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Molecular and Morphological Identification of Local Sand Fly Species (Diptera: Psychodidae) in Saudi Arabia

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Abstract.- The identification of sand fly species is important for prediction of disease risks. A method of typing *Sergentomyia* and *Phlebotomus* taxa in three areas in Saudi Arabia was investigated by establishing a polymerase chain reaction (PCR) and direct partial sequence of 18S ribosomal RNA (rRNA) gene using specific designed primers. Morphological investigation of adults sand fly showed that Phlebotomine flies are identified into five species, three of them belong to genus *Phlebotomus* (*P. papatasi*, *P. bergeroti*, *P. sergenti*), and two belong to genus *Sergentomyia* (*S. antennata* and *S. clydei*). Several direct PCR – amplified sequences of each species analyzed using Maximum Composite Likelihood (MCL), Neighbor-Joining (NJ) and MEGA 5 methods revealed phylogenetic relationships of taxa. The obtained data of 18S subunits of rRNA gene showed moderate degree of interspecific variability between species of the same genus and among species of different genera. These results provide a powerful tool for further molecular retyping of *Phlebotomus* and *Sergentomyia* species in endemic areas of Saudi Arabia which may help planning for appropriate epidemiological surveillance programs that could be used to detect natural infections of sand fly vectors with pathogens, and hence, designing the appropriate control measure that limit spreading of such serious vectors.

Key Words: *Sergentomyia, Phlebotomus,* PCR, direct sequencing, **s**and fly, molecular and morphological characteristics, 18S rRNA, Saudi Arabia.

INTRODUCTION

Phlebotomine sand flies (Diptera: Psychodidae) are small–sized blood sucking insects feeding on a wide range of hosts and potentially acting as vectors of pathogens responsible for human and animal diseases worldwide. Out of over 800 sand fly species that have been described to date, approximately 10% are proven or suspected vectors of bacteria (*Bartonella bacilliformis*), viruses (*Phlebovirus*, *Vesiculvirus*) as well as *Leishmania* spp. protozoa, the causative agent of *leishmaniasis* (Yahia *et al*., 2004; Kato *et al*., 2008; Depaquit *et al*., 2009; Kato *et al*., 2010). It is suggested that only a restricted number of specific sand fly species can support the development and transmission of specific species of *Leishmania* (Munstermann, 2004; Kato *et al.,* 2007). *P. papatasi* is the most prevalent vector of *Leishmania major*, the causative agent of zoonotic *Cutaneous leishmaniasis* in North Africa and Middle East (Lane and Crosskey, 1993). It has been also implicated in the transmission of *Phlebovirus* viruses in Europe (Depaquit *et al*., 2009). The occurrence of sand fly species in a given region may represent a risk for *L. major* transmission or even the introduction of a non endemic parasite species from overseas into the Saudi Arabian regions provided that the reservoir host for such species is present in the area. Thus**,** identifications of both sand fly and *Leishmania* spp. are of great importance for predicting expansion of the disease in endemic areas, and also help in designing new strategic programs that limit spreading of such serious vectors (Kato *et al*., 2007; Fujita *et al*., 2012). Recently both *L. major* and *L. tropica* from *Phlebotomus papatasi* and *Phlebotomus sergenti*, respectively are identified using the semi-nested PCR method against kDNA and the Internal Transcribed Spacer 1 Polymerase Chain Reaction Restriction Fragment Length Polymorphism (ITS1-

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PCR-RFLP) in Al-madinah Al-munawarah province of Saudi Arabia (El-Beshbishy *et al*., 2013).

The most abundant phylogenetic analysis of morphological characters led to the conclusion that the Subfamily Phlebotominae comprises six genera: *Phlebotomus*, *Sergentomyia* and *Chinius* in the Old World and *Lutzomyia*, *Brumptomyia* and *Warileya* in the New World (Lane and Crosskey, 1993). Six subgenera of the genus *Phlebotomus* (*Phlebotomus*, *Paraphlebotomus*, *Synphlebotomus*, *Euphlebotomus*, *Adlerius*, *Larroussius*) and more than 90 species were identified depending on morphological criteria including terminal genitalia of males and internal structures of females, such as spermatheca and cibarium and also pharynx in the head region (Belkaid *et al*., 2000 ; Lango, 2005; Singh and Philips-Singh, 2010). This traditional method of identification requires refined storage conditions for samples, highly skilled techniques, taxonomic expertise, presence of intraspecific variability (Aslamkhan and Aslamkhan, 2000 ; Kuwahara *et al.,* 2009), time consuming (Mukhopadhyay *et al.,* 2000; Barroso *et al.,* 2007) as well as expert analysis of cryptic species (Bauzer *et al.,* 2007). Thus**,** the development of alternative molecular data sets have been introduced only recently as powerful tools for the identification of sand flies is an important issue. Modern techniques that are based on DNA identification of sand flies are generally not limited to specific developmental stages or to specific sex but also extend to intraspecific variability (Collins and Paskewitz, 1996).

Of these techniques, the ribosomal RNA (rRNA) gene architecture and the highly conserved sequences of certain domains of the gene have been used to reevaluate higher level relationships within the family Phlebotominae and within genera *Phlebotomus* and *Sergentomyia* (Depaquit *et al.,* 1988; Aransay *et al.,* 2000). The rRNA gene is a multi-copy gene of tandem repeated transcription units. Each transcription unit includes regions that produce three of the major rRNA subuints in insects (18S, 5.8S and 28S) after processing (Burton, 2008). The transcribed unit, which vary in copy number in different insect species, are highly similar to each other in a particular individual, although not necessarily identical.

The 18S rRNA subunit was used to identify and differentiate between several sand fly species based on RFLP-PCR. Additionally**,** studying the18S rRNA subunit sequence of sand fly species was investigated by Fujita *et al.* (2012) to differentiate between 13 sand fly species in Peruvian Andes. Also the sequence of 18S rRNA gene was used to identify different *Lutzomyia* species captured in different areas in Ecuador (Terayama *et al.*, 2008).

The current study was conducted to assess the appropriate genetic diversity among phlebotomine sand fly species collected from three different regions in Saudi Arabia including Riyadh, Al-Madinah Al-Munawarah and Assir provinces, and comparing their phylogenetic relationships with those available and registered in the GeneBank.

MATERIALS AND METHODS

Sand flies collection and identification

Sand fly adults of both female and male (n=250) were collected during the year 2012 from field localities including Riyadh, Al-Madinah Al-Munawarah and Assir (Table I) the endemic areas of *Cutaneous leishmaniasis,* the most prevalent type of *leishmaniasis* in Saudi Arabia that is caused by *L. major* and *L. tropica.* Flies populations were analyzed and identified morphologically according to Killick-Kendrick *et al.* (1985) and Al-Dawood *et al.* (2004).

Immediately after sand fly collection using sticky and light traps, specimens were preserved in 70% ethanol. Each fly was dissected into two parts**;** the first part consists of the head and terminalia and used for morphological identification according to the morphological taxonomic keys of (Alves *et al.,* 2008; Singh and Philips-Singh, 2010). While the other body part was stored at -80˚C and used for the molecular identification.

DNA extraction

Individual ethanol-fixed specimens were homogenized and lysed using a DNA extraction kit (DNeasy tissue kit, Qiagen, California, USA) following manufacturer's instructions. Then 5µl portions of the DNA extracts were subjected to the PCR gene amplification.

PCR - gene amplification and sequencing

Several sequences from GeneBank were used

as references to design primers binding to highly conserved sequences within the 18S ribosomal RNA gene and flanking a short and highly variable region to discriminate between the five species for which sequence data were undertaken in this study**.** Primers
SandF1: 5′-AGGCTCATTCAGTCGCTTTC-3′and
SandR1: 5′-TGCAAGCTTATGACTCACACTT-3′
were designed to bind within highly conserved regions. A region of 540 bp was amplified, of which a central 200 bp portion was predicted to be diagnostic. Polymerase chain reactions (PCRs) were performed in a final volume of 50μl which includes 5μl PCR buffer, 7μl $MgCl_2$, 10μl Q solution, 1μl dNTPs, 0.5μl Taq polymerase, 1.25μl forward, 1.25μl reverse primers, 19μl distilled water and 5μl DNA. PCR conditions (PCR palm cycler R Corbett research, USA) were as follows: 40 cycles: denaturation at 94°C for 1min, annealing at 55°C for 1min, extension at 72°C for 1min and final extension at 72°C for 7min. Analysis of PCR products. Five micro liters of PCR products were analyzed using electrophoresis on 2% agarose/1X TAE buffer gels in the presence of 0.5 μg/ml ethidium bromide and visualized under UV transilluminator then submitted directly for sequencing with the same F1 and R1 primers using the sequencing kit, Big Dye terminator V3.1 cycle (cycle sequencing kit, Applera, Foster city, California, USA). Sequences were analyzed using ABI 3700 DNA analyzer (Applied Biosystem, Foster city, California, USA) (Aransay *et al*., 1999). Direct sequence editing and assembly were performed using ChromasPro. MEGA 5 program and Neighbor-Joining (NJ) methods with bootstrap values of 1000 replicates. The consensus sequences for the Saudi Arabian species of the genus *Phlebotomous* (*P. papatasi*, *P. bergeroti* and *P. sergenti*) and genus *Sergentomyia* (*S. antennata* and *S. clydei*) have been deposited in GeneBank, with accession numbers JQ920354, JQ929127, JQ929126, JQ929128 and JQ929125, respectively.

Data analysis

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-716.9640) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach (Kato *et al.,* 2008) and then selecting the topology with superior log likelihood value. There were a total of 384 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.,* 2011).

RESULTS

Morphological results

All specimens were identified morphologically according to the keys of Killick-Kendrick *et al.* (1985) and Al-Dawood *et al*. (2004) into 5 species belonging to two genera, *Phlebotomus* and *Sergentomyia*.

Genus Phlebotomus

Of the collected specimens 178 were identified belonging to genus *Phlebotomus*, where all individuals were characterized by having hairs with round sockets, unarmed cibarium and male style carrying four to five spines. *Phlebotomus* (*Phlebotomus*) *papatasi* was the most abundant species (52.8%) collected from all the studied areas. *Phlebotomus* (*Phlebotomus*) *bergeroti* constituted 14.4% of the total collected sand flies. Ten specimens were identified as *Phlebotomus* (*Paraphlebotomus*) *sergenti. Sergentomyia* (*Sergentomyia*) *antennata* represented about 8% of the total collected sand flies. *Sergentomyia* (*Sintonius*) *clydei* accounted for 20.8% of the total collected sand flies.

The PCR amplified products showed bands of variable molecular weights ranging from 480-550bp for each of the random chosen individuals (Fig. 1).

PCR- amplicons of several direct sequences of 18S subunit of rRNA for each species that are submitted in GeneBank under the accession numbers: JQ929125, JQ929126, JQ929127, JQ929128 and JQ920354 for *P. papatasi, P. Bergeroti*, *P. sergenti*, *S. antennata* and *S. clydei*, respectively.

Analysis of individual sequences belonging to *P. papatasi* species showed little differences in the 18S rRNA subunit among the randomly chosen twelve individual sequences and found to match to

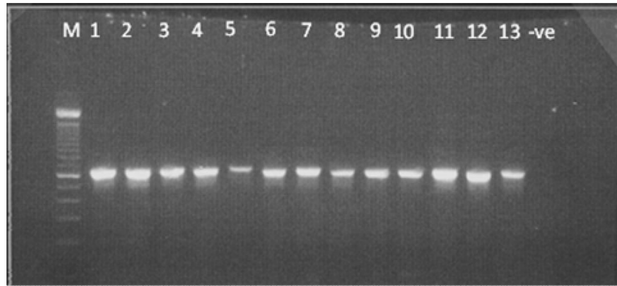

Fig. 1. Agarose gel electrophoresis of PCR products after amplification of 18S rRNA gene. Lane M: Molecular weight marker (100bp), Lanes 1 to 13 represent different random samples of variable sandfly species, last lane : negative control (PCR product without DNA template)

the GeneBank reference AJ244414.1 only in the region between nucleotides 1 to 20. However, significant variations were observed among individuals belonging to *P. sergenti* and *P. bergeroti*. Most of these variations were detected in the region of nucleotides from 1 to 45 compared to the reference sequence AJ244405.1 or those of nucleotides in the range from 304 to 465 as compared to the GeneBank reference *P. bergeroti.1* sequence. The calculated inter species variations of the genus *Phlebotomus* (*P. papatasi*, *P. bergeroti* and *P. sergenti*) ranged from 3.6 to 4.1%. However, these variations were significantly higher when species belonging to genus *Sergentomyia* (*S. clydei* and *S. antennata*) compared to each other, reaching up to 7.6%.

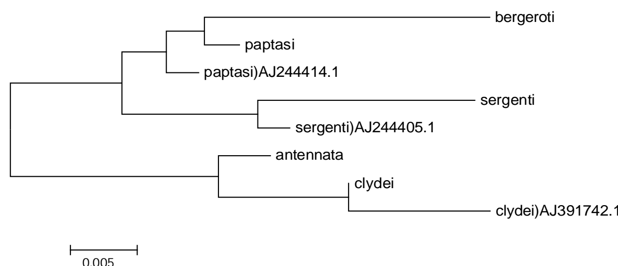

Fig. 2. Cladogram of different collected species based on Maximum Likelihood method. AJ244414.1, AJ244405.1, AJ391742.1 are reference sequences.

These sequences were used for construction of phylogentic trees using ChromasPro. MEGA 5 program and Neighbor-Joining (NJ) and Maximum Likelihood methods. Phylogenetic trees (Figs. 2, 3) revealed moderate degrees of interspecific variability between species of the same genus and among species of different genera.

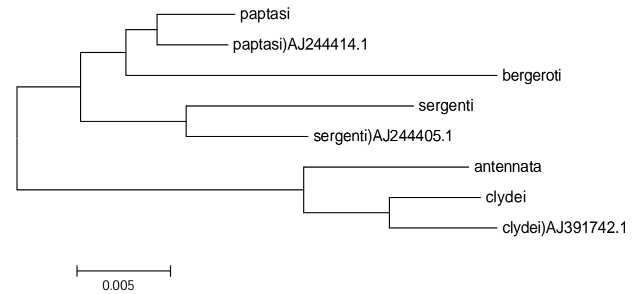

Fig. 3. Phenetic tree of different collected species based on Neighbor-Joining method. AJ244414.1, AJ244405.1, AJ391742.1 are reference sequences.

Since no reference gene sequence was available in the GenBank database, the partial sequence of 18S rRNA of a randomized individual from the present work was chosen and used as a reference sequence for the other eleven corresponding ones.

DISCUSSION

The morphological classification requires considerable skill as well as taxonomic expertise. In addition, the presence of intraspecific variation and cryptic species frequently complicates classifications based on morphological features (Bauzer *et al*., 2007). Sometimes damage caused by improper storage makes the identification more difficult. Molecular characteristic markers therefore, have been explored in the present investigation for the development of simpler and more accurate techniques to confirm and retype the morphologically identified sand flies.

The adult morphological features confirmed that the identified phlebotomine sand flies include *P. papatasi*, *P. bergeroti*, *P. sergenti*, *S. sergenti* and *S. clydei* species (Young and Duncan, 1994; Munstermann, 2004).

Genetic variability based on 18S rRNA in the present work was highly significant among

individuals belonging to the genus *Sergentomyia* (7.6%). However, insects belonging to the genus *Phlebotoumus* showed very little specific variability (3.6-4.1%). Individuals related to *P. sergenti* species showed more intra-individual differences than other species. Similarly 12S and 18S rRNA genes have been analyzed to study the phylogeny of some *Lutzomyia* species (Beati *et al*., 2004). Moreover, the discrepancies in the classification of some *Lutzomyia* species between generally accepted morphologic groupings and the phylogentic relationships of the sequence of 18S rRNA gene have been investigated by Terayama *et al.* (2008). It has also been previously proven that 18S rRNA gene of arthropods contains some of the slowest evolving sequences (conserved) in living organisms and therefore, used for examining ancient evolutionary phylogenetic status (Hill and Dixon, 1991; McClintock *et al*., 1991).

The complete sequence of rRNA genes of *Drosophila melanogaster* (Tautz *et al.,* 1988) and Phlebotomine sand flies (Aransay *et al.,* 2000) have also been investigated for interpretation of the phylogenetic relationships.

The genotyping method has been shown to be more accurate and easy-to-use for identification of sand fly species, requiring less expertise and less risk of different interpretations than the conventional morphology-based classification. Besides that damage to samples, which affects the morphologic classification in many cases, does not affect the genotyping analysis (Lane and Crosskey, 1993; Barroso *et al.,* 2007; Terayama *et al.,* 2008).

Additionally, the phylongenetic tree of different Phlebotomine species based on Neighbor-Joining (NJ) and Maximum Likelihood methods included in the present investigation (Figs. 2, 3) shows that each species is much related to the same species reported as reference species in the GenBank.

CONCLUSION

In conclusion, the sequence analysis of the 18S region of rRNA gene is a valuable tool in studying inter-species variability in organisms related to the genus *Sergentomyia.* However, it is not suggested in studying the intra-species variability among different sand fly species.

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