 2010). The highest numbers of sand flies were distributed in Abha, Jazan and Najran regions in the southwestern part of Saudi Arabia, while the lowest distribution was found in Al- Jouf and Tabouk regions (Alahmed *et al.,* 2010). Surveillance of prevalent sand fly species in endemic areas are important for prediction of the risk of transmission and expansion of the disease (Bates, 2007).

Sand fly species were identified principally based on morphological characteristics using internal structures such as spermatheca, cibarium, and pharynx in females and terminal genitalia in males (Munstermann, 2004). The morphological classification requires considerable skills and taxonomic expertise. Furthermore, it is difficult in most cases where the intraspecific variation and sibling species frequently complicate classifications based on morphological criteria (Aransay *et al.,* 1999; Testa *et al.,* 2002; Torgerson *et al.,* 2003; Beati *et al.,* 2004; Kato *et al.,* 2005; Barroso *et al.,* 2007). Therefore, molecular techniques with several genetic markers have been used to study population analysis within species and identification of sand fly species (Testa *et al.,* 2002; Torgerson *et al.,* 2003; Kato *et al.,* 2005; 2007; 2008; Depaquit *et al.,* 2008; Terayama *et al.,* 2008; Kuwahara *et al.,* 2009). These techniques include Random Amplified Polymorphic DNA-PCR (RAPD-PCR) (Mukhopadhyay *et al.,* 2000; Balbino *et al.,* 2006; Rocha *et al.,* 2007; Hamarsheh *et al.,* 2007; Al-Ajmi *et al.,* 2013), PCR- Restriction Fragment Length Polymorphism (PCR-RFLP) (Aransay *et al.,* 1999; Barroso *et al.,* 2007; Terayama *et al.,* 2008; Latrofa *et al.,* 2011; Alam *et al.,* 2012; Minter *et al.,* 2013; Hughes *et al.,* 2014; Bounamous *et al.,* 2014), and DNA sequencing of PCR-amplified segments for identifying sand flies (Di Muccio *et al.,* 2000; Barón *et al.,* 2008; Depaquit *et al.,* 2008; Dantas-Torres *et al.,* 2010; Zapata *et al.,* 2012; Al-Ajmi *et al.,* 2015a). Nevertheless, each of these techniques requires the use of additional reagents and equipment and thus adds to the complexity of the molecular taxonomy of sand flies.

LSSP-PCR (low-stringency single specific primer PCR) is a rapid and simple technique that involves a simple repetition of the PCR process with one of the two specific primers used in the first amplifications (Pena *et al.,* 1994). It detects sequence variations in DNA fragments by amplification under very low-stringency conditions

with a single primer specific for one of the extremities of the template. The end result of the LSSP-PCR analysis is a complex, heterogeneous, and sequence dependent pattern of amplified products that is called a 'gene signature profile' (Pena *et al.*, 1994).

LSSP-PCR technique has been broadly used for the detection of mutations in human genetic diseases (Pena *et al.*, 1994), sequence variations in human mitochondrial DNA (Barreto *et al.*, 1996; Marino *et al.*, 1996) and for genetic typing of infectious agents such as papilloma virus (Villa *et al.*, 1995). It has also been extensively used to genetically characterize populations of *Leptospira* species (Oliveira *et al.*, 1994; 1995; 2003), *Trypanosoma cruzi* (Vago *et al.*,1996; Mejía-Jaramillo *et al.*, 2011; Segatto *et al.*, 2013), *Trypanosoma rangeli* (Cuervo *et al.*, 2006; Marquez *et al.*, 2007) *Leishmania (Viannia) braziliensis* (Ferreira *et al.*, 2007; Baptista *et al.*, 2009; Oliveira *et al.*, 2010; 2013) *Leishmania infantum* (Alvarenga *et al.*, 2012).

The aim of this study was to show, for the first time, the applicability of LSSP-PCR technique based on 18S rRNA region to identify the genetic variability among and within phlebotomine sand fly species collected from Al-Madinah and Asir regions in Saudi Arabia.

2. Materials and Methods

2.1. Study area

Sand flies have been collected from different regions in western province (Al-Madinah Al- Munawarah) and southern western province (Asir) of Saudi Arabia.

Al-Madinah Al-Munawarah region is located at eastern part of Al Hijaz region in the Kingdom of Saudi Arabia. Its geographical coordinates are 24° 28' 7" North, 39° 36' 51" East. It has a typical desert climate, which is cold rainy in winter and hot dry in summer. Sand flies were collected from five villages on the outskirts of Al-Madinah: Mondasa, Agol, Almaliliah, Al-Yutamah and Abyar Al-Mashy.

Asir region is a mountainous area and lies in the south western part of Saudi Arabia. Its geographical coordinates are 18° 12' 59" North, 42° 30' 19" East. Depending on the geographical characteristics, it is divided into three distinct topographical zones: Sarawat Asir, Asir Plateau and Tihama lowlands. Sand flies were collected from six areas of Asir region: Al-Farsh, Al-Magarda, Al-Ajra, Al-Marsad, Al-Birk and Mohayel.

2.2. Sand flies collection

Sand flies were collected during the period of sand fly peak activity, May to July 2014, using sticky traps (A4 sheets of white papers soaked in castor oil) (Rioux *et al.*, 1967). Sticky traps were fixed at wall cracks and crevices in front of rodents'

burrows and at the bottom of large trees before sunset (18:00 pm) and collected in the following morning before sunrise (06:00 am), according to the methods described by Izri and Belazzoug (1993). Flies collected were removed from sticky traps with a needle, rinsed in water, placed in a labeling vial containing 70% ethanol, and kept in refrigerator for further analysis.

2.3. Dissection, mounting and morphological identification of sand flies

Each sand fly was dissected by removing the head and last three abdominal segments that were cleared by 10% potassium hydroxide. The specimen was mounted on microscope slides in Puri's medium (Smart *et al.*, 1965). These slides were used for morphological identification based on external and internal morphology using several taxonomic keys (Lewis and Büttiker 1980; Lewis 1982; Büttiker and Lewis 1983; Lane 1986; Young and Duncan, 1994). The remainder of the body (Thorax, abdomen, legs and wings) was stored in 96% ethanol at -20˚C for molecular identification (Martine-Sánchez *et al.*, 2000; Parvizi *et al.*, 2003).

2.4. DNA extraction

For DNA extraction, individual sand flies were homogenized in a sterile 1.5 ml Eppendorf tubes within lysis buffer using sampler tip of 1.5 ml homogenization pestles. The homogenates were lysed in animal tissue lysis buffer (Qiagen) containing proteinase K. DNA was extracted from the homogenate using the Qiagen DNA mini kit (DNeasy tissue kit, Qiagen, California, USA) according to the manufacturer instructions. The extracted DNA was stored at −20˚C until use for further analysis.

2.5. Polymerase Chain Reaction (PCR) amplification

For amplification of the 18S rRNA gene, PCR was performed with two specific primers, SandF1:5′-AGGCTCATTCAGTCGCTTTC-3′ and SandR1:5′-TGCAAGCTTATGACTCACAC-TT-3′ (Macrogene, Korea) (Al-Ajmi *et al.*, 2015b), that amplified approximately 700 base pair (bp) fragment of the 18S gene.

PCR was carried out in a volume of 50µl that contain: 10µl of 5X PCR buffer, 0.5µl of (5U/µl) Taq DNA polymerase (Promega, USA), 2µl of (10mM) deoxynucleoside triphosphate (dNTPs), 5µl of (25 mM) $MgCl_2$, 0.3µl of (100 pmol/µl) of each primer and 5µl of template genomic DNA (50 ng/ µl). A negative control containing all reactants but no DNA was always included. Amplification was performed in a T100 thermocycler (Bio Rad, UK). After an initial denaturation step at 95˚C for 5 min, the specific PCR program consisted of 40 amplification cycles of denaturation at 95˚C for 1min, annealing at 53˚C for 1min, extension at 72˚C for 2 min, followed by a final extension at 72˚C for 10 min. The amplification products were analyzed by electrophoresis on 2% agarose gel with TAE (Tris-acetic acidEDTA) buffer at 100 volts for 1 hour, detected by staining with ethidium bromide (Sigma) and visualized under ultraviolet (UV) light, to confirm the presence of an

~700 bp product. The fragment sizes were determined by the Gene Ruler TM 100bp DNA Ladder (Thermo Scientific) which was added in each agarose gel. The PCR products were stored at −20°C for further analysis.

2.6. Low-Stringency Single Specific Primer- PCR (LSSP-PCR) analysis

LSSP-PCRs were performed by amplifying products obtained from the PCR step with the SandR1 primer following the protocol described by Pena *et al.* (1994) with slight modifications. The reactions were carried out in a final volume of 20µl which contain: 4µl of 5X PCR buffer, 0.6µl of (5U/µl) Go Taq DNA polymerase (Promega, USA), 0.5 µl of (10mM) dNTPS, 1.2µl of (25 mM) $MgCl_2$, 0.7µl of (100 pmol/ µl) SandR1 primer and 2µl of PCR product. Amplification was performed in a T100 PCR thermocycler (Bio Rad, UK) as follows: an initial denaturation step at 95°C for 5 min, followed by 40 amplification cycles of denaturation at 95°C for 1min, annealing at 34°C for 1min, extension at 72°C for 1min, followed by a final extension at 72°C for 10 min. The products were analyzed on 2% agarose gels (High Resolution, Sigma) at 50V for 4h, visualized with ethidium bromide under UV light. The fragment sizes were determined by using the Gene RulerTM 50bp DNA Ladder (Thermo Scientific).

2.7. Data Analysis

LSSP-PCR reactions were performed in duplicate, and only the consistent bands were taken into account to build a profile of each sand fly population. The multiband profiles obtained by LSSP-PCR were scored visually, and each band was scored as present (1) or absent (0).

2.7.1. Phylogenetic analysis

The similarity coefficients for all possible pairs of strains based on the fingerprint groups were estimated according to the Dice method (Dice, 1945). Dendograms were constructed from the similarity coefficient data using the unweighted pair group method with arithmetic average (UPGMA) clustering (Sneath and Sokal, 1973). To evaluate the robustness of the groupings formed, the binary data set was subjected to 1000 bootstrapping value, using the Past 3.04 software program (Hammer *et al.*, 2001).

2.7.2. Population genetic analysis

Genetic variation within and among species was analyzed using POPGENE software, version 1.32 (Yeh *et al.*, 1997).

Molecular diversity within each species was assessed by calculating the percentage of polymorphic fragments (P%), the number of observed alleles (na) and the effective number of alleles (ne). Gene diversity (h) and the Shannon diversity index (I) were estimated according to Nei (1973); Shannon and Weaver (1949),

respectively. Genetic diversity within each species was calculated for each locus and then averaged over all loci and populations. Also, estimates of genetic differentiation between species were assessed. Genetic diversities in subpopulations (Hs) and in the total population (Ht) were calculated according to Nei (1978). The extent of differentiation between subpopulations, called the coefficient of gene differentiation (Gst) (Nei, 1973), was also calculated. Corresponding estimates of gene flow (Nm), i.e., the average effective number of migrants exchanged between populations in each generation, were calculated as Nm= 0.5 (1-Gst)/Gst according to McDermott and McDonald (1993). Based on pair-wise Nei's standard distances (Nei, 1978), the results were subjected to UPGMA clustering analysis to assess the genetic distances between species.

3. Results

A total number of 483 sandflies (236 females and 247 males) were collected from two geographically different areas in Saudi Arabia viz., Al- Madinah Al-Munawarah (257) and Asir (226). Based on external and internal morphological characters by using several taxonomic keys (Lewis and Büttiker, 1980; Lewis, 1982; Büttiker and Lewis, 1983; Lane, 1986; Young and Duncan, 1994), sand flies were differentiated into seven species, three of them were belonging to genus *Phlebotomus* (*P. papatasi, P. sergenti* and *P. bergeroti*), and the remaining four were belonging to genus *Sergentomyia* (*S. clydei*, *S. antennata*, *S. fallax* and *S. schwetzi*). *P. papatasi* was the most abundant species in Al-Madinah where 250 (97.28%) specimens were identified. *S. clydei* was the prevalent species in Asir region where 150 (66.37%) specimens were recorded.

3.1. PCR amplification and LSSP-PCR analysis

To study the genetic variability of the collected insects, 18S rRNA region was amplified using specific primers, SandF1 and SandR1. The amplification products resulted in amplicons of the same size (~700bp) for all studied species (Fig. 1). Non-specific bands were not observed as well as no amplification products were obtained from negative control samples.

The seven morphologically identified sand flies species were subjected to LSSP- PCR analysis to evaluate their inter- and intra-specific and genetic diversity. The PCR products of 18S rRNA gene (700bp band) were used as templates for the LSSP-PCR. The analysis obtained with the primer SandR1 was more informative than SandF1. The reproducibility of the method was confirmed when identical electrophoresis profiles were observed in the assays on repeated analysis under the same conditions. The LSSP-PCR profiles were reproducible in the evaluated banding range of 70–1300bp and showed inter- and intra-specific and genetic variability among and within the seven analyzed species, with clear variable patterns for some

species. All analyzed species displayed some differences in amplicons that produced distinct genetic signatures among sand flies from different genomic species (Fig. 2).

3.2. Data analysis

3.2.1. Phylogenetic analysis

The main constitutive bands generated from LSSP-PCR analysis were used to investigate the phylogenetic relationships among species and individuals of sand fly species from Al-Madinah and Asir. The obtained data were employed to build phylogenetic tree (Fig.3). The genetic similarity matrix generated using LSSP analysis ranged from 0.58 to 1 with an average of 0.79. The results of the consensus tree indicated that the dendrogram separated sand fly species into four main clusters (A,B,C and D) at 75% similarity. Cluster (A) consisted of two species, *S. fallax* and *S. schwetzi* with 81% similarity. The samples of *S. clydei* were involved in the second cluster (B) at approximately 77.5% genetic similarity. The third cluster (C) was the largest one and included many sub-clusters at 76% similarity. It showed a considerable overlap between *P. papatasi*, *P. bergeroti* and *P. sergenti* species. In the fourth cluster (D), all *S. antennata* samples were clustering together and showed 89.5% genetic similarity

3.2.2. Population genetic analysis

The genetic analysis of each sand fly species under study, inferred from the LSSP profiles, is presented in Table (1). The number of polymorphic loci and their percentages varied among species. It ranged from 8.33% in *P. sergenti* and *S. fallax* to 83.33% in *P. papatasi* with an average of 32.14%. Similarly, the observed number of alleles (na) had similar trend where both *P. sergenti* and *S. fallax* had the least value, while *P. papatasi* had the highest value. The effective number of alleles (ne) was the highest in *S. clydei* and *P. papatasi*, and the lowest in *P. sergenti* and *S. fallax*. The Nei's gene diversity (h) ranged between (0.027) for *S. fallax* and (0.145) for *P. papatasi*. Similarly, Shannon's diversity index (I) ranged between (0.042) in *S. fallax* to (0.246) in *P. papatasi*.

Sand fly species showed significant differences in eleven LSSP polymorphic loci (91.67%) among all species examined (Table 2). The average number of observed alleles (na) was (1.917) and effective alleles (ne) was (1.403) in all sand fly species. The number of effective nuclear alleles was slightly lower than the total number of alleles, showing that most alleles contributed to the variation. The genetic diversity (h) and Shannon's diversity index (I) across all populations were(0.246) and (0.385), respectively. These results revealed that the genetic diversity within species level was relatively low, while the total genetic diversity among the species level was high. For the two genera, *Phlebotomus* and *Sergentomyia*, the percentage of polymorphic loci (P%) was (91.67 %) and the observed number of alleles (na) was (1.917). On the other hand, the effective number of alleles (ne), the Nei's gene diversity (h), and

Shannon's diversity index (I) for *Sergentomyia* spp. were (1.319), (0.212) and (0.342), respectively, which were higher than that for *Phlebotomus* spp.

The overall genetic diversity (Ht), the average gene diversity within a species (Hs) and the coefficient of gene differentiation (Gst) of the studied species were calculated (Table 3). The average total genetic diversity (Ht) overall species was (0.250) which was higher than the genetic diversity within populations (Hs) (0.076). This means, not all the alleles were present in every population. The average proportion of genetic diversity stemming from the differences between species (Gst) was significant (0.694), indicating that most of the gene variability occurred between species. The corresponding average value of gene flow (Nm) between all the seven species analyzed was estimated with a value of (0.221). Additionally, the total genetic diversity (Ht), the average of gene diversity within a species (Hs) and the genetic differentiation (Gst) were calculated to investigate the genetic diversity between *Phlebotomus* spp. and *Sergentomyia* spp. The total diversity (Ht) for *Sergentomyia* (0.238) was higher than that for *Phlebotomus* (0.154), while the average of genetic diversity within a species of *Sergentomyia* (Hs) was (0.072) which lower than that for *Phlebotomus* (0.083). The coefficient of genetic differentiation (Gst) between populations of *Sergentomyia* was higher (0.670) than that for *Phlebotomus* (0.461), which revealed that *Sergentomyia* showed genetic diversity between species more than that for *Phlebotomus* species. This indicates that a high proportion (67%) of diversity is observed between species of *Sergentomyia*, while proportion of diversity between species of *Phlebotomus* (46%) was lower. The estimated gene flow over generations (Nm) was (0.586) and (0.215) for *Phlebotomus* and *Sergentomyia*, respectively.

Nei's genetic distances (D) and genetic identity between the studied sand fly species were investigated (Table 4). The closest distance was observed between *S. fallax* and *S. schwetzi* (0.0626) followed by (0.0756) between *P. papatasi* and *P. bergeroti*. The largest distances was detected between *P. papatasi* and *S.antennata* (0.5320). A cluster analysis based on Nei's standard distance matrix (Nei, 1978) reflects the genetic distances and shows the phylogenetic relationships among the studied sand fly species, based on the UPGMA method (Fig.4). On the basis of allele frequencies, the differences in genetic structures show a high degree of genetic divergence between *S. antennata* and all the other species of sand fly that are located in different clusters. The clade of the other species showed great similarities between *P. papatasi* and *P. bergeroti*.

4. Discussion

This study was conducted to collect and identify the sand fly species in two regions in Saudi Arabia. The major sand fly species detected were identical to those previously reported from Saudi Arabia. The predominant species of Al- Madinah region was *P. papatasi* (El-Badry *et al.*, 2008; El-Badry *et al.*, 2009; Alahmed *et al.*, 2010), while *S. clydei* was dominant in Asir region (Ibrahim and Abdoon, 2005; Doha, 2009; Doha and Samy, 2010; Alahmed *et al.*, 2010).

Morphological characteristics are the most reliable and practical method for identification and characterization of sand fly species. However, molecular methods are preferred due to their accuracy and reproducibility. Therefore, 700 bp of the 18S rRNA gene was targeted in this study. This amplicon was examined for each sand fly species where it showed no variability in terms of size and could not be used for identification purposes as found by Al-Ajmi *et al.* (2015b). In the present study, an attempt was carried out to evaluate the efficacy of LSSP-PCR for characterization and differentiation of the seven sand fly species collected from Al- Madinah and Asir regions and detailed information on intraspecific DNA polymorphisms was produced. Compared to other molecular technical approaches, LSSP-PCR is simple, reliable and a sensitive method for the molecular identification of sand fly species.

LSSP-PCR method uses a single primer that interbreeds with high specificity to its complementary sequence in the amplicons during the first round of PCR and also with low specificity to multiple sites within the amplified amplicon but in a sequence dependent manner during the second round. Thus, the reaction produces a large number of products that can be resolved by electrophoresis (Segatto *et al.*, 2013). Any variation in a single base can be distinguished by the LSSP-PCR assay (Pena *et al.*, 1994).

Earlier investigators used LSSP-PCR technique to analyze human mitochondrial DNA gene (Barreto *et al.*, 1996). Also, it has been used to molecular characterization of populations of many organisms including *Leptospira* species (Oliveira *et al.*, 1994; 1995; 2003), *Trypanosoma* (Vago *et al.*,1996; Cuervo *et al.*, 2006; Marquez *et al.*,2007; Mejía-Jaramillo *et al.*, 2011; Segatto *et al.*, 2013), and *Leishmania* (Ferreira *et al.*, 2007; Baptista *et al.*, 2009; Oliveira *et al.*, 2010; 2013; Alvarenga *et al.*, 2012). So far, LSSP-PCR technique has not been used previously for genetic characterization of insects. To the best of our knowledge, this is the first study to apply LSSP-PCR molecular technique to identify the sand fly species which reflect not only the large genetic diversity frequently observed between the species, but also the intraspecific variability between individuals.

A phylogenetic analysis of the seven sand fly species in this study was performed based on LSSP-PCR analysis of 18S rRNA gene, to observe the pattern of species diversification (Fig. 3). The phylogenetic tree agreed with the traditional morphological taxonomy of the phlebotomine sandflies collected from Al- Madinah

and Asir, Saudi Arabia, where all individuals of each species are grouped together. These data is consistent with those found in other studies used sequencing approach with ITS2 gene (Di Muccio *et al.*, 2000; Al-Ajmi *et al.*, 2015a) and with mitochondrial Cytb gene (Bounamous *et al.*, 2014).

Furthermore, the intra-population genetic diversity, within the seven sand fly species using LSSP-PCR analysis of the 18S rRNA gene displayed average diversity of (0.077) for Nei's gene diversity (h) and (0.122) for Shannon's index (I), and the mean proportion of polymorphic loci (P%) was 32.14%; While for Nei's gene diversity (h) and Shannon's diversity index (I) across all species was (0.246) and (0.385), respectively. These values showed that the genetic diversity among the species level was higher than that within the species. The coefficient of genetic differentiation between species (Gst) was significant (0.694), indicating that most of the gene variability (69%) occurred between species. These results are in agreement with a previous study on genetic differentiation of sandflies, using other marker (ITS2), which reported that the difference in ITS2 sequence between sand fly species was 25.1%, while the intra-specific variability ranged from 0.2% to 4.9% (Latrofa *et al.*, 2011).

Apparently, our molecular data showed low levels of gene flow for the studied sand fly species. The estimated gene flow (Nm) from Gst between all the seven species analyzed was (0.221). Nm value was < 1.0, which indicated that genetic flow between all species did not exist or was very low, implying that the high differentiation among species could have occurred through random genetic drift (Slatkin, 1985; 1987). Genetic drift causes random changes in allele frequencies and causes differentiation because random genetic changes occur differently in each subpopulation (Milgroom and Lipari, 1995). Gene flow (Nm) decreases with the increase of (Gst), as shown in the results because greater differentiation between populations corresponds to lower levels of gene flow (Freeland *et al.*, 2011). This restricted gene flow and high differentiation among species could result from the fact that the sand flies are generally considered to be poor fliers, not traveling far away from their breeding and resting sites (Killick-Kendrick, 1990).

On an intraspecific level, the results showed that *P. papatasi* had the highest value of genetic diversity, (0.145) for Nei's gene diversity (h) and (0.246) for Shannon's index (I) when compared to values of other species. The high level of variation among *P. papatasi* populations was reported by Hamarsheh *et al.* (2007) who detected significant genetic variation among *P. papatasi* from the West Bank. Similar results were also reported by Ghosh *et al.* (1999) and Hamarsheh *et al.* (2006), in which isoenzyme electrophoresis and microsatellite markers were used to detect polymorphisms in a group of *P. papatasi* sand flies from Egypt and Palestine. In contrast, the data revealed low levels of genetic diversity among *P. sergenti*, h= 0.034 and I= 0.050. This result disagreed with those obtained by the ITS2 region of *P. sergenti* individuals studied in different regions (Depaquit *et al.*, 2002; Barón *et al.*, 2008). The difference in the results could be attributed to different types of genetic

markers used and the difference in the habitats of the populations. The increase in the number of the samples (new populations, more specimens per population) could perhaps help to resolve this point.

Genetic diversity based on 18S rRNA signatures generated from LSSP data in the present work was highly significant among species belonging to the genus *Sergentomyia* which reached up to (Ht= 0.238) (24%). However, species belonging to the genus *Phlebotoumus* have lower genetic diversity (Ht= 0.154) (15%). Although the gene flow (Nm) between the species of *Phlebotomus* (0.586) was higher than that for *Sergentomyia* (0.215), these values of Nm was <1.0, indicating low levels of gene flow and limited genetic exchange among populations of each genus. This is in agreement with a previous finding which utilized the sequencing of ITS2 region in rRNA gene to study a genetic variation of sand fly species belonging to *Sergentomyia* and *Phlebotoumus* who reported that the genetic variability was highly significant among individuals in the genus *Sergentomyia* (Al-Ajmi *et al.*, 2015a).

Genetic distance analysis of the LSSP-PCR profiles showed a low genetic distance (7.6%) between *P. papatasi* and *P. bergeroti*, indicating a close relation between them and appear as sister species. Though *P. papatasi* and *P. bergeroti* appear to be closely related, *P. papatasi* has a distribution area much greater than that of *P. bergeroti*, probably because *P. papatasi* is a less thermophilic and low-land species than *P. bergeroti*. These results are concurrent with Depaquit *et al.* (2008) who reported that the difference in ITS2 sequence between *P. papatasi* and *P. bergeroti* to be 3 to 5.5% while the difference in mtND4 sequence between them was 4–5%.

In conclusion, we consider that the LSSP-PCR technique employed in the present work represents a simple and new molecular tool to perform genetic characterization of the phlebotomine species. Further studies using LSSP-PCR technique should be performed with specimens from more diverse geographical areas and with other genetic markers. This is necessary to obtain more detailed information on intraspecific DNA polymorphisms for different sand fly species. Due to the simplicity of its execution, its speed and its capacity to produce highly reproducible and informative genetic signatures, we recommend the use of LSSP-PCR for the identification of sand flies and as a valuable tool for the molecular and epidemiological study of phlebotomine species.

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References

Alahmed AM, Kheir SM and Al-Khereji MA (2010). Distribution of Sandflies (Diptera : Psychodidae) in Saudi Arabia. Res. Bult., No. (171), Food Sci. & Agric. Res. Center, King Saud Univ. 5 -23.

Al-Ajmi R, Al-Jaser M and Al-Qahtani A (2013). The application of random amplified polymorphic DNA for sand fly species identification in Saudi Arabia. Egypt Acad. J. Biolog. Sci. 5(1): 43-51.

Al-Ajmi RA, Ayaad TH, Al-Anazi M, Al-Ahdal MN and Al-Qahtani AA (2015a). Molecular identification of natural sand fly species populations inferred from ITS2 rRNA gene in Saudi Arabia. J. of Environ. Biol. 36: 627-631.

Al-Ajmi RA, Ayaad TH, Al-Enazi M and Al-Qahtani AA (2015b). Molecular and morphological identification of common sand fly species in Saudi Arabia regions inferred from partial sequence of 18S ribosomal RNA gene (accepted for publication). Pakis. J. Zool.

Alam MS, Kato H, Fukushige M, Wagatsuma Y and Itoh M (2012). Application of RFLP-PCR-Based identification for sand fly surveillance in an area endemic for Kala-Azar in Mymensingh, Bangladesh. J. Parasitol. Res. doi: 10.1155/2012/467821.

Alvarenga JSC, Ligeiro CM, Gontijo CMF, Cortes S, Campino L, Vago AR and Melo MN (2012). KDNA Genetic Signatures Obtained by LSSP-PCR Analysis of *Leishmania (Leishmania) infantum* Isolated from the New and the Old World. PLoS ONE. 7(8): e43363.

Aransay AM, Scoulica E, Chaniotis B and Tselentis Y (1999). Typing of sand flies from Greece and Cyprus by DNA polymorphism of 18S rRNA gene. Insect Mol. Biol. 8: 179–184.

Balbino VQ, Coutinho-Abreu IV, Sonoda IV, Melo MA, Andrade PP, Castro JAF, Rebelo JMM and Santos MSC (2006) Genetic structure of natural populations of the sand fly *Lutzomyia longipalpis* (Diptera: Psychodidae) from the Brazilian northeastern region. Acta. Trop. 98: 15-24.

Baptista C, Schubach AO, Madeira MF, Leal CA, Pires MQ, Oliveira FS, Conceicao-Silva F, Rosalino CM, Salgueiro MM and Pacheco RS (2009). *Leishmania (Viannia) braziliensis* genotypes identified in lesions of patients with atypical or typical manifestations of tegumentary leishmaniasis: evaluation by two molecular markers. Exp. Parasitol. 121: 317-322.

Barón S, Martín-Sánchez J, Gállego M, Morales-Yuste M, Boussaa S and Morillas-Márquez F (2008). Intraspecific variability (rDNA ITS and mtDNA Cyt b) of *Phlebotomus sergenti* in Spain and Morocco. Acta. Trop. 107: 259-267.

Barreto G, Vago AR, Ginther C, Simpson AJG and Pena SDJ (1996). Mitochondrial D-Loop "Signatures" Produced by Low-Stringency Single Specific Primer PCR Constitute a Simple Comparative Human Identity Test. Am. J. Hum. Gene. 58: 609–616.

Barroso PA, Marco JD, Kato H, Tarama R, Rueda P, Cajal SP, Basombrío MA, Korenaga M, Taranto NJ and Hashiguchi Y (2007). The identification of sand fly species, from an area of Argentina with endemic leishmaniasis, by the PCR-based analysis of the gene coding for 18S ribosomal RNA. Ann. Trop. Med. Parasitol. 101: 247–253.

Bates PA (2007). Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies. Int. J. Parasitol. 37(10): 1097–1106.

Beati L, Caceres AG, Lee JA and Munstermann LE (2004). Systematic relationships among *Lutzomyia* sand flies (Diptera: Psychodidae) of Peru and Colombia based on the analysis of 12S and 28S ribosomal DNA sequences. Int. J. Parasitol. 34: 225–234.

Birtles RJ (2001). Carrion's disease. In: Service, M.W. (Ed.), The Encyclopaedia of Arthropod Transmitted Infections. CABI Publishing, Wallingford, 104–106.

Bounamous A, Lehrter V, Hadj-Henni L, Delecolle J-C and Depaquit J (2014). Limits of a rapid identification of common Mediterranean sandflies using polymerase chain reaction-restriction fragment length polymorphism. Mem. Inst. Oswaldo Cruz, Rio de Janeiro. 109(4): 466-472.

Büttiker W and Lewis DJ (1983). Some ecological aspects of Saudi Arabian Phlebotominae sandflies (Diptera: Psychodidae). Fauna of Saudi Arabia, 5: 479-528.

Cuervo C, Lo´pez MC and Puerta C (2006). The *Trypanosoma rangeli* histone H2A gene sequence serves as a differential marker for KP1 strains. Infect. Genet. Evol. 6:, 401-409.

Dantas-Torres F, Latrofa MS and Otranto D (2010). Occurrence and genetic variability of *Phlebotomus papatasi* in an urban area of southern Italy. Parasit. Vectors. 3: 77.

Depaquit J, Ferte H, Leger N, Lefranc F, Alves-Pires C, Hanafi H, Maroli M, Morillas-Marquez F, Rioux JA, Svobodova M and Volf P (2002) ITS2 sequences heterogeneity in *phlebotomus sergenti* and *phlebotoums similis* (Diptera, psychodidae): Possible consequences in their ability to transmit *Leishmania tropica*. Int. J. Parasitol. 32: 1123-1131.

Depaquit J, Grandadam M, Fouque F, Andry PE and Peyrefitte C (2010). Arthropod-borne viruses transmitted by Phlebotomine sandflies in Europe: a review. Euro. Surveill. 15: 19507.

Depaquit J, Lienard E, Verzeaux-Griffon A, Ferté H, Bounamous A, Gantier JC, Hanafi HA, Jacobson RL, Maroli M, Moin-Vaziri V, Müller F, Ozbel Y, Svobodova M, Volf P and Léger N (2008). Molecular homogeneity in diverse geographical populations of *Phlebotomus papatasi* (Diptera, Psychodidae) inferred from ND4 mtDNA and ITS2 rDNA epidemiological consequences. Infect. Genet. Evol. 8: 159-170.

Di Muccio T, Marinucci M, Frusteri L, Maroli M, Pesson B and Gramiccia M (2000). Phylogenetic analysis of *Phlebotomus* species belonging to the subgenus *Larroussius* (Diptera, psychodidae) by ITS2 rDNA sequences. Insect Biochem. Mol. Biol. 30: 387-393.

Dice LR (1945). Measurement of the amount of ecologic association between species. Ecology. 26: 297-302.

Doha S (2009). Phlebotomine sand flies (Diptera : Psychodidae) in different localities of Al-Baha province , Saudi Arabia. Egypt Acad. J. biology. Sci. 1 (1): 31- 37.

Doha SA and Samy AM (2010). Bionomics of phlebotomine sand flies (Diptera : Psychodidae) in the province of Al-Baha, Saudi Arabia. Mem. Inst. Oswaldo. Cruz, Rio de Janeiro. 105(7): 850-856.

El-Badry A, Al-Juhani A, El-Kheir I and Al-Zubainy S (2009). Sandflies Distribution and Bionomics in Al- Madinah Al-Munawwarah Region, Western of Saudi Arabia. Parasitol. Res. (4)1: 1-11.

El-Badry A, Al-Juhani A, El-Kheir I and Al-Zubiany S (2008). Distribution of sand flies in El-Nekheil province, in Al-Madinah Al-Munawwarah region, western of Saudi Arabia. Parasitol. Res. 103: 151–156.

Ferreira GA, Soares FC, Vasconcellos SA, Rodrigues EH, Werkhäuser RP, Brito ME and Abath FG (2007). Discrimination of *Leishmania braziliensis* variants by kDNA signatures produced by LSSP-PCR. J. Parasitol. 93(3): 712-4.

Freeland JR, Petersen SD and Kirk H (2011). Molecular Ecology. 2nd edition. Chichester, UK: Wiley-Blackwell.

Ghosh KN, Mukhopadhyay H, Guzman H, Tesh R and Munstermann L (1999). Interspecific hybridization and genetic variability of *Phlebotomus* sand flies. Med. Vet. Entomol. 13:78-88.

Hamarsheh O, Presber W, Abdeen Z, Sawalha S, Al-lahem A and Schoenian G (2006). Isolation and characterization of microsatellite loci in the sand fly *Phlebotomus papatasi* (Diptera: Psychodidae). Mol. Ecol. Notes. 6: 826-828.

Hamarsheh O, Barghouthi S, Al-Jawabreh A, Zayed A, Azmi K, Amro A and Abdeen Z (2007). Genetic Variability of Sand Fly *Phlebotomus papatasi* Populations (Diptera: Psychodidae) Originating from the West Bank, Palestine. J. Entomol. 4(6): 425-434.

Hammer Ø, Harper DAT and Ryan PD (2001). PAST: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontol Electron. 4(1): 9pp.

Hughes GL, Samuels SK, Shaikh K and Rasgon JL (2014). Scientific Note Discrimination of the *Plasmodium mexicanum* vectors *Lutzomyia stewarti* and *Lutzomyia vexator* by a PCR-RFLP assay and *Wolbachia* infection. J. Vector Ecol. 39(1): 224–227.

Ibrahim AA and Abdoon AMO (2005). Distribution and Population Dynamics of *Phlebotomus* sandflies (Diptera: Psychodidae) in an endemic area of Cutaneous Leishmaniasis in Asir Region, Southwestern of Saudi Arabia. J. Entomol. 2 (1): 102-105.

Izri MA and Belazzoug S (1993). *Phlebotomus (Larroussius) perfiliewi* naturally infected with dermotropic *Leishmania infantum* at Tenes, Algeria. Trans. R. Soc. Trop. Med. Hyg. 87: 399.

Kato H, Uezato H, Katakura K, Calvopiña M, Marco JD, Barroso PA, Gomez EA, Mimori T, Korenaga M, Iwata H, Nonaka S and Hashiguchi Y (2005). Detection and identification of *Leishmania* species within naturally infected sand flies in the andean areas of Ecuador by a polymerase chain reaction. Am. J. Trop. Med. Hyg. 72: 87–93.

Kato H, Uezato H, Gomez EA, Terayama Y, Calvopiña M, Iwata H and Hashiguchi Y (2007). Establishment of a mass screening method of sand fly vectors for *Leishmania* infection by molecular biological methods. Am. J. Trop. Med. Hyg. 77: 324-329.

Kato H, Cáceres AG, Gomez EA, Mimori T, Uezato H, Marco JD, Barroso PA, Iwata H and Hashiguchi Y (2008). Molecular mass screening to incriminate sand fly vectors of Andean-type cutaneous leishmaniasis in Ecuador and Peru. Am. J. Trop. Med. Hyg. 79: 719-721.

Kato H, Gomez EA, Cáceres AG, Uezato H, Mimori T and Hashiguchi Y (2010). Molecular epidemiology for vector research on leishmaniasis. Int. J. Environ. Res. Public Health. 7(3): 814–826.

Killick-Kendrick R (1990). Phlebotomine vectors of the leishmaniasis: a review. Med. Vet. Entomol. 4: 1–24.

Kuwahara K, Kato H, Gomez EA, Uezato H, Mimori T, Yamamoto YI, Calvopiña M, Cáceres AG, Iwata H and Hashiguchi Y (2009). Genetic diversity of ribosomal RNA internal transcribed spacer sequences in *Lutzomyia* species from areas endemic for New World cutaneous leishmaniasis. Acta Trop. 112: 131-136.

Lane RP (1986). Recent advance in the systematics of phlebotomine sand flies. Insect Sci. Appl. 7: 225 -230.

Latrofa MS, Dantas-Torres F, Weigl S, Tarallo VD, Parisi A, Traversa D and Otranto D (2011). Multilocus molecular and phylogenetic analysis of phlebotomine sand flies (Diptera: Psychodidae) from Southern Italy. Acta Trop. 119: 91-98.

Lewis D and Büttiker W (1980). Insects of Saudi Arabia, Diptera Fam. Psychodidae, Subfam. Phlebotominae. Fauna of Saudi Arabia, 2: 252-285.

Lewis DJ (1982). A taxonomic review of the genus *Phlebotomus*. Bull Brit. Mus. Nat. Hist. Entomol. 45: 121-209.

Marino MA, Weaver KR, Tully LA and Girard JE (1996). Characterization of mitochondrial DNA using low- stringency single specific primer amplification analyzed by laser induced fluorescence – capillary. Electrophoresis, 17:1499-1504.

Marquez DS, Ramirez LE, Moreno J, Pedrosa AL and Lages-Silva E (2007). *Trypanosoma rangeli*: RAPD-PCR and LSSP-PCR analyses of isolates from southeast Brazil and Colombia and their relation with KPI minicircles. Exp. Parasitol. 117: 35-42.

Martín-Sánchez J, Gramiccia M, Pesson B and Morillas-Márquez F (2000). Genetic polymorphism in sympatric species of the genus *Phlebotomus*, with special reference to *Phlebotomus perniciosus* and *Phlebotomus longicuspis* (Diptera, Phlebotomidae). Parasite. 7: 247-254.

McDermott JM and McDonald BA (1993). Gene flow in plant pathosystems. Annu. Rev. Phytopathol. 31: 353-373.

Mejía-jaramillo AM, Fernández GJ, Palacio L and Triana-chávez O (2011). Gene expression study using real-time PCR identifies an NTR gene as a major marker of resistance to benznidazole in *Trypanosoma cruzi*. Parasit. Vectors. 4(1): 169.

Milgroom MG and Lipari SE (1995). Population Differentiation in the Chestnut Blight Fungus, *Cryphonectria parasitica*, In Eastern North America. Genetics. 85(2): 155-160.

Minter LM, Yu T, Florin DA, Nukmal N, Brown GC and Zhou X (2013). Molecular identification of sand flies (Diptera: Psychodidae) in eastern North America by using PCR-RFLP. J. Med. Entomol. 50: 920-924.

Mukhopadhyay J, Ghosh K and Braig HR (2000). Identification of cutaneous Leishmaniasis vectors, *Phlebotomus papatasi* and *P. duboscqi* using random amplified polymorphic DNA. Acta Trop. 76(3): 277-283.

Munstermann LE (2004). Phlebotomine sand flies, the Psychodidae. In Biology of Disease Vectors, 2nded.; Marquardt WC, Black WC, Freier, JE, Hagedorn HH, Hemingway J, Higgs S, James AA, Kondratieff B, Moore CG, Eds.; Elsevier: San Diego, CA, USA. 141-151.

Nei M (1973). Analysis of gene diversity in subdivided populations. Proc. Natl. Acad. Sci. USA. 70: 3321-3323.

Nei M (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics. 89: 283-290.

Oliveira FS, Valete-Rosalino CM, Schubach AO and Pacheco RS (2010). kDNA minicircle signatures of *Leishmania (Viannia) braziliensis* in oral and nasal mucosa from mucosal leishmaniasis patients. Diagn. Microbiol. Infect. Dis. 66: 361-365.

Oliveira MAA, Hartskeerl R, Caballero OLSD and 8 other authors (1994). LSSP-PCR: a methodology for *Leptospira* species determination using gene fragments derived during PCR based diagnosis. In Annals of the VIIIth Meeting of European Leptospira Workers. 1: 53-55.

Oliveira MAA, Caballero OLSD, Dias Neto E, Koury MC, Romanha AJ, Carvalho J, Hartskeerl RA and Simpson AJG (1995). Use of nondenaturing silver-stained polyacrylamide gel analysis of polymerase chain reaction amplification products for the differential diagnosis of *Leptospira interrogans* infection. Diagn. Microbiol. Infect. Dis. 22: 343-348.

Oliveira MAA Caballero L, Vago AR, Assunc MA, Harskeerl RA, Romanha AJ, Simpson AJG and Koury MC (2003). Low-stringency single specific primer PCR for identification of *Leptospira*. J. Med. Microbiol. 52: 127-135.

Oliveira GM, Madeira MDF, Oliveira FS, Pires MQ and Pacheco S (2013). Canine Cutaneous Leishmaniasis : Dissemination and Tissue Tropism of Genetically Distinct *Leishmania (Viannia) braziliensis* Populations. Vet. Med. Int. Article ID 982183.

Parvizi P, Benlarbi M and Ready PD (2003). Mitochondrial and Wolbachia markers for the sandfly *Phlebotomus papatasi*: little population differentiation between peridomestic sites and gerbil burrows in Isfahan province, Iran. Med. Vet. Entomol. 17(4): 351-362.

Pena SDJ, Barretot G, Vagot AR, Marco LDE, Reinach FC, Netoii ED and Simpsonii AJG (1994). Sequence-specific "gene signatures," can be obtained by PCR with single specific primers at low stringency. Proc. Natl. Acad. Sci. USA, 91: 1946-1949.

Rioux JA, Golvan YJ, Croset H, Houin R, Juminer B, Bain O and Tour S (1967). Ecologie des leishmanioses dans le Sud de France. Echantillonnage. Ethologie. Ann. Parasitol. Hum. Comp. 42: 561-603.

Rocha LS Falqueto F, Santos C, Grimaldi JR and Cupolillo E (2007). Genetic Structure of *Lutzomyia (Nyssomyia) Intermedia* Populations from two Ecologic Regions in Brazil Where Transmission of *Leishmania (Viannia) Braziliensis* Reflects Distinct Eco-Epidemiologic Features. Am. J. Trop. Med. Hyg. 76(3): 559-565.

Segatto M, Rodrigues CM, Machado CR, Franco GR, Danilo S, Pena J and Macedo AM (2013). LSSP-PCR of *Trypanosoma cruzi* : how the single primer sequence affects the kDNA signature. BMC Res. Notes. 6:174.

Shannon CE and Weaver W (1949). The mathematical theory of communication. University of Illinois Press, Urbana, Illinois.

Slatkin M (1985). Gene flow in natural populations. Ann Rev Ecol. Syst. 16: 393-430.

Slatkin M (1987). Gene flow and the geographic structure of natural populations. Science. 236: 787-792.

Smart J, Jordan K and Whittick RJ (1965). Insect of medical importance, 4th ed., British Museum Natural History, Adlen Press Oxford, 286-288.

Sneath PHA and Sokal RR (1973). Numerical Taxonomy. San Francisco, CA, USA: WH Freeman and Co.

Terayama Y, Kato H, Gomez EA, Uezato H, Calvopi˜na M, Iwata H and Hashiguchi Y (2008). Molecular typing of sand fly species (Diptera, Psychodidae, Phlebotominae) from areas endemic for Leishmaniasis in Ecuador by PCR-RFLP of 18S ribosomal RNA gene. J. Vet. Med. Sci. 70: 907–913.

Testa JM, Montoya-Lerma J, Cadena H, Oviedo M and Ready PD (2002). Molecular identification of vectors of *Leishmania* in Colombia: mitochondrial introgression in the *Lutzomyia townsendi* series. Acta. Trop. 84: 205–218.

Torgerson DG, Lampo M, Velazquez Y and Woo PT (2003). Genetic relationships among some species groups within the genus *Lutzomyia* (Diptera: Psychodidae). Am. J. Trop. Med. Hyg. 69: 484–493.

Vago AR, Macedo AM, Oliveira RP, Andrade LO, Chiari E, Galvão LM, Reis D, Pereira ME, Simpson AJ, Tostes S and Pena SD (1996). Kinetoplast DNA signatures of *Trypanosoma cruzi* strains obtained directly from infected tissues. Am. J. Pathol. 149: 2153–2159.

Villa LL, Caballero OL, Levi JE, Pena SD and Simpson AJ (1995). An approach to human papillomavirus identification using low stringency single specific primer PCR. Mol. Cell. Probes. 9:45-48.

Yeh FC, Yang RC, Boyle TBJ, Ye ZH and Mao JX (1997). POPGEN Ver. 1.32. The user-friendly software for population genetic analysis. Molecular Biology and Bio-technology Center, University of Alberta, Alberta, Canada.

Young DG and Duncan MA (1994). Guide to the Identification and Geographic Distribution of *Lutzomyia* sand flies in Mexico, the West Indies, Central and South America (Diptera:Psychodidae), Mem. Am. Entomol. Inst. (Gainesv). 881 pp.

Zapata S, Mejı´a L, Le Pont F, Leo´n R, Pesson B, Ravel C, Bichaud L, Charrel R, Cruaud C, Trueba G and Depaquit J (2012). A study of a population of *Nyssomyia trapidoi* (Diptera: Psychodidae) caught on the Pacific coast of Ecuador. Parasit Vectors. 5: 144.

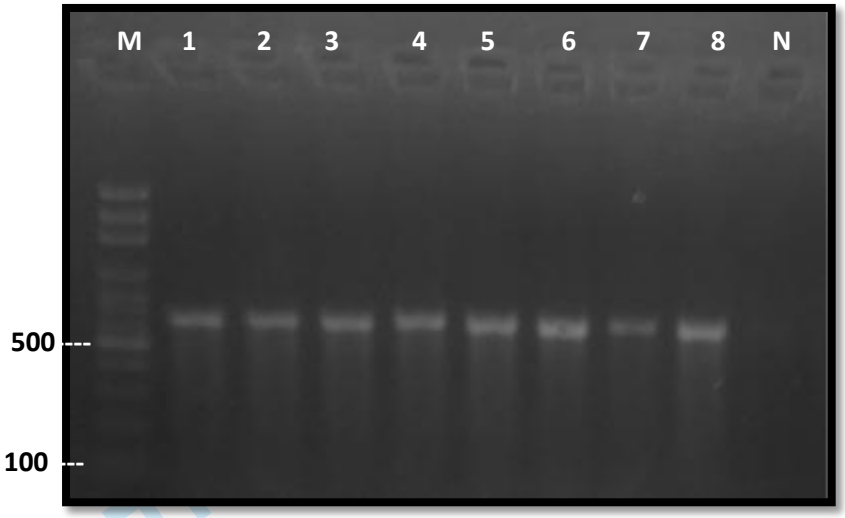

Fig. (1): Agarose gel electrophoresis of PCR amplification of 700bp fragment of 18S rRNA gene.M:100bp DNA marker, lanes 1-8: random samples of variable sandfly species, N: negative control.

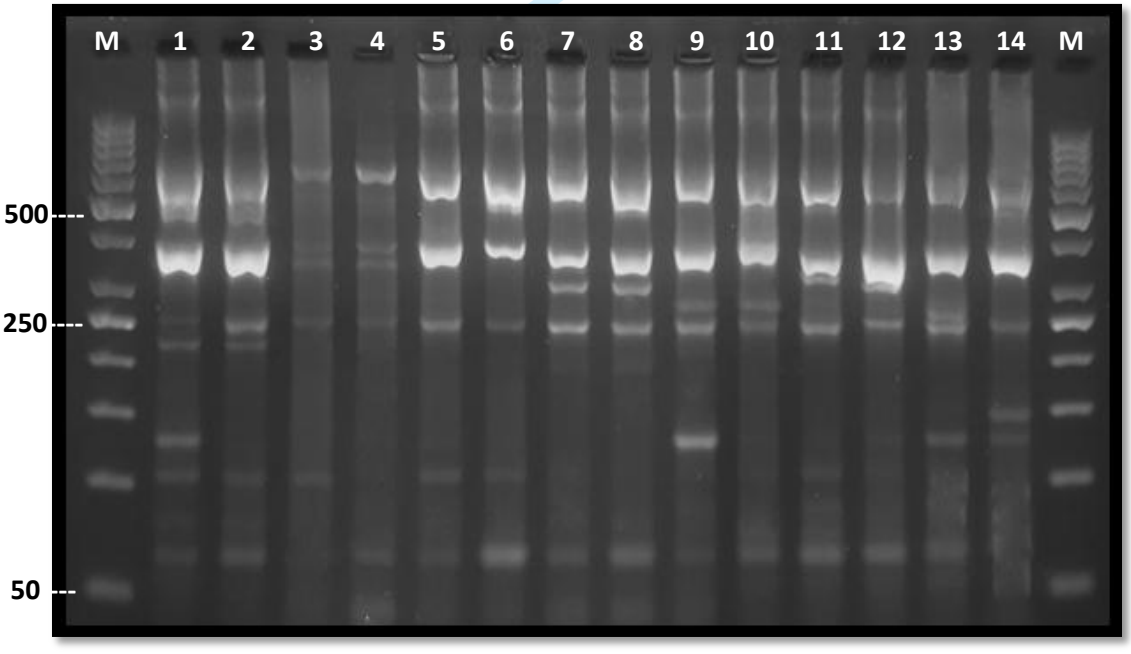

Fig.(2): LSSP-PCR profiles of 18S rRNA fragment of the seven studied species of sand fly. Five µl of the LSSP-PCR reaction products, were loaded in each lane of a 2% high resolution agarose gel and ethidium bromide-stained. M: DNA marker, lanes 1-2: *P. papatasi*, lanes 3-4: *P. sergenti*, lanes 5-6: *P. borgeroti*, lanes 7-8: *S. clydei*, lanes 9-10: *S. antennata*, lanes 11-12: *S. fallax*, and lanes 13-14: *S. schwetzi*.

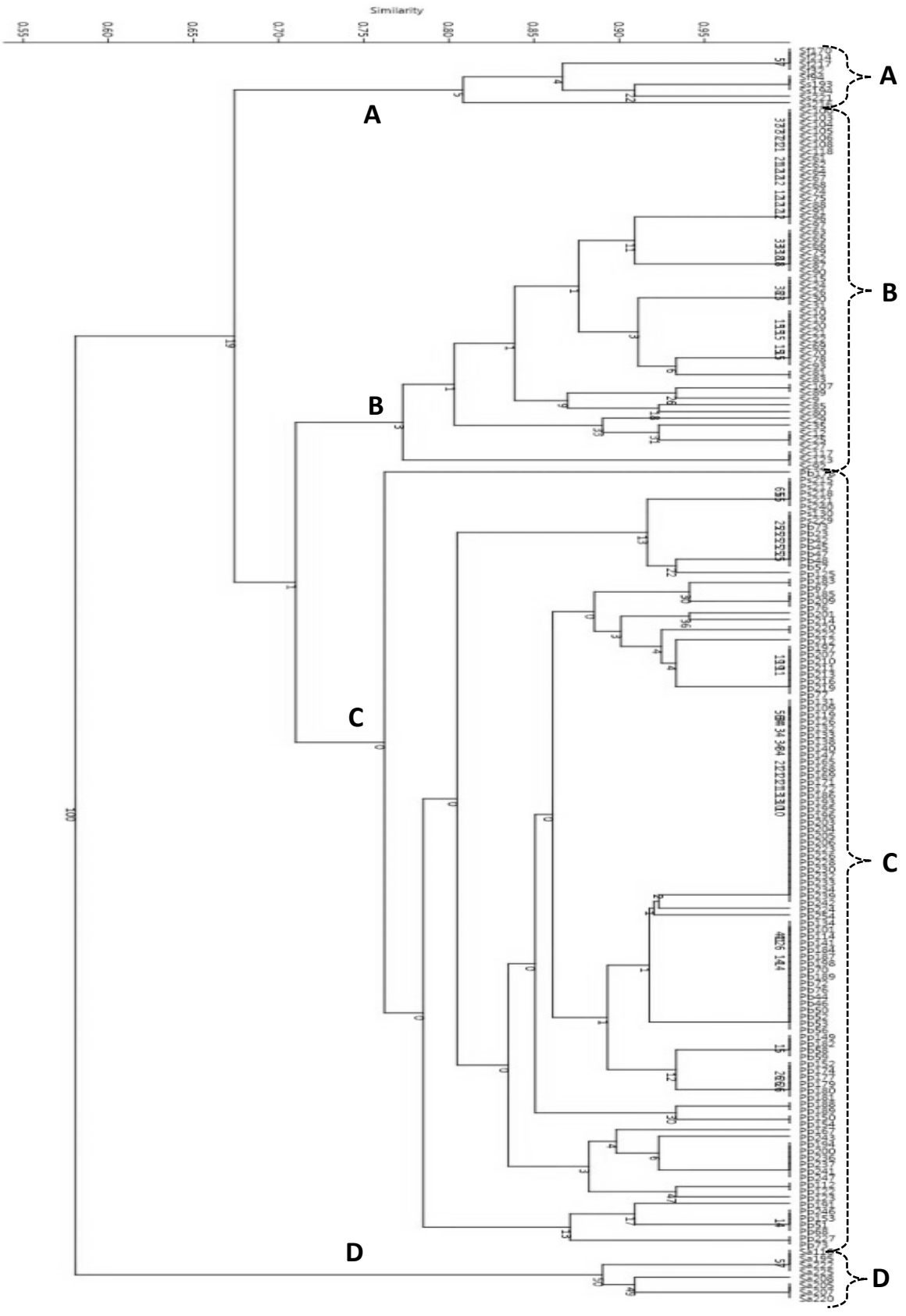

Fig.(3): Genetic similarity obtained by LSSP-PCR analysis of the 18S rRNAgene for 194 phlebotomine sand fly collected from Al- Madinah and Asir. A dendrogram was constructed using the unweighted pair group method with arithmetic average clustering (UPGMA). Values on branches represent the results of bootstrap analysis with 1000 iterations using the Past 3.04 software program (Hammer *et al.*, 2001).

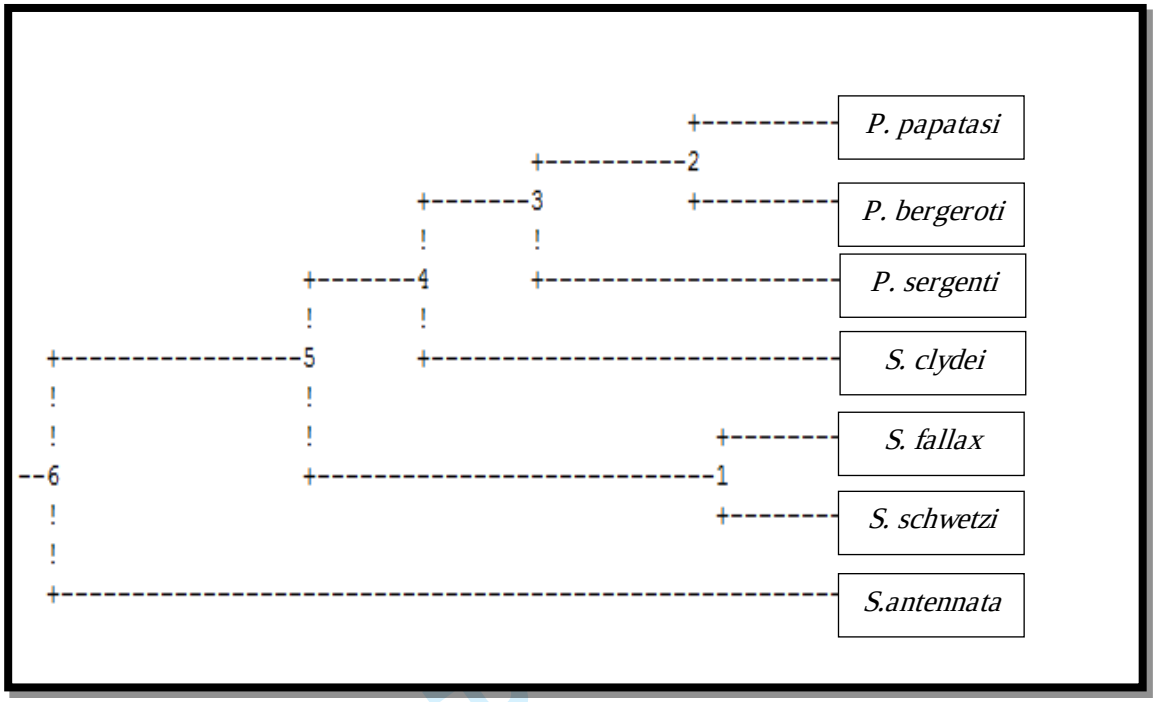

Fig. (4): UPGMA phenogram of the genetic relationships among seven sand fly species collected from Al-Madinah Al-Munawarah and Asir.

Table (1): Genetic analysis within species of sand fly collected from Saudi Arabia generated by the LSSP-PCR profiles.

Species	Genetic analysis (Mean±SD)					
	Npol[a]	P%[b]	na[c]	ne[d]	h[e]	I[f]
P. papatasi	10	83.33	1.833±0.389	1.208±0.238	0.145±0.148	0.246±0.215
P. sergenti	1	8.33	1.083±0.289	1.058±0.199	0.034±0.118	0.050±0.173
P. bergeroti	3	25.00	1.250±0.452	1.112±0.261	0.069±0.148	0.108±0.219
S. clydei	8	66.67	1.667±0.492	1.214±0.316	0.137±0.167	0.225±0.238
S.antennata	2	16.67	1.167±0.389	1.107±0.293	0.060±0.152	0.089±0.219
S. fallax	1	8.33	1.083±0.289	1.039±0.136	0.027±0.092	0.042±0.145
S. schwetzi	2	16.67	1.167±0.389	1.100±234	0.063±0.146	0.094±0.219
Mean	3.857	32.14	1.321±0.302	1.120±0.068	0.077±0.047	0.122±0.081

Mean± SD: Mean ± Standard Deviation
[a.]Npol= Number of polymorphic LSSP loci.
[b.]P%= Percentage of polymorphic fragments.
[c.]na = Observed number of alleles.
[d.] ne = Effective number of alleles.
[e.] h = Nei's gene diversity.
[f.]I = Shannon's diversity index.

Table (2): Genetic analysis among species of sand fly collected from Saudi Arabia generated by the LSSP profiles.

Species	Genetic analysis (Mean±SD)					
	Npol[a]	P%[b]	na[c]	ne[d]	h[e]	I[f]
Among all species	11	91.67	1.917±0.289	1.403±0.364	0.246±0.178	0.385±0.231
Phlebotomus spp.	11	91.67	1.917±0.289	1.236±0.273	0.161±0.150	0.272±0.211
Sergentomyia spp.	11	91.67	1.917±0.289	1.319±0.287	0.212±0.155	0.342±0.216

Mean± SD: Mean ± Standard Deviation
[a.]Npol= Number of polymorphic LSSP loci.
[b.]P%= Percentage of polymorphic fragments.
[c.]na = Observed number of alleles.
[d.] ne = Effective number of alleles.
[e.] h = Nei's gene diversity.
[f.]I = Shannon's diversity index.

Table (3): Nei's Analysis of genetic diversity for *Plebotomus* spp., *Sergentomyia* spp and overall species generated by the LSSP profiles.

Species	Ht[a]	Hs[b]	Gst[c]	Nm[d]
Overall species	0.250±0.027	0.076±0.003	0.694	0.221
Phlebotomus spp.	0.154±0.030	0.083±0.005	0.461	0.586
Sergentomyia spp.	0.238±0.039	0.072±0.005	0.670	0.215

[a.]Ht= Total genetic diversity overall species.
[b.] Hs= Genetic diversities within a species.
[c.]Gst= Coefficient of gene differentiation.
[d.] Nm= Estimate of gene flow from Gst.

Table (4): Nei's genetic identity (above diagonal) and genetic distance (below diagonal) in seven studied species (Nei, 1978).

Species	*P. papatasi*	*P. sergenti*	*P. bergeroti*	*S. clydei*	*S. antennata*	*S. fallax*	*S. schwetzi*
P. papatasi	-	0.8058	0.9272	0.7888	0.5875	0.7631	0.7261
P. sergenti	0.2160	-	0.9266	0.8632	0.8039	0.8200	0.7678
P. bergeroti	0.0756	0.0762	-	0.8114	0.7038	0.8186	0.7651
S. clydei	0.2372	0.1471	0.2090	-	0.6731	0.8000	0.7605
S. antennata	0.5320	0.2183	0.3513	0.3959	-	0.6623	0.7486
S. fallax	0.2703	0.1984	0.2001	0.2231	0.4120	-	0.9393
S. schwetzi	0.3201	0.2642	0.2678	0.2738	0.2896	0.0626	-