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Individual Variations in *Phlebotomus papatasi* Collected from Different Localities in Saudi Arabia

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ABSTRACT

A method of typing *Phlebotomus* taxa in three areas in Saudi Arabia (Riyadh, Madinah and Asir) using morphological characteristics revealed that the phlebotomine species is *Phlebotomus papatasi*. This identification was confirmed by establishing a polymerase chain reaction (PCR) and direct partial sequences of 18S ribosomal RNA (rRNA) gene using specific designed Primers SandF1:5′-AGGCTCATTCAGTCGCTTTC-3′ and SandR1:5′-TGCAAGCTTATGACTCACACTT-3′. Morphological individual variations were also observed among some collected specimens. Nevertheless, genetic analysis confirmed that these specimens were also *P. papatasi*. PCR-amplified amplicons using ChromasPro. MEGA 5 program and neighbor joining (NJ) methods revealed several direct sequences for *P. papatasi* that were submitted in GenBank under the accession number JQ929125. In conclusion, the obtained results establish a powerful tool for the molecular taxonomy of *Phlebotomus* spp. in endemic areas to plan appropriate epidemiological surveillance programs that could be used to detect natural infections of sand fly vectors with pathogens.

Keywords: Phlebotomine sand flies, PCR, 18S ribosomal RNA gene, direct sequences, morphological characters, molecular characters, taxonomy.

INTRODUCTION

Zoonotic cutaneous leishmaniasis is caused by the parasitic protozoan *Leishmania major*, which is transmitted by the phlebotomine sand fly *Phlebotomus papatasi* in North Africa and Middle East (Lane, 1993). Also *P. papatasi* has been implicated in the transmission of *L. arabica* in Saudi Arabia (Peters *et al*., 1986; Kllick-Kendrick, 1990), and of arboviruses in many countries (Javadian *et al*., 1977; Tesh *et al*., 1977). It is postulated that successful establishment of the disease in an endemic area is the outcome of a close association between the *Leishmania* parasite and its natural sand fly vector (Hamarsheh, 2011). Thus, identification of both sand fly and *Leishmania* is of great importance for predicting expansion of the disease in endemic areas, and also it helps in designing new strategic programs that limit spreading of such serious vectors (Kato *et al*., 2007; Fujita *et al*., 2012).

Sand fly identification based on morphological characters includes terminal genitalia of males and internal structures of females, such as spermatheca and cibarium and also pharynx in the head region (Lango, 2005; Singh and Philips-Singh, 2010). On the other hand, the development of alternative molecular data has been recently introduced as tools for the identification of sand flies. Among these techniques are the ribosomal RNA (rRNA) gene architecture and the highly conserved sequences of certain domains of the gene. These rRNA gene sequences have been used to reevaluate higher level relationships within the subfamily Phlebotominae and within the genera *Phlebotomus* and *Sergentomyia* (Aransay, 2000). The rRNA gene is a multicopy gene of tandem repeated transcription units. Each transcription unit

includes regions that after processing produce three of the major rRNA subuints; those in insects include 18S, 5.8S, and 28S subunits) (Burton, 2008).

Therefore, the present study aims at identifying the phlebotomine sand flies collected from three localities in Saudi Arabia (Riyadh, Madinah, and Asir) using morphological and molecular characters (direct sequence of 18S rRNA gene subunit).

MATERIALS AND METHODS

Sand Flies Collection and Identification

Sand fly adults of both female and male sexes (N = 250) were collected, using sticky and light traps, during the year 2012 from three field localities (Riyadh,Al-Madinah, and Asir) (Table 1 and Fig.1). These localities were chosen based on the fact that they are the endemic areas of cutaneous leishmaniasis in Saudi Arabia (Lewis and Buttiker, 1980; Killick-Kendrick *et al*., 1985). Populations were identified morphologically based on the previous morphological studies (Lewis and Buttiker, 1980; Killick-Kendrick *et al*., 1985; El-Sibae and Eesa, 1993; Al-Dawood *et al*., 2004). Immediately after sand fly collection, specimens were preserved in 70% ethanol. Each fly was dissected into many parts; the first part consists of the head and terminalia which was used for morphological identification according to the morphological taxonomic keys of (Lango, 2005; Carvalho and Jose, 2006; Alves *et al*., 2008; Singh and Philips-Singh, 2010). While the other body parts of each fly were stored at -80˚C for the molecular identification.

Fig. 1: Saudi Arabia political map showing Riyadh, Madinah and Asir regions (www. mapsofworld.com) (updated on, 16th May, 2012).

Table 1: *Phlebotomus papasi* prevalence in some Saudi Arabian localities during the year 2012.

Locality	Males	Females
Central region of Riyadh and its surrounding areas	30	2
Western region of Madinah and its surrounding areas	25	10
Southern region of Asir and its surrounding areas	58	7
Total	113	19

DNA Extraction

Individual ethanol-fixed specimens were homogenized and lysed in a DNA extraction kit (DNeasy tissue kit, Qiagen, California, USA) following manufactures instructions. Then, 5 µL portions of the DNA extracts were subjected to the polymerase chain reactions (PCRs) amplification.

PCR-Gene Amplification and Sequencing

The primers SandF1:5′-AGGCTCATTCAGTCGCTTTC-3′ and SandR1:5′-TGCAAGCTTATGACTCACACTT-3′ was 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. PCRs were performed in a final volume of 50 µL. The products were resolved on a 2% (w/v) agarose gel and visualized under UV transilluminator and 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). The consensus sequences for the Saudi Arabian *P. papatasi* have been deposited in GenBank, with accession number JQ929125.

RESULTS

Morphological Characteristics

Of the collected sand fly specimens, 132 specimens (52.8%) were identified to belong to the *Phlebotomus papatasi* (Table 1), where all individuals are characterized by having hairs with round sockets (Fig. 2). *P. papatasi* was the most abundant species and it was collected from the three studying areas. Males belonging to this species have long style with five spines, three terminal spines, and two lateral. The distance between the lateral spines is less than that between the lateral and terminal ones (Fig. 3a). Aedagus with round tip where the sperm duct pass through it (Fig. 3a). Paramere is trilobed, lateral lobes are shorter than the coxite and each of them bears two terminal spines (Fig. 3a). Female pharynx with narrow anterior and wide posterior ends, and armed with longitudinal ridges and several rows of teeth in the posterior part (Fig. 3b). Females are also characterized by having unarmed cibarium (Fig. 3c). Spermatheca is divided into ten segments. The terminal segment is the largest and the rest of the preceding ones become smaller that terminate with long segmented spermathecal duct (Fig. 3d).

However, 15 male specimens showed individual variations; (eight) specimens collected from (Al- Madinah) showed styles, each with six spines; three terminal, and three lateral spines (Fig. 4a). However, (seven) specimens colllected from (Asir) exhibited two lateral lobes with different numbers of terminal spines. The formula of these spines for the two lobes was 2 + 3 (Fig. 4b), 3 + 3 (Fig. 4c), and 2 + 1 (Fig. 4d), respectively.

Molecular Characteristics

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 in match with the GenBank reference AJ244414.1 in the region between nucleotides 1 to 20 only (Fig. 5). PCR-amplified amplicons using ChromasPro. MEGA 5 program and neighbor-joining (NJ) methods revealed direct sequences for *P. papatasi* that are submitted in GenBank under the accession number JQ929125.

DISCUSSION

The morphological features of sand fly adults such as the chaetotaxy on the style and lateral lobes of terminal genitalia in males, as well as the internal features such as the spermatheca, cibarium, and pharynx in females confirmed that the identified

phlebotomine sand fly species is *P. papatasi*. These characteristics are in agreement with the previous morphological identification of *P. papatasi* (Lewis and Buttiker, 1980; Killick-Kendrick *et al*., 1985; El-Sibae and Eesa, 1993; Al-Dawood *et al*., 2004). Nevertheless, damage caused by improper storage of specimens and mounting may make the process of identification difficult or can cause misidentification.

Therefore, molecular characteristics markers have been explored in the present investigation for the development of more accurate techniques to confirm the morphologically identified sand flies collected from different localities in Saudi Arabia. The results of genetic analysis in the present study confirmed the accepted classification based on morphological characteristics even with the presence of morphological individual variations.

However, discrepancies existing between the two tools of classification, suggesting the necessity for careful reconsideration of the sand fly taxonomy. Therefore, genotyping method was shown to be accurate and easy-to-use for the identification of sand fly species, requiring less expertise and less risk of different interpretations among researchers than the conventional morphology-based classification. It is important to note that this DNA-based technique does not require special storage conditions for the specimens, and different methods of preservation such as drying and the use of 70% ethanol or liquid nitrogen, which may affect the quality of the results. In addition, damage of samples, which should affect the morphologic classification in many cases, does not affect the genotyping analysis (Kato *et al*., 2007; Baroso *et al*., 2007; Terayama *et al*., 2008).

In conclusion, the obtained results establish a powerful tool for the molecular taxonomy of *Phlebotomus* spp. in endemic areas to plan appropriate epidemiological surveillance programs that could be used to detect natural infections of sand fly vectors with pathogens.

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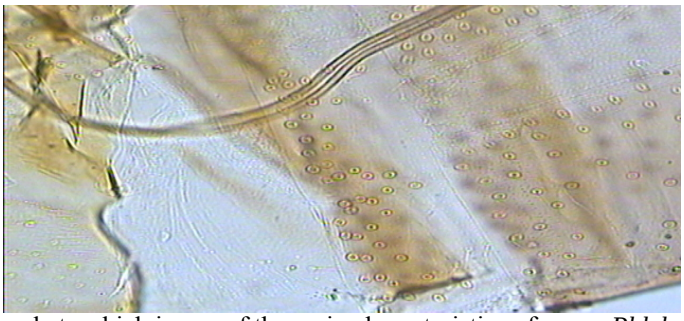

Fig. 2: Round hair sockets which is one of the main characteristics of genus *Phlebotomus* (40 ×).

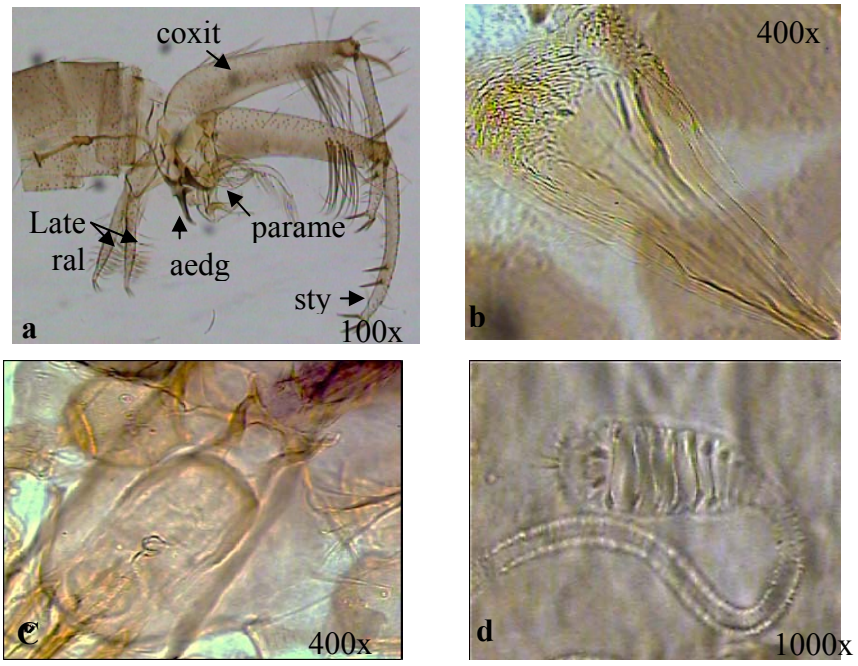

Fig. 3: Photomicrograph showing *P. paptasi* adult: (a) male terminalia; (b) female pharynx; (c) female cibarium; (d) female spermatheca.

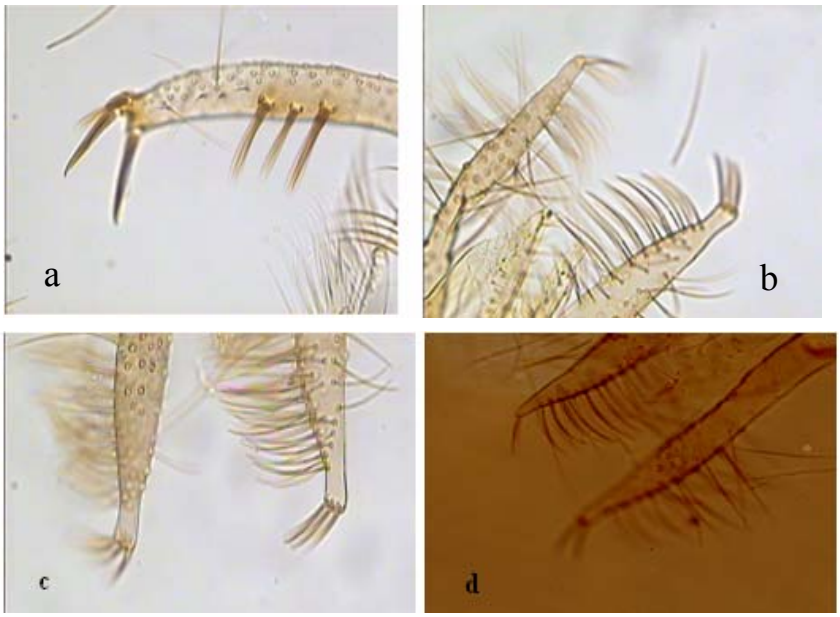

Fig. 4: Individual variations in *P. papatasi* males: (a) style with 6 spines, 3 terminal and 3 lateral (40 ×); (b) lateral lobes, one with two terminal spines and the other with three terminal spines (40 ×); (c) lateral lobes, each with three terminal spines (40 ×); (d) lateral lobes, one with two lateral spines and the other with only one lateral spine (40 ×).

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10        20        30        40        50        60
          ....|....|....|....|....|....|....|....|....|....|....|....|

AJ244414.1  AGCTTTTGGGCTCCGGGGGAAGTATGGTTGCAAAGCTGAAACTTAAAGGAATTGACGGAA
P.pap1      .ATCC.G.CA...A-C...G........................................
P.pap2      CAT...G.......--....G........................................
P.pap3      --A.......GA.TC....G.....................-..................
P.pap4      .AAG..G.......--....G........................................
P.pap5      ...G..........--....G........................................
P.pap6      G.............--....G........................................
P.pap7      CA.A..........--....G........................................
P.pap8      -AACG.G.......--....G........................................
P.pap9      -.AC..........-....G............-...........................
P.pap10     --GAC.........--....G........................................
P.pap11     -.ACG.........--....G.....................-..................
P.pap12     -CACG.........--....G........................................

                70        80        90       100       110       120
          ....|....|....|....|....|....|....|....|....|....|....|....|

AJ244414.1  GGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAAACTTACCAG
P.pap1      ............................................................
P.pap2      ............................................................
P.pap3      ............................................................
P.pap4      ............................................................
P.pap5      ............................................................
P.pap6      ............................................................
P.pap7      ............................................................
P.pap8      ............................................................
P.pap9      ............................................................
P.pap10     ............................................................
P.pap11     ............................................................
P.pap12     ............................................................

                130       140       150       160       170       180
          ....|....|....|....|....|....|....|....|....|....|....|....|

AJ244414.1  GTCCGAACATAAATGAGTAAGACAGATTGATAGCTCTTTCTCGAATCTATGGGTGGTGGT
P.pap1      ............................................................
P.pap2      ............................................................
P.pap3      ............................................................
P.pap4      ............................................................
P.pap5      ............................................................
P.pap6      ............................................................
P.pap7      ............................................................
P.pap8      ............................................................
P.pap9      ............................................................
P.pap10     ............................................................
P.pap11     ............................................................
P.pap12     ............................................................


                190       200       210       220       230       240

          ....|....|....|....|....|....|....|....|....|....|....|....|

AJ244414.1  GCATGGCCGTTCTTAGTTCGTGGAGTGATTTGTCTGGTTAATTCCGATAACGAACGAGAC
P.pap1      ............................................................
P.pap2      ............................................................
P.pap3      ............................................................
P.pap4      ............................................................
P.pap5      ............................................................
P.pap6      ............................................................
P.pap7      ............................................................
P.pap8      ............................................................
P.pap9      ............................................................
P.pap10     ............................................................
P.pap11     ............................................................
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P.pap12     ..................................................
               250       260       270       280       290       300

            ....|....|....|....|....|....|....|....|....|....|....|....|

AJ244414.1  TCAAACTTTTTTAAATAGATGCTTGCAGGACTATGACGCTGAAGCCATGTGTATGTTGGT
P.pap1      ..................................................
P.pap2      ..................................................
P.pap3      ..................................................
P.pap4      ..................................................
P.pap5      ..................................................
P.pap6      ..................................................
P.pap7      ..................................................
P.pap8      ..................................................
P.pap9      ..................................................
P.pap10     ..................................................
P.pap11     ..................................................
P.pap12     ..................................................

               310       320       330       340       350       360
            ....|....|....|....|....|....|....|....|....|....|....|....|

AJ244414.1  TGCTCTGTTTTGCTTTCGGGTTAAAATGGAGTGGTTTGATGTATATATGGTGGAGTCATA
P.pap1      ..................................................
P.pap2      ..................................................
P.pap3      ..................................................
P.pap4      ..................................................
P.pap5      ..................................................
P.pap6      ..................................................
P.pap7      ..................................................
P.pap8      ..................................................
P.pap9      ..................................................
P.pap10     ..................................................
P.pap11     ..................................................
P.pap12     ..................................................

               370       380       390       400       410       420
            ....|....|....|....|....|....|....|....|....|....|....|....|

AJ244414.1  CCTGTTTGGTTTGTCTTAAGAGGACACTTTGCTTCTTAAATGGACAAAATGCGTCTAGCA
P.pap1      ..................................................
P.pap2      ..................................................
P.pap3      ..................................................
P.pap4      ..................................................
P.pap5      ..................................................
P.pap6      ..................................................
P.pap7      ..................................................
P.pap8      ..................................................
P.pap9      ..................................................
P.pap10     ..................................................
P.pap11     ..................................................
P.pap12     ..................................................


               430       440       450       460       470       480
            ....|....|....|....|....|....|....|....|....|....|....|....|

AJ244414.1  TTAATGAGATTGAGCAATAACAGGTCTGTGATGCCCTTAGATGTCCTGGGCTGCACGCGC
P.pap1      ..................................................
P.pap2      ..................................................
P.pap3      ..........................................G.........S.C.
P.pap4      ..................................................
P.pap5      ..................................................
P.pap6      ..................................................
P.pap7      ..................................................
P.pap8      ..................................................
P.pap9      ..................................................
P.pap10     ..................................................
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P.pap11     ..................................................
P.pap12     ..................................................


                 490       500       510       520       530       540

             ....|....|....|....|....|....|....|....|....|....|....|....|

AJ244414.1  GCTACAATGAAGGTATCAATGTGTTATTATCCTTGACCGAGAGGTATGGGTAAACATTGA
P.pap1      ............................................................
P.pap2      ............................................................
P.pap3      C...........................................................
P.pap4      ............................................................
P.pap5      ............................................................
P.pap6      ............................................................
P.pap7      ............................................................
P.pap8      ............................................................
P.pap9      ............................................................
P.pap10     ............................................................
P.pap11     ............................................................
P.pap12     ............................................................

                550       560       570       580       590       600
            ....|....|....|....|....|....|....|....|....|....|....|....|

AJ244414.1  ACCACCTTCATGGTTGGGATTGTGGACTGTAACTGTTCACATGAACCCGGAATTCCCAGT
P.pap1      ............................................................
P.pap2      ............................................................
P.pap3      ...........................................................-..
P.pap4      ............................................................
P.pap5      ............................................................
P.pap6      ............................................................
P.pap7      ............................................................
P.pap8      ............................................................
P.pap9      ............................................................
P.pap10     ............................................................
P.pap11     ............................................................
P.pap12     ............................................................

           ....|...
AJ244414.1  AAGTGTGA
P.pap1      ........
P.pap2      ........
P.pap3      ........
P.pap4      ........
P.pap5      ........
P.pap6      ........
P.pap7      ........
P.pap8      ........
P.pap9      ........
P.pap10     ........
P.pap11     ........
P.pap12     ........
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Fig. 5: 18S rRNA sequences alignment of *P. papatasi* individuals. Identities are denoted by dots and gaps by dashes. AJ244414.1 is a reference sequence.

ARABIC SUMMARY

الاختلافات الفردية في ذبابة الرمل التى Phlebotomus papatasi تم جمعها من مواقع مختلفة في المملكة العربية السعودية

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أوضحت طريقة تقسيم أنواع جنس Phlebotomus المنتشرة في ثلاث مناطق بالمملكة العربية السعودية ممثلة بالرياض والمدينة وعسير باستخدام الخصائص المورفولوجية أن نوع Phlebotomus papatasi هو أكثر الانواع إنتشارا.

وقد أكد ذلك التصنيف باستخدام تفاعل البلمرة المتسلسل المباشر(PCR) لسلاسل جزئية من الجين المشفر للحمض النووي الرايبوزى الريبوزومي 18S rRNA وذلك من خلال إستخدام بوادئ مصممة متخصصة (S and F1:5′-AGGCTCATTCAGTCGCTTTC-3′ and S and R1:5′-TGCAAGCTTATGACTCACACTT-3′). هذا وقد لوحظ بعض الاختلافات الشكلية (المورفولوجية) الفردية بين بعض العينات التى تم تجميعها.

ومع ذلك أكد التحليل الجينى أن تلك العينات تنتمى الى نوع Phlebotomus papatasi . وقد كشفت الطرق التصنيفية الاحصائية والتقنية الحيوية المستخدمة [ChromasPro. MEGA 5 program and Neighbor-Joining (NJ)] تتابع جينى تسلسلى للجين المشفر للحمض النووى الرايبوزى الريبوزومى 18S rRNA والذى تم تسجيله فى الجين بنك برقم JQ929125.

وعليه فإن النتائج التي تم الحصول عليها تعد نواة لأداة قوية للتصنيف الجزيئي لنوع Phlebotomus تجدد الاهتمام فيما يتعلق بتأثير إدراج هذه الأنواع في المناطق الموبوءة على خطة المراقبة الوبائية والبرامج المناسبة التي يمكن استخدامها للكشف عن الإصابة بذباب الرمل الناقل للجراثيم المسببة للامراض الوبائية المختلفة .