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Molecular typing of phlebotomine sand flies in al-madinah and asir regions, Saudi Arabia using PCR–RFLP of 18S ribosomal RNA gene

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Abstract Studies on the distribution of sand flies are important for the control of leishmaniasis in endemic and neighboring areas. In the present study polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) was used to identify the distribution of sand flies in Al-Madinah and Asir Regions of Saudi Arabia using PCR–RFLP of 18S ribosomal RNA gene. Based on the morphological characteristics, the sand flies were differentiated into seven species viz., *Phlebotomus papatasi*, *Phlebotomus sergenti*, *Phlebotomus bergeroti*, *Sergentomyia clydei*, *Sergentomyia antennata*, *Sergentomyia fallax* and *Sergentomyia schwetzi*. PCR–RFLP of 18S ribosomal RNA (rRNA) genes with eight different restriction enzymes resulted in species-specific agarose gel electrophoresis banding patterns. Of the eight restriction enzymes used, not a single restriction enzyme by itself could separate species belonging to the same genera (like *P. papatasi* and *P. sergenti* by *AseI*) as well as those belonging to different genera (like *P. papatasi* and *S. clydei* by *AseI*). We therefore conclude that the genetic diversity within sand fly species based on PCR–RFLP technique was nonspecific. Studies are in progress to study the viability of alternate techniques like low-stringency single specific primer polymerase chain reaction which can be used for molecular typing. © 2016 The Authors. Production and hosting by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

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1. Introduction

Leishmaniasis is caused by protozoan *Leishmania* parasites which are transmitted to mammals including human beings by the bite of infected phlebotomine sand flies (Reithinger and Dujardin, 2007). An estimated 1.3 million new cases and 20,000–30,000 deaths have been reported annually (World Health Organization, 2015). Leishmaniasis has been reported to be endemic in 98 countries and around 1.3 million new cases

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are reported every year (Alvar et al., 2012). It is spread through the bite of female phlebotomine sandflies by nearly 20 different species of *Leishmania* (Salam et al., 2014). The vectors of *Leishmania* in the Middle East are *Plebotomous papatasi* (*P. papatasi*) which cause zoonotic cutaneous leishmaniasis with rodent species *Psammomys obesus*, *Meriones libycus*, *Nesokia indica*, and *Rhombomys opimus* serving as nonhuman reservoirs. Zoonotic visceral leishmaniasis is caused by *Leishmania infantum* (*L. infantum*) spread through *Psammomys galilaeus*, *Psammomys syriacus*, *Psammomys tobbei*, *Psammomys shalensis*, and the dog species of *Canis familiaris* as nonhuman reservoirs (Salam et al., 2014). Anthroponotic cutaneous leishmaniasis (ACL) caused by *Leshmania tropica* and spread through *Phlebotomus sergenti* affects only humans. Anthroponotic visceral leishmaniasis (AVL) caused by *Leshmania donovani* spreads through *Psammomys alexandri* without any non human reservoir (Salam et al., 2014).

Cutaneous leishmaniasis (CL) is the most common form of the disease present in Saudi Arabia and is commonly known as okhet, dommal, nafra, and Elmohtafara (Salam et al., 2014). Cutaneous leishmaniasis is prevalent in the Al-Hasa Oasis (eastern province of Saudi Arabia) (Amin et al., 2012). *Leshmania major* has been reported to be widely distributed in Saudi Arabia and has been identified as the species causing zoonotic cutaneous leishmaniasis with *P. papatasi* as its vector and *Meriones spp.* and *P. obesus* as its reservoir hosts. *L. tropica* was identified from only two foci with *P. sergenti* as its vector (World Health Organization, 2015).

Traditional diagnostic procedures include microscopy, culture and immunological techniques. PCR-based assays currently constitute the main molecular diagnostic approach of researchers and health professionals (Reithinger and Dujardin, 2007). In the present study an attempt was made to use PCR-RFLP technique to study the distribution of sand flies in Saudi Arabia.

2. Materials and methods

2.1. Study area

Sand flies were collected from different regions in Western Province (Al-Madinah Al-Munawarah) and Southern Province (Asir) of Saudi Arabia. Al-Madinah Al-Munawarah region is located at the Eastern part of the Al Hijaz region in the Kingdom of Saudi Arabia. Its geographical coordinates are 24°28'7" North, 39°36'51" East. It lies 2050 feet (625 m) above sea level on a fertile oasis. It has a typical desert climate, which is cold rainy winter and hot dry summer. Sand flies were collected from five villages on the outskirts of the Al-Madinah Al-Munawarah: (Mondasa, Agol, Almalilah, Al-Yutamah and Abyar Al-Mashy). These areas are semi-cultivated inhabited deserts with many farms having sheep and poultry breeding houses which are irrigated by underground water. The desert surrounding the villages has small caves inhabited by the desert rodent (*P. obesus*) which is the main reservoir of cutaneous leishmaniasis.

Asir region lies in the southwestern part of the Kingdom of Saudi Arabia. Its geographical coordinates are 18°12'59" North, 42°30'19" East. The region is a mountainous area and is divided into 3 distinct topographical zones depending on the geographical characteristics: Sarawat Asir, Asir Plateau

and Tihama lowlands. Sand flies were collected from six areas of Asir region viz., Al-Farsh, Al-Magarda, Al-Ajra, Al-Marsad, Al-Birk and Mohayel).

2.2. Collection of sand flies

Sand flies were collected during the period of their peak activity, which is usually from May to July using sticky traps. Sticky papers of A4 size were smeared with castor oil (Rioux et al., 2013) and held vertically on wooden dowels to permit ground clearance and rotation with wind direction. They were fixed at a height of 20 cm above ground level in different habitats and biotopes (wall cracks and crevices in front of rodents' burrows and at the bottom of large trees). Traps were placed at the site of collection before sunset (18:00 h) and collected the following morning before sunrise (06:00 h) according to the methods described by Izri and Belazzoug (1993).

2.3. Morphological identification of sand flies

Each sand fly specimen was washed in sterile distilled water, dissected under a binocular microscope, by removing the head and last three abdominal segments with a pair of sterilized entomological needles and forceps. These parts of each fly were cleared by placing in 10% potassium hydroxide overnight. The specimens were washed three to four times in water and mounted on microscope slides in Puri's medium (Smart et al., 1965) with the head ventral-side up and the reminder of the specimen placed laterally under a single cover slip (Sawalha et al., 2003).

The slides of the head and terminalia were used for morphological identification using morphological characters such pharyngeal armature, spermathecal shape and number of segments, length of spermathecal neck, palpal and ascoids formula, using several taxonomic keys (Lewis and Büttiker, 1980; Lewis, 1982; Lane, 1986; Young and Duncan, 1994).

2.4. DNA extraction from individual flies

Ethanol-fixed individual sand flies were homogenized in DNA extraction 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 to be used for further analysis.

2.5. Polymerase chain reaction (PCR) amplification

18S rRNA gene from various phlebotomine sand flies was amplified by PCR. PCR was performed with primers Sand F1:5'-AGGCTCATTCAGTCGCTTTC-3' and Sand R1:5'-TGCAAGCTTATGACTCACACTT-3' (Macrogene, Korea) to produce amplicons of 750 base pair (bp) fragment of the 18S gene. PCR was carried out in a volume of 50 µl of PCR buffer (Promega, USA), Go taq® Flexi DNA polymerase (Promega, USA), Deoxynucleotide Triphosphates mixture (Thermo Scientific), MgCl₂, forward and reverse primer and template genomic DNA. After initial denaturation at 95 °C for 5 min, amplification was performed with 40 cycles consisting of denaturation at 95 °C for 1 min, annealing at 53 °C for

1 min, extension at 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. A negative control containing all reactants except DNA was included.

PCR products were analyzed by electrophoresis on 2% agarose gel with TAE (Tris-acetic acid-EDTA) 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 base pair (bp) product. The fragment sizes were determined by comparing with markers in the Gene Ruler TM 100 bp DNA Ladder (Thermo Scientific).

2.6. Restriction fragment length polymorphism (RFLP)

PCR products were digested with eight restriction endonucleases (New England BioLabs, Inc.), viz., *HaeIII*, *HinfI*, *RsaI*, *PstI*, *AseI*, *MnII*, *BbvI* and *BstNI*.

The RFLP assay was performed, according to the manufacturer's instructions with slight modifications. The reaction mix in a total volume of 20 µl contained 5 µl of the PCR product, 5 units of the restriction enzyme, 2 µl of 10× NE buffer and sterile distilled water. Samples were digested for 15 min at 37 °C. The digested PCR products were separated by electrophoresis on 2–3% agarose gelatin gel at 80 V for 2 h to produce a DNA fragment pattern which was detected by staining with ethidium bromide (Sigma) and visualized under ultraviolet light. Band sizes were determined and compared with markers in the Gene Ruler TM 50 bp DNA Ladder.

3. Results

3.1. Classification of sand flies based on morphology

A total 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 the morphological criteria, they were differentiated into seven species, three of them are belonging to genus *Phlebotomus* (*P. papatasi*, *P. sergenti* and *P. bergeroti*), and the remaining four are belonging to genus *Sergentomyia* (*S. clydei*, *S. antennata*, *S. fallax* and *S. schwetzi*). The identities of the species were confirmed using external and internal morphological characters of the head and abdominal terminalia and other taxonomic characteristics. *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. The only species of *Phlebotomus* genus collected from Asir was *P. bergeroti*.

3.2. Genetic variability of sand fly 18S rRNA gene

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 (~700 bp) for all studied species (Fig. 1). No non-specific bands were observed and no amplification was obtained for the negative control samples.

3.3. PCR-RFLP of 18S rRNA gene of sand fly species

In order to establish a genetic basis for the precise taxonomic identity of phlebotomine sand fly species, analysis of the 18S rRNA gene was carried out using PCR-RFLP assay.

The digestion of 18S rRNA gene PCR products with various restriction enzymes (*HaeIII*, *BstNI*, *HinfI*, *RsaI*, *PstI*, *AseI*, *BbvI* and *MnII*) produced DNA fragments of different sizes (Table 1).

HaeIII and *BstNI* digested 18S rRNA gene PCR products of all the studied species produced two (200 and 500 bp) and four (50, 100, 160 and 400) fragmented bands, respectively. They were identical in all analyzed samples (Figs. 2 and 3).

Digestion of 18S rRNA gene PCR products with *HinfI* produced two different patterns. *P. papatasi*, *S. antennata* and *S. schwetzi* showed similar RFLP-pattern producing three fragments (80, 140 and 300 bp) whereas *P. sergenti*, *P. bergeroti*, *S. clydei* and *S. fallax* almost had an identical pattern and produced four fragments (30, 80, 140 and 300 bp) (Fig. 4).

With *RsaI* only *S. schwetzi* species 18S rRNA gene PCR products were cleaved to produce two fragments (150 and 550 bp) while there was no digestion in 18S rRNA gene PCR products from other species (Fig. 5).

PstI-digestion of 18S rRNA gene PCR products showed variable fragments in *Phlebotomus* spp. and *Sergentomyia* spp. All specimens of *S. clydei*, *S. antennata*, *S. fallax* and *S. schwetzi* produced two fragments (320 and 380 bp). There was no digestion of the 18S rRNA gene amplified from *P. papatasi*, *P. sergenti* and *P. bergeroti* (Fig. 6).

By using *AseI* restriction enzyme, the RFLP-patterns were divided the seven species into two groups; All *Phlebotomus* spp. and *S. clydei* specimens were digested into two fragments (200 and 500 bp), while other species samples viz., *S. antennata*, *S. fallax* and *S. schwetzi* were not digested by this enzyme (Fig. 7).

Treatment with *BbvI* revealed an identical pattern of the RFLP-analysis for four species, *P. papatasi*, *S. clydei*, *S. antennata* and *S. fallax*. Their 18S rRNA gene was cut into two fragments (200 and 500 bp). The treatment with *BbvI* also gave the same pattern for other three species *P. sergenti*, *P. bergeroti* and *S. schwetzi*, produced four bands (50, 100, 200 and 350 bp) (Fig. 8).

When *MnII* enzyme was tested, different forms of the RFLP-patterns were observed. *P. bergeroti* showed a unique pattern and produced four fragments (70, 110, 150 and 400 bp), while the other six species were divided into 2 groups: *P. papatasi*, *P. sergenti*, *S. clydei* and *S. fallax* specimens were almost identical because they were cut into three fragments (100, 150 and 500 bp), and *S. antennata* was indistinguishable

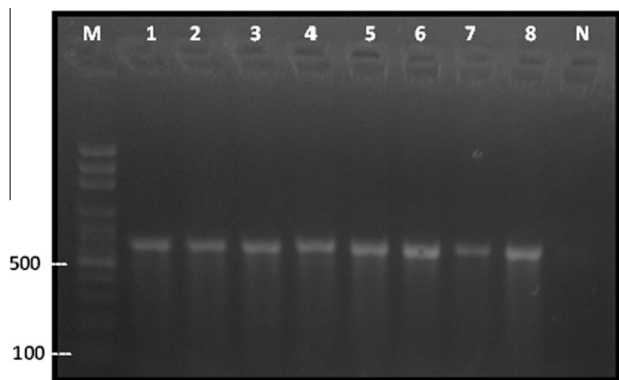
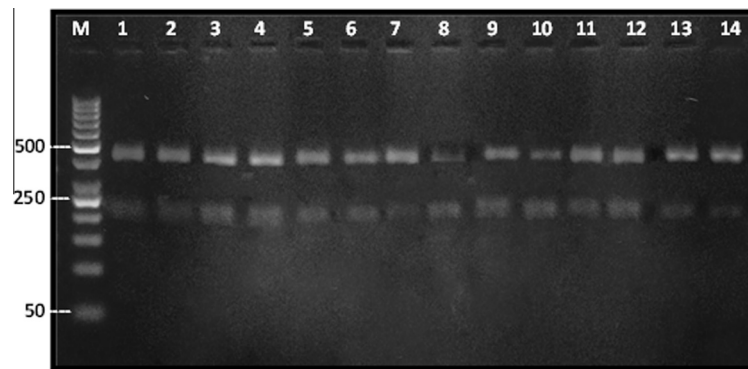
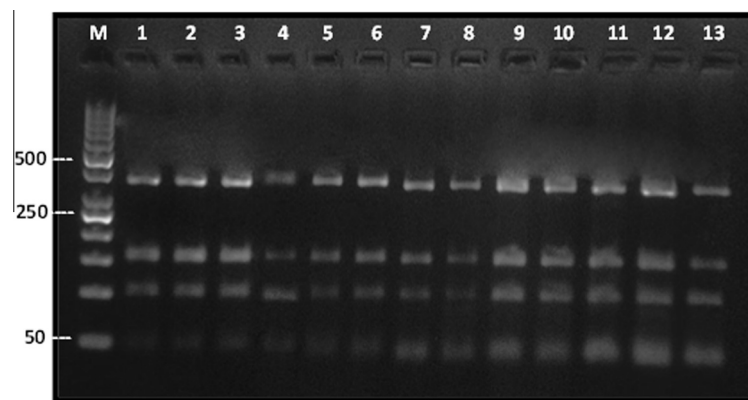


Figure 1 Agarose gel electrophoresis of PCR amplification products of 18S rRNA gene. M: 100 bp DNA marker; lanes 1–8: random samples of various sand fly species, N: negative control.

Table 1 Species of sand flies and the cut sites of restriction enzymes from restriction fragment length polymorphism (PCR–RFLP) analysis of 18S rRNA gene.

Species of sand fly	Restriction bands (bp)							
	HaeIII	BstNI	HinfI	RsaI	PstI	AseI	BbvI	MnII
<i>P. papatasi</i>	200, 500	50, 100, 160 400	80,140, 300	–	–	200, 500	200,500	100, 150, 500
<i>P. sergenti</i>	200, 500	50, 100, 160 400	30, 80, 140, 300	–	–	200, 500	50, 100, 200, 350	100, 150, 500
<i>P. bergeroi</i>	200, 500	50, 100, 160 400	30, 80, 140, 300	–	–	200, 500	50, 100, 200, 350	70,110, 150, 400
<i>S. clydei</i>	200, 500	50, 100, 160 400	30, 80, 140, 300	–	320, 380	200, 500	200,500	100, 150, 500
<i>S. antennata</i>	200, 500	50, 100, 160 400	80, 140, 300	–	320, 380	–	200,500	250, 500
<i>S. fallax</i>	200, 500	50, 100, 160 400	30, 80, 140, 300	–	320, 380	–	200,500	100, 150, 500
<i>S. schwetzi</i>	200, 500	50, 100, 160 400	80, 140, 300	150, 550	320, 380	–	50, 100, 200, 350	250, 500

**Figure 2** PCR–RFLP analysis of 18S rRNA gene digested by *HaeIII* restriction enzyme. *P. papatasi* (lanes 1,2), *P. sergenti* (lanes 3,4), *P. bergeroi* (lanes 5,6), *S. clydei* (lanes 7,8), *S. antennata* (lanes 9,10), *S. fallax* (lanes 11,12) and *S. schwetzi* (lanes 13,14). M: DNA marker.**Figure 3** PCR–RFLP analysis of 18S rRNA gene digested by *BstNI* restriction enzyme. *P. papatasi* (lanes 1,2), *P. sergenti* (lanes 3,4), *P. bergeroi* (lanes 5,6), *S. clydei* (lanes 7,8), *S. antennata* (lanes 9,10), *S. fallax* (lanes 11,12) and *S. schwetzi* (lane 13). M: DNA marker.

from *S. schwetzi* specimens which formed two fragments (250 and 500 bp) (Fig. 9).

4. Discussion

In the present study an attempt was made to establish a simple and reliable method for identification of sand flies captured

from different regions in Western Province (Al-Madinah Al-Munawarah) and Southern Province (Asir) of Saudi Arabia. The 18S rRNA gene was targeted and PCR amplicons were determined for seven species in the areas. The 18S rRNA gene was targeted and PCR amplicons for each phlebotomine sand fly species here examined showed no variability in terms of size and could not be used for identification purposes as found by

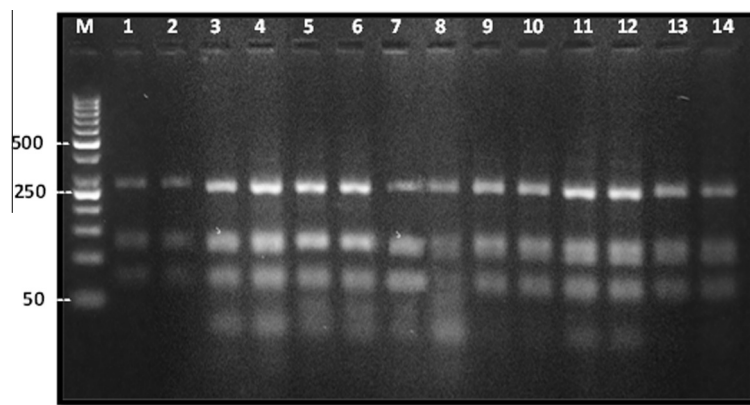


Figure 4 PCR–RFLP analysis of 18S rRNA gene digested by *HinfI* restriction enzyme. *P. papatasi* (lanes 1,2), *P. sergenti* (lanes 3,4), *P. bergeroti* (lanes 5,6), *S. clydei* (lanes 7,8), *S. antennata* (lanes 9,10), *S. fallax* (lanes 11,12) and *S. schwetzi* (lanes 13,14).

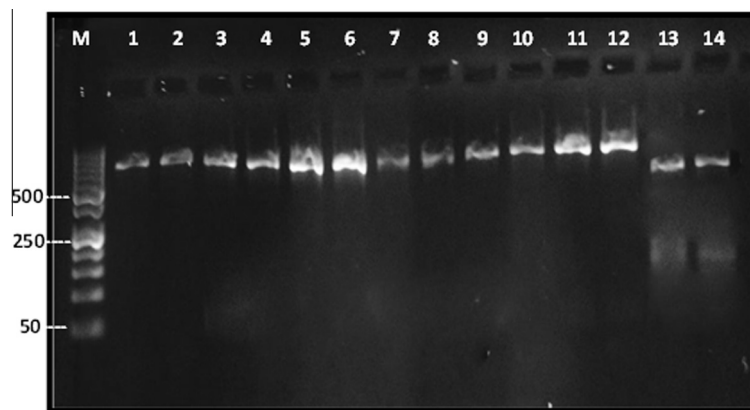


Figure 5 PCR–RFLP analysis of 18S rRNA gene digested by *RsaI* restriction enzyme. *P. papatasi* (lanes 1,2), *P. sergenti* (lanes 3,4), *P. bergeroti* (lanes 5,6), *S. clydei* (lanes 7,8), *S. antennata* (lanes 9,10), *S. fallax* (lanes 11,12) and *S. schwetzi* (lanes 13,14). M: DNA marker.

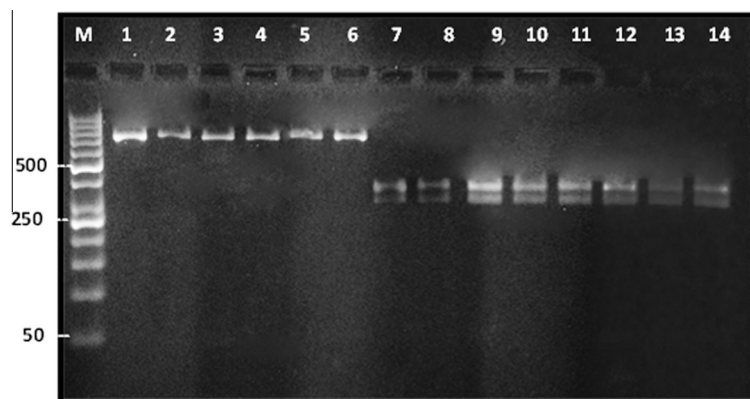


Figure 6 PCR–RFLP analysis of 18S rRNA gene digested by *PstI* restriction enzyme. *P. papatasi* (lanes 1,2), *P. sergenti* (lanes 3,4), *P. bergeroti* (lanes 5,6), *S. clydei* (lanes 7,8), *S. antennata* (lanes 9,10), *S. fallax* (lanes 11,12) and *S. schwetzi* (lanes 13,14). M: DNA marker.

Al-Ajmi et al. (2015). RFLP-patterns of 18S rRNA genes PCR products with the restriction enzymes *HaeIII* and *BstNI* yielded identical fragments as reported in earlier studies (Terayama et al., 2008). No genetic diversity was observed in the studied species. *HinfI* showed a 30 bp band in *P. sergenti*, *P. bergeroti*, *S. clydei* and *S. fallax* whereas this band was absent in other species studied. *RsaI* cut only *S. schwetzi* genotype at two places and no restriction sites were observed in the

other species. *PstI* could not cut *P. papatasi*, *P. sergenti* and *P. bergeroti* but cut all other species at two places to produce identical fragments. Similarly *AseI* cut *P. papatasi*, *P. sergenti*, *P. bergeroti*, *S. clydei* to produce two similar fragments but could not cut other species. *BbvI* showed similar genotypes in *P. papatasi*, *S. clydei*, *S. antennata* and *S. fallax* while similarity was observed in *P. sergenti*, *P. bergeroti* and *S. schwetzi*. *MnII* showed similarity of genetic pattern in *P. papatasi*, *P. sergenti*,

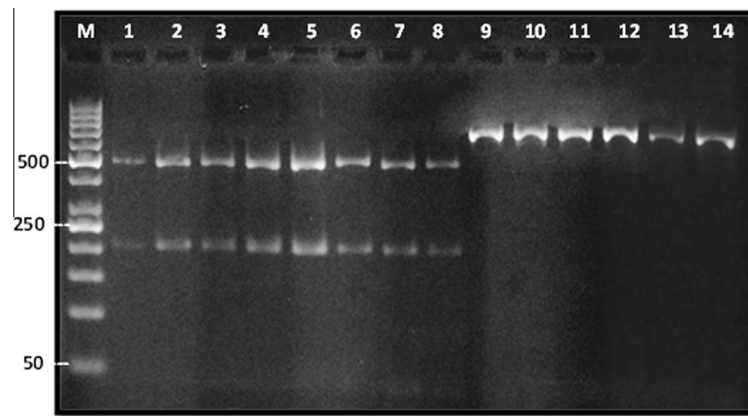


Figure 7 PCR–RFLP analysis of 18S rRNA gene digested by *AseI* restriction enzyme. *P. papatasi* (lanes 1,2), *P. sergenti* (lanes 3,4), *P. bergeroti* (lanes 5,6), *S. clydei* (lanes 7,8), *S. antennata* (lanes 9,10), *S. fallax* (lanes 11,12) and *S. schwetzi* (lanes 13,14). M: DNA marker.

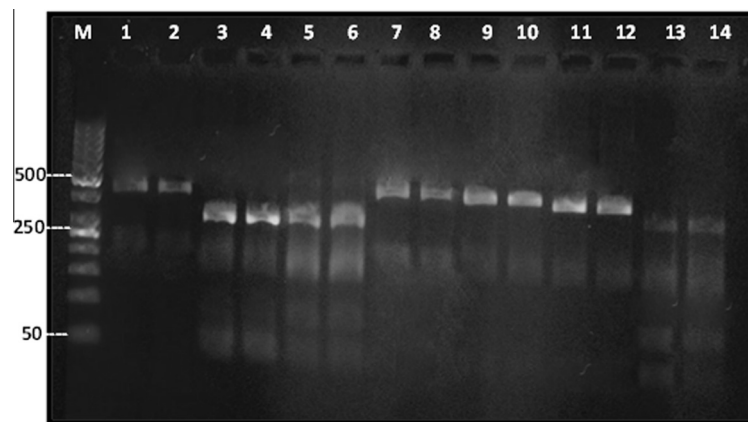


Figure 8 PCR–RFLP analysis of 18S rRNA gene digested by *BbvI* restriction enzyme. *P. papatasi* (lanes 1,2), *P. sergenti* (lanes 3,4), *P. bergeroti* (lanes 5,6), *S. clydei* (lanes 7,8), *S. antennata* (lanes 9,10), *S. fallax* (lanes 11,12) and *S. schwetzi* (lanes 13,14). M: DNA marker.

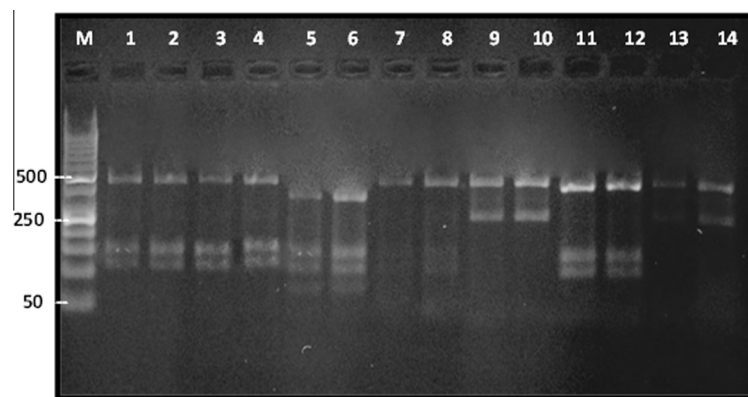


Figure 9 PCR–RFLP analysis of 18S rRNA gene digested by *MnlI* restriction enzyme. *P. papatasi* (lanes 1,2), *P. sergenti* (lanes 3,4), *P. bergeroti* (lanes 5,6), *S. clydei* (lanes 7,8), *S. antennata* (lanes 9,10), *S. fallax* (lanes 11,12) and *S. schwetzi* (lanes 13,14). M: DNA marker.

S. clydei and *S. fallax* while a completely different pattern was found in *P. bergeroti*, *S. antennata* and *S. schwetzi* showed identical fragments with *MnlI*. Interspecific differences were observed in 18S rRNA gene PCR products digested with various restriction endonucleases. The results confirm the earlier studies of Aransay et al. (2000) who used PCR–RFLP of 18S rRNA gene, with changes in the kinds of the restriction

enzymes used, to identify seven *Phlebotomus* and three *Sergentomyia* species distributed in Greece and Cyprus.

Double digestion of 18S rRNA gene amplicons resulted in very small fragments which were difficult to analyze through agarose gel electrophoresis (unpublished data). Similar results have been reported by Barroso et al. (2007) and Terayama et al. (2008). We therefore conclude that single digestion of

amplicons of 18S rRNA gene may be suitable for typing the seven sand fly species identified in Saudi Arabia.

The usefulness of PCR–RFLP profiles depends on the number of the restriction enzyme employed (Cortes et al., 2006) and also the restriction sites of these enzymes. It is difficult to classify all the species of sand flies solely by PCR–RFLP analysis. This leads to the conclusion that morphological examination continues to be an indispensable tool for classification. The result of PCR–RFLP analysis can be used to confirm the results of morphological taxonomy.

The present study demonstrated that the digestion of PCR products by one enzyme cannot separate some species belonging to the same genera (like *P. papatasi* and *P. sergenti* by *AseI*) as well as those belonging to different genera (like *P. papatasi* and *S. clydei* by *AseI*), due to the conservation of some DNA sequences. Consequently, PCR–RFLP molecular technique was not able to distinguish all of the common phlebotomine sandflies in the studied regions, where these insects have minute species-specific morphological characters. This method required the use of many restriction enzymes to distinguish between the species which exacerbates the cost and resources. Therefore there is a need to explore alternate techniques like low-stringency single specific primer polymerase chain reaction which can be used for molecular typing.

5. Conclusion

Morphological examination continues to be an indispensable tool for classification. PCR–RFLP molecular technique used in this study was not able to distinguish all of the common phlebotomine sandflies in the studied regions.

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