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The application of random amplified polymorphic DNA for sandfly species identification in Saudi Arabia

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ABSTRACT

Sandflies are of great medical and economic importance as vectors of disease agents such as viruses, bacteria and protozoan parasites. Because of the great importance of these insects in the Kingdom, The present work has been undertaken to collect and identify samples from different regions of the Kingdom of Saudi Arabia, which included the Central Province (Riyadh and Qassim), the East Province (El Ehsa), the West Province (AlMadinah AlMunawarh) and South Province (Abha and Assir). Samples were divided into two parts: the first included the head and terminalia, which were used for morphological taxonomy, and the second part included the rest of the body which was used for molecular taxonomy. Standard keys of morphological taxonomy were used for the identification and classification of the sandflies. The collected sandflies were found to belong to five species and two genera. Of these, three species belonged to the genus *Phlebotomus,* these were *Phlebotomus* (*P.*) *papatasi*, *P. bergeroti* and *P. sergenti*. The other species belong to genus *Sergentomyia,* these were *Sergentomyia* (*S.*) *antennata*, *S. clydei*. *P. papatasi* was the most common species in all of the collection areas (56.37%), *S. clydei* was the second common (23.58%) and *S. antennata* was the third common species (8.4%) followed by *P. sergenti* (7.86%), then *P. bergeroti* (2.71%) . The second part of each fly, including the thorax, anterior part of the abdomen and wings, were used for DNA extraction. The DNA was amplified by the RAPD-PCR method using two different arbitrary primers, Opa-2 and Ap-16. Species-specific banding patterns were obtained by this method. Slight differences were observed in the banding pattern within the species which suggested that there were individual diversity or that these variations were owing to the presence of subspecies or sibling species in the same species.

Keywords: Sandflies- DNA- Saudi Arabia

INTRODUCTION

Sandflies (Diptera, Psychodidae) are among the most medical important insects since they transmit several species of pathogenic bacteria (Beati *et al*., 2004), viruses and protozoan parasites, the agents of both cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) (Robert & Janovy, 1996). CL is present in Bisha (Lewis and Buttiker,1980); Al-Kharj, Al-Ahsa (killick –Kindrick *et al*., 1985) ; Hail (Al-Zaharani *et al*.,1988b); Makkah (Lane & Fritz, 1986); Rfha, Najran, Taif, Asir and Jizan (Killick-Kindrick *et al*., 1985; Al-Zahrani *et al*., 1988b); Riyadh (Al-Dawood *et al*., 2004). *Leishmania major*, the causative agent of CL in Kingdom of Saudi Arabia (KSA) is transmitted by female of *Phlebotomous papatasi* (Abou El-Ela *et al*., 1995; Al-Dawood *et al*., 2004) while the other causative agent of CL, *Leishmania tropica*, is transmitted by the females of *Phlebotomus sergenti* (Al-Zaharani, 1988; El-Sibae and Eesa, 1993). VL, caused by *Leishmania*

donovani is suggested to be transmitted by females of *Phlebotomus bergeroti* (Al-Zaharani *et al*., 1988a) and has been reported only from the southwest part of KSA (Buttiker, 1979; Lewis and Buttiker, 1980).

Sandflies are classified into six genera: Phlebotomus, Sergentomyia and Chinius in the Old World and Lutzomyia, Warileya and Brumptomyia in the New World (Lewis *et al*., 1971; Lane and Crosskey, 1993). In Saudi Arabia, 21 species have been identified. Of these, eight species belong to the genus *Phlebotomus* and thirteen species to the genus *Sergentomyia* (Leiws and Buttiker, 1982; Al-Dawood *et al*., 2004).

External and internal morphological characters have been used to study sand fly in Saudi Arabia. However, this method is time-consuming (Mukhopadhyay *et al*., 2000) and requires well-experienced researchers for accurate characterization. In addition, individuals belonging to different species were found to be morphologically identical (Ward *et al*., 1981; Anez *et al*., 1997). Also differentiation between subspecies and sibling species are extremely difficult using morphological features (Williams *et al*., 1990; Black, 1993). Recently more accurate techniques have been developed in identifying sandflies based on molecular markers. For instance, Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) has been successfully used to differentiate between sandfly species (Hardys *et al*., 1992; Kernodle *et al*., 1993; Edelberto *et al*., 1998; Mukhopadhyay *et al*., 2000; Margonari *et al*., 2004a&b; Balbino *et al.,* 2006; Hamarsheh *et al*., 2007). RAPD-PCR utilizes small arbitrary DNA primer to amplify DNA fragments from nearly any DNA template. The resulting amplified DNA fragments are then analyzed by electrophoresis which results in a specific banding pattern that is similar and characteristic for individuals of the same species and differs from one species to another (Welsh and Mc-Celland, 1990; Martin-Sanchez *et al*., 2000). The aim of this study is to introduce the technique of RAPD-PCR to identify different species of sandflies collected from various localities of KSA.

MATERIALS AND METHODS

Sandflies of both sexes were collected during the years 2002-2003 from different regions of SA (Table 1). The collected sandflies were preserved in 70% ethanol (Lane and Crosskey, 1993; Torgerson *et al*., 2003).

Table 1: Showing regions, date, trap type and number of collected specimens

Region	Date of collection	Type of traps	Number of collected specimens		
			Female	Male	Total
1- Central province	2002/11/13	Sticky	9	40	49
- Riaydh		&			
- Al-Qssim	2003/6/21	light	25	53	78
2- Eastern province	24-3-2003	Sticky			
- Al-Hessa	1-4-2003		53	59	112
3- Western province	24-2-2002	Sticky			
- AlMadinah AlMonawarah			29	131	160
4- Southern province	2/5-2003	Sticky			
- Abha					
- Assir			111	141	252
- Bisha					
- Najran					

The head and terminalia of each sandfly were dissected and used for morphological identification, while the other body parts of unfed males and

females were used for molecular identification (Martine-Sanchez *et al*., 2000; Parivizi *et al*., 2003). Sandflies were identified morphologically according to the keys of Theodor (1948), Lewis (1973), Lewis and Buttiker (1980, 1986), Lane and Crosskey (1993) and El-Hossary (2001).

DNA extraction

DNA was extracted using the method of Sunnucks and Hales (1996). Individual specimen was homogenized in 1.5 eppendorf tube by adding 150 ml of TNES solution, 3ml of 20mg/ ml protinase K and incubated at 55 °C for one hour. Then adding 54µl of 5M NaCl vortex and centrifuged at 13000 rpm for 5 minutes. The supernatant was transferred into a sterile eppendorf tube containing 100% cold ethanol (v/v) and centrifuged for 5 minutes. The extracted DNA was washed and centrifuged with 250µl of 70% ethanol. The precipitated DNA was left for 15 minutes. 20µl of TE (Tris - EDTA) was then added.

RAPD-PCR

Two arbitrary primers, Opa-2 (5-TGCCGAGCTG-3) (Mokhopadhyay *et al*., 2000) and Ap-16 (5-CAGCACCCAC-3) (Sreenivas *et al*., 2004) were used in the present study with some modifications for optimizing the PCR conditions. Opa-2 primer was performed in the following buffer: 1xPCR buffer, 2.5 mM MgCl2, 0.4 mM dNTPs mix, 0.5 µM primer, 2U taq polymerase, 100 ng DNA template and double distilled water in the total reaction volume per tube (25 µl). The PCR machine was programmed for 44 cycles; each include a hot start at 95°C for 5min, denaturation at 94°C for 1min, annealing at 49° for 2 min, Extension at 72°C for 3 min and final extension at 72°C for 10 min.

Ap-16 primer was used following the procedure of Sreenivas *et al*. (2004) with some modifications. PCR was performed in the following buffer: 1x PCR buffer, 2.5 mM MgCl2, 0.4 mM dNTPs mix, 0.5 µM primer, 2U taq polymerase, 100 ng DNA template and double distilled. The total reaction volume per tube was 25µl. PCR was programmed as follows: hot start 94 °C for 5min, denaturation at 95°C for 2 min, annealing at 45°C for 2min, extension at 72°C for 2min and final extension at 72°C for 10min. The PCR Products were analyzed using 1% agarose gel electrophoresis and ethidium promide then visualized under UV light.

RESULTS

Based on morphological identification the collected sandflies were classified into five species: *P. papatasi*, *P. sergenti*, *P. bergeroti*, *S. antennata* and *S.clydei* (Table 2).

Table 2: Showing identified species.

Genus	Species	Percentages
Phlebotomus	*P. papatasi*	56.37%
	P. bergeroti	2.71%
	P. sergenti	7.86%
Sergentomyia	*S. antennata*	8.4%
	S. clydei	23.58%

Results using the Opa-2 primer

Agarose gel electrophoresis of *P.papatasi* species revealed five high intensity bands (Fig.1). The sizes of the bands were 470bp, 600bp, 700bp, 900bp and 1240bp as shown clearly in samples, number 2,7,8,9 and 12 with different intensities. However, individual variations and inter-sample differences, for example, the 1240 bp band was detected in samples number 7,8 and 10. In addition to the 1950 bp band that was detected in samples number 3, 8, 9, 11

and 12. Also a band of 1500 bp was added in samples number 6, 10 and 12.

P. sergenti

Two bands of 460 bp and 700 bp were detected in individuals of *P. sergenti* species with different molecular weight intensities. The 1000 bp band appeared only in samples number 5 and 7 (Fig.1).

P. bergeroti

Two bands of 350 bp and 600bp were present in all individuals of this species. But in sample number 5 the 600bp band was disappear and two different bands of length 700bp and 900bp were detected. However sample number 4 give different banding pattern compared to the other individuals (Fig.1).

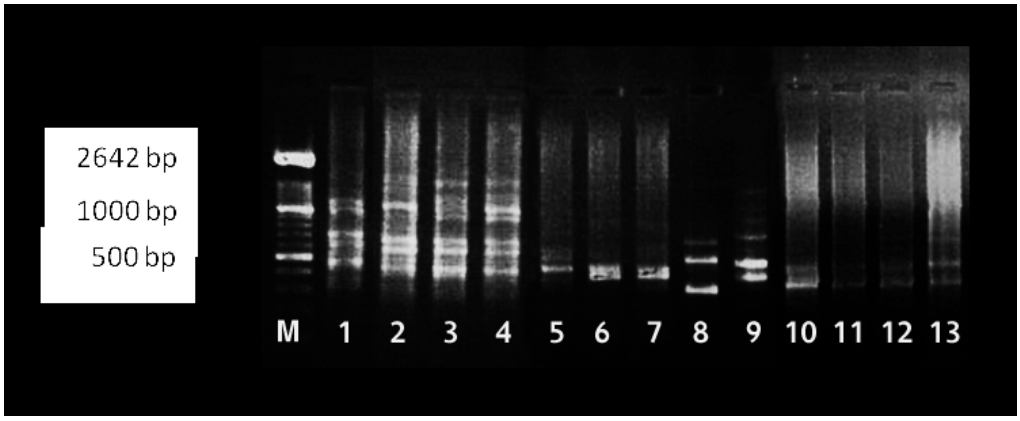

Fig.1: Agarose gel electrophoresis. M: marker; (1-4): *P. papatasi*; (5-9): *P. sergenti* ; (10-13): *P. bergeroti* using Opa-2 primer.

S. antennata

Individuals of this species showed five bands of variable intensities: 470;700;800;1000 and 1850 bp as detected in samples number 1, 2,3 and an extra band was detected with a length of 300bp (Fig. 2).

S. clydei

Three bands with different intensities and variable lengths (500bp, 700bp and 1200bp) were detected in samples number 4, 5and 7 belonging to this species (Fig. 2).

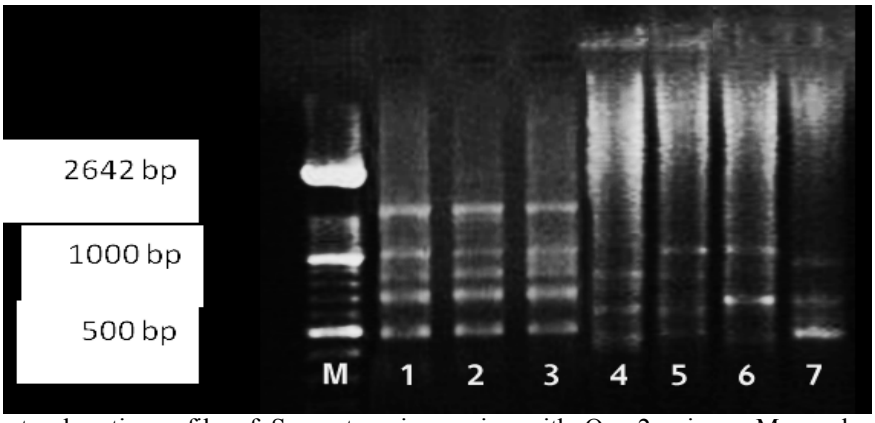

Fig. 2: Electrophoretic profile of Sergentomyia species with Opa-2 primer. M: marker; (1-3): *S. antennata*; (4-7): *S. clydei*.

Results using Ap-16 primer

P. papatasi

Individuals of this species showed three main bands of lengths 750 bp, 1600

bp and 1800 bp sample number 1,2 and 3 (Fig.3).

P. bergeroti

No bands were detected in samples belonging to this species.samples 4and 5 (Fig.3).

P. sergenti

Three main bands were detected 750, 1600 and 1800 bp as in samples number 1,2 and 3 of *P. papatasi*. In sample number 6 the band of length 470 bp was not clear, however, the band of 1200 bp was detected. In sample number 7 bands of 1600 bp and 1800bp with low intensities were detected (Fig.3).

S. clydei

Two main bands of 800 bp and 1870 bp were observed in samples belong to this species. Samples number 9, 10 and 11. However, two bands with low intensities and length of 1200and 2000 bp were also detected. Negative control is represented in sample 15 (Fig.3).

S. antennata

Individuals of this species showed two main bands of different intensities 470bp and 1000bps. However a low intensity band of length 900 bp was detected in all samples. Samples number 12 and showed a band of 470 bp. Another pale band of 600 bp was detected in samples number 13 and 14. Sample number 15 is a negative control (Fig.3).

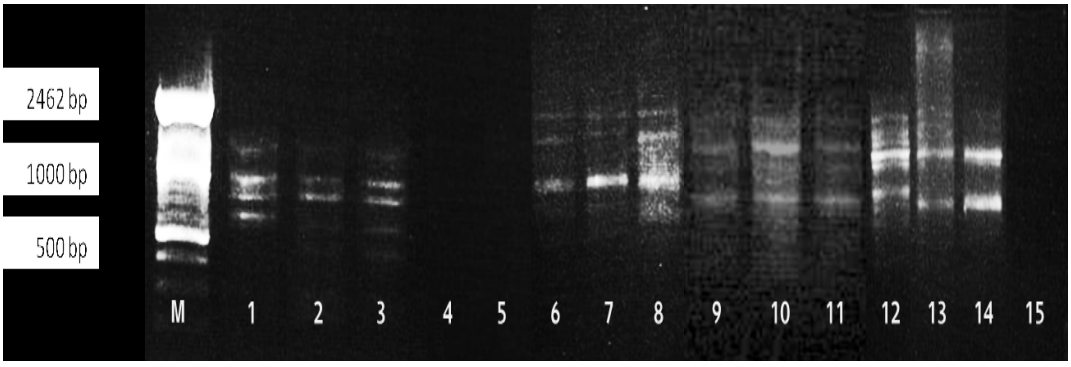

Fig. 3: Electrophoretic profile of Sandfly samples with Ap-16 primer .M: marker; (1-3): *P. papatasi*; (4,5): *P. bergeroti*; (6-8): *P. sergenti*; (9-11): *S. clydei*; (12-14): *S. antennata*; 15: negative control.

DISCUSSION

Sand fly is one of the most medically and economically important insect. Taxonomists classify in an approach to control its spreading. Sandflies were identified by different methods the most widely accepted method is by using external morphology and some internal organs. In this study we follow the classical morphological taxonomy and the advanced molecular method in identifying sandflies collected from different regions of Saudi Arabia. In the present investigation the most common species was *P. papatasi* and this agreed with Buttiker (1979), Lewis and Buttiker (1980, 1986), El-Sibae and Eesa (1993) and Al-Dawood *et al*. (2004). Coincided results are also detected in Egypt (El-Okbi *et al*., 1989) and in Portugal, Morocco and India (Lane & Fritz, 1986).

Recently, several modern techniques have been introduced to integrate with the classical taxonomical data. RAPD-PCR is one of these techniques which is independent of the previous DNA sequence information (Williams *et al*., 1990; Posso *et al*., 2003). It is an easy and fast way compared to other molecular techniques (Adamson *et al*., 1993; Black, 1993 and Abo El-Ela *et al*., 1995).

The RAPD-PCR involving the amplification of DNA via using PCR of random segment of genomic DNA by using a single primer of arbitrary nucleotide sequence (Hardys *et al*., 1992; Adamson *et al*., 1993; Kernodle *et al*., 1993; Wilkerson *et al*., 1995). This method was used by Adamson *et al*. (1993) to identify sand fly and produce a species-specific genetic marker to distinguish between two species of sandfly Lutzomyia (*L.young* and *L. spinicrass* in Venzulla. Also Edelberto *et al*. (1998) use the same method to differentiate between four sibling species of sand flies. Margonari *et al*. (2004a) used RAPD-PCR to study the genetic polymorphism between individuals of sand fly species *L. whitamni*. The same technique was used by Mukhopadhayay *et al*. (2000) in the differentiation between two sandfly species (*P. papatasi*) and *P. duboscqi.* RAPD-PCR technique has been used to study the genetic variability of sandfly *Phlebotomus papatasi* in west bank, Palastine (Hamarsheh *et al*., 2007). In the present investigation it was found that each species has a specific banding pattern which is characteristic to other species. The intensities of each band was also variable between different species and between individuals of the same species. These suggestive results are comparable to the result obtained by (William *et al*., 1990; Kernodle *et al*., 1993). Although each species had its own banding pattern, some bands of certain individuals are different compared to the other individuals of the same species. Also the intensity of the bands were variable within individuals of the same species that may indicate intraspecific variability (Williams *et al*., 1990; Adamson *et al*., 1993; Black, 1993; Kernodle *et al*., 1993). Individual variations of the same species may also predict subspecies or sibling species (Edlelberto *et al*., 1998). Collectively all the resulting banding patterns showed molecular weights varying form 470 bp to 1870 bp and this was agreed with Black (1993) who found that by using arbitrary primer the resulting bands are varying between 200bp and 2000bp. Moreover using two different arbitrary primers resulted in banding pattern variable in the same species with the different primer (Williams *et al*.,1990; Margonari *et al*., 2004b).

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ARABIC SUMMERY

تطبيق تقنية تضخيم عشوائي ل DNA متعددالأشكال لتحديد انواع ذبابة الرمل في المملكة العربية السعودية

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حشرات ذباب الرمل (الفواصد) ذات أهمية طبية واقتصادية نظرا لما تنقله من مسببات الأمراض الفيروسية و البكتيرية و الطفيلية. و لأهمية هذه الحشرة في نقل العديد من مسببات الأمراض في المملكة العربية السعودية تم تجيمع ذباب الرمل من عدة مناطق من المملكة العربية السعودية شملت المنطقة الوسطى (الرياض و القصيم) و المنطقة الشرقية (الإحساء) و المنطقة الغربية (المدينة المنورة و ضواحيها) و المنطقة الجنوبية (أبها و ضواحيها). وقد قسمت كل عينة من العينات المجموعة إلى جزأين، يتألف الجزء الأول من الرأس و نهاية الجسم لإستخدامه في التصنيف الشكلي (المورفولوجي). أما الجزء الثاني الذي يشمل باقي جسم الحشرة فقد إستخدم في التصنيف الجزيئي. وقد صنفت العينات شكليا بإستخدام مفاتيح تصنيفية عالمية، واعتمدت التفرقة بين الأنواع على الأشكال المميزة لكل من الأعضاء التناسلية كالقلم (stylus) والصفيحة الحرقفية (coxite) وعضو الإمساك (paramere) وعضو السفاد (aedeagus) ومضخة النطاف (genital pump) في الذكور وأشكال حافظة النطاف (spermatheca) وقناة النطاف (spermathecal duct) والقناة الجامعة (common spermathecal duct) في الإناث. وتبعا لذلك صنفت العينات المجموعة لخمسة أنواع تابعة لجنسين، ثلاثة أنواع منها تتبع جنس *Phlebotomus* وهي *P. papatasi* و*P.bergeroti sergenti* . *P*. أما الأنواع الأخرى فتبعت جنس *Sergentomyia* وهي *S.antennata* و *S.clydei* وقد كان النوع *P. papatasi* الأكثر انتشارا في جميع مناطق الجمع (بنسبة 56.37%) ثم تلاه النوع *S. clydei* (بنسبة 23.58%)، وجاء النوع *S. antennata* (بنسبة 8.4%) في المرتبة الثالثة ثم تلاه النوع *P. sergenti* (بنسبة 7.86%) ثم جاء النوع *P. bergeroti* في المرتبة الخامسة (بنسبة 2.71%) .وقد كان ذباب الرمل أكثر تنوعا في المنطقتان الوسطى و الجنوبية حيث جمع منهما الخمسة أنواع فمن المنطقة الوسطى جمعت الأنواع *P. papatasi* و *P. sergenti* و *S. antennata* و *S. clydei* .أما من المنطقة الجنوبية فجمعت الأنواع *P. papatasi* و *P. sergenti* و *P. bergeroti* و *S. antennata* و *S. clydei* . وجاءت المنطقة الشرقية في المرتبة الأخيرة حيث جمع منها النوعان *P. papatasi* و *P. bergeroti* فقط.

أما بالنسبة للدراسة الجزيئية فقد تم استخلاص الحامض النووي DNA من الصدر ومقدمة البطن والأجنحة، وتم تضخيم DNA باستخدام طريقة RAPD-PCR بواسطة بادئين عشوائيين (arbitrary primer) هما Opa-2 وAp-16. وقد أظهرت هذه الطريقة أن لكل نوع من أنواع ذباب الرمل نمط مميز من حزم الحامض النووي species-specific banding pattern خاص به مع وجود بعض الاختلافات الطفيفة في عينات النوع الواحد قد ترجع إلى اختلافات وراثية فردية أو إلى وجود تحت أنواع أو أخوة الانواع.