JEB
Journal of Environmental Biology

Journal Website : www.jeb.co.in
E-mail : editor@jeb.co.in
ISSN: 0254-8704 (Print)
ISSN: 2394-0379 (Online)
CODEN: JEBIDP

Molecular identification of natural sand fly species populations inferred from ITS2 rRNA gene in Saudi Arabia

Reem A. Al-Ajmi[1], **Tahany H. Ayaad**[1,2]*, **Mashael Al-Anazi**[3], **Mohammed N. Al-Ahdal**[3] **and Ahmed A. Al-Qahtani**[3, 4]

[1]Zoology Department, Faculty of Science, King Saud University, Riyadh, 2457,11451, Saudi Arabia

[2]Entomology Department,Faculty of Science ,Cairo University,Giza,11613,Egypt

[2]Department of Infection and Immunity, Research Center, King Faisal Specialist Hospital and Research Center, Riyadh,3354, 11211, Saudi Arabia

[3]Liver Disease Research Center, King Saud University, Riyadh, 2925,11461, Saudi Arabia

*Corresponding Author's E-mail: ayaadmtah@gmail.com

Publication Info

Paper received:
10 February 2014

Revised received:
19 September 2014

Re-revised received:
29 November 2014

Accepted:
08 December 2014

Abstract

Sandflies are blood-sucking insects of great medical importance and are transmission vectors for several organisms that are infectious to humans. Three hundred fourteen adult sandflies were collected and morphologically identified as five species: *Phlebotomus papatasi*, *P. bergeroti*, *P. sergenti*, *Sergentomyia antennata* and *S. clydei*. *P. papatasi* was the most abundant species (22.29%) of central region of Saudi Arabia, while *S. clydei* was the most abundant species (18.79 %) of southwestern region. Molecular classification of sandflies was done based on ribosomal RNA (rRNA) gene Internal Transcribed Spacer 2 (ITS2) sequences. ITS2 region was amplified using specific primers designed from a conserved region. Several clones from each individual were sequenced and analyzed using different DNA sequence analysis software. The results showed moderate variations (0.06%) between clones of same *P. papatasi* individual. However, *P. sergenti* individuals were highly divergent (0.207%). A phylogenetic tree, based on neighbor-joining analysis, showed that morphologically identical individuals of same species exhibited significant variations at the molecular level. Cloned *P. bergeroti* sequences were highly related to *P. papatasi* sequences (0.05%). Further, *S. antennata* species sequences were similar to *S. clydei* species sequences. In conclusion, molecular typing of sand fly species from Saudi Arabia based on ITS2 region may aid in differentiating between species and, possibly, sub-species.

Key words

ITS2, Ribosomal RNA gene, Sandfly

Introduction

Phlebotomine sand flies (Diptera: Psychodidae) are blood-sucking insects of great medical importance. They act as transmission vectors for bacteria and viruses that cause human disease and transmit the protozoan parasite *Leishmania*, which causes Leishmaniasis in humans (Depaquit *et al.,* 2010). Estimates indicate that leishmaniasis approximately affects 12 million people worldwide, and approximately 20 *Leishmania* species that infect humans cause severe disease in different body parts (Choi and Lerner, 2001). Sandflies have traditionally been identified on the basis of morphological characteristics, mainly internal structures, such as spermatheca, cibarium and pharynx in females and terminal genitalia in males (Munstermann, 2005). However, morphological classification is not always reliable in closely related species and intra-species variations (Kuwahara *et al.,* 2009). Therefore, such techniques have been successfully utilized to study different aspects of classification, evolution and forensics in insects (Wells and Stevens, 2008). These methods include random amplified polymorphic DNA-PCR (RAPD-PCR) (Mukhopadhyay *et al.,* 2000; Hamarsheh *et al.,* 2007). Further, DNA sequencing of PCR-amplified segments for portions of nuclear and mitochondrial DNA has been used to accurately identify and classify sandflies (Di Muccio *et al.,* 2000; Depaquit *et al.,* 2008).

Genes that encode rRNA contain conserved and variable regions, and both can be used for molecular classification and taxonomy. The conserved region has been used to identify organisms across species or even genera, and variable regions

Online Copy

© Triveni Enterprises, Lucknow (India)

were used to identify sub-species differences. For example, direct sequencing of rRNA ITS1 and ITS2 regions was used to study heterogeneity in *P. sergenti* and *P. similis* (Depaquit *et al.,* 2008). Moreover, cloning and sequencing PCR products from the ITS2 sequence was successfully used to study genetic diversity the *Lutzomyia* (Kuwahara *et al.,* 2009) and *Phlebotomus* species (Latrofa *et al.,* 2011). Recently, semi-nested PCR method was compared with (kinetoplast DNA) kDNA and ITS1-PCR-RFLP analysis, as useful tools, for molecular identification of both *L. major* and *L. tropica* in the prevalent sandflies from the Al-Madinah Al-Munawarah province in KSA (El-Beshbishy *et al.,* 2013)

In the present study, molecular analyses were performed to identify sandflies collected from different localities in Saudi Arabia. DNA sequence for ITS2 region of rRNA in several sandfly species was investigated, and the results showed significant variations in sequence among different species and individual variations within the same species. To elucidate the phylogenetic pattern of sand fly species diversification, a molecular phylogenetic study based on the ITS2 sequences to reliably distinguish members of this taxon was carried out. The morphologically distinguished taxa were compared with the molecular data below genera level.

Materials and Methods

Sandflies were caught using sticky and light traps from the following Saudi Arabian provinces: Riyadh, Al-Madinah Al-Munawarah and Assir. Traps were placed at rat burrow entrances. The collected specimens were preserved in 70% ethanol and frozen at – 80 °C until use. Each fly was cut into two parts; the first part consisted of the head and terminalia and was used for morphological identification, based on standard taxonomical keys as indicated in (Kakarsulemankhel,2007), while the second part was used for molecular techniques.

DNA extraction and analysis : DNA was extracted from each ethanol-fixed sandfly individual using a DNA extraction kit (DNeasy tissue kit, Qiagen, California, USA) following the manufacturer's instructions.

PCR, cloning and sequencing of ITS2 of rRNA gene : To amplify the ITS2 region from various phlebotomine individuals that belong to different species, PCR was performed in a 50 µl volume using the following primers: forward (C1a) 5'-CCTGGTTAGTTTCTTTTCCTCCGC T-3' and reverse (JTS3) 5'-CGCAGCTAACTGTGT GAA ATC-3'. PCR products were cloned using TA cloning kit (Invitrogen, California, USA). Sequencing was performed using M13 primer with the sequencing kit Big Dye terminator V3.1 cycle (Applera, Foster City, CA, USA), and the results were analyzed using ABI 3700 DNA Analyzer (Applied Biosystem, Foster City, CA, USA)

All sequences were aligned with sequences from GenBank using ClustalW (Thompson *et al.,* 1994). A phylogenetic tree was constructed using MEGA 5 (Tamura *et al.,* 2011), and genetic distances were calculated using Kimura 2-Parameter (K2P) model. The robustness of branching pattern in NJ analysis was assessed using 1,000 bootstrap replicates. All positions containing gaps and missing data were eliminated from the dataset. ITS2 sequence of *Anopheles lesteri* (accession number AJ620902) was obtained from GenBank and used as an out group. The ITS2 nucleotide sequence data obtained from *P. papatsi*, *P. sergenti*, *P. bergeroti* and *S. clydei,* reported in this study, were deposited in GenBank database and assigned accession numbers JN172917.1,JN172919.1, JN230424 and JN172918.1, respectively.

Results and Discussion

Identification and classification of insect vectors are important in medical entomology field as they are responsible for transmitting and spreading several disease-causing organisms, including bacteria (e.g., *Bartonella bacilliformis*), viruses (e.g., *Phlebovirus, Vesiculovirus*) and protozoa *Leishmania* spp. Molecular markers are invaluable tools in studies on population genetics and evolution because the levels of genetic variability observed are typically higher than the variability produced based on morphological and isoenzyme markers (Balbino *et al.,* 2006). A number of DNA repeat families are potentially good targets for molecular insect identification, including rRNA genes.

In the present study, 314 sandflies were collected from three different regions of Saudi Arabia, namely Riyadh, Al-Madinah Al-Munawarah and Assir (Table 1). The sandflies collected were morphologically identified as five species out of them; three belonged to genus *Phlebotomus* (*P. papatasi*, *P. bergeroti*, *P. sergenti*) and two belonged to genus *Sergentomyia* (*S. antennata* and *S. clydei*). *P. papatasi* was the most abundant

Table 1 : Number and species of sandflies collected from each region of Saudi Arabia

Sampling regions	*P. papatasi*		*P. bergeroti*		*P. sergenti*		*S. clydei*		*S. antennata*	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Riyadh	45	8	5	-	-	-	4	1	5	6
*Al-Madinah Al-Munawarah	60	10	1	-	-	1	-	-	-	-
**Assir	24	19	38	1	8	5	44	15	10	3

*Central region; **South western region

Online Copy

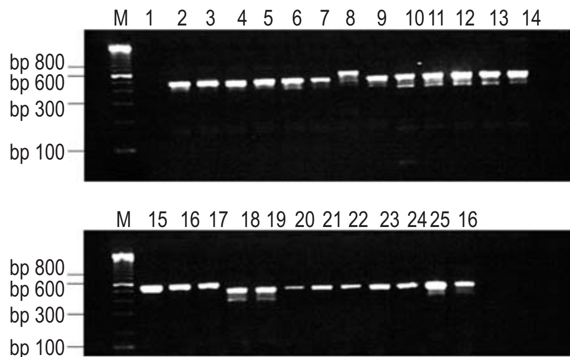

Fig. 1 : PCR products after amplification of ITS2. Lane M: DNA marker (100bp), Lane 1: negative control, Lanes 2 to 26 represents different samples of variable sand fly species

species in the Riyadh and Al-Madinah Al-Munawarah regions; *S. clydei* was the most prevalent species in the Assir region.

To study the genetic variability of sandfly individuals collected, ITS2 region was amplified using specific primers. The amplification products showed bands with variable molecular weights ranging from 480-550 bp. Certain individuals exhibited PCR products with multiple bands (Fig. 1). PCR products from twelve representative samples were randomly selected from each species, cloned and sequenced using M13 primers. Subsequently, the sequences were compared to the reference sequences from GenBank data base.

Few variations were observed in *P. papatasi,* in contrast, significant variations were evident among individuals that belonged to *P. sergenti*. Most of the changes were observed in the region between nucleotides 270 to 405 of the reference sequence (AF462328.1). To our knowledge, this is the first study that has investigated ITS2 sequence variations in *S. clydei* and *S. antennata*. No reference sequence was observed in GenBank database for these species; therefore, ITS2 sequence of a randomly selected individual collected in the present study was used as reference sequence.

Nucleotide sequences were used to perform combined intraspecies and interspecies genetic distance comparisons (Table 2). The smallest genetic distance was observed between members of *P. papatasi* (0.06%), and the greatest sequence diversity was observed between members of *S. antennata* (0.972%). The amplified fragment sizes were species-dependent and were of approximately of the same size for each individual investigated from the same species; however, certain individuals produced more than one amplified product. Similar results have earlier been reported using same experimental procedures to study sandflies from different species of different countries (Depaquit *et al.,* 2008; Latrofa *et al.,* 2012).

ITS2 sequence analyses showed that genetic differentiation among species was often pronounced. Although NJ phylogenetic tree showed high identification success rate at genus level, the position of certain organisms were anomalous (Fig. 2). For example, one *S. clydei* isolate (isolate A 58) was positioned with *S.antennata* (isolate 68). *Phlebotomus* species were more closely related; however, individuals of *Sergentomyia* species were distinctly separate. The bootstrap values showed fairly strong support for the majority of nodes throughout phylogeny. Most clades were clearly monophyletic, except for certain species. This level of differentiation might be greater than morphological differentiation among subspecies. Molecular and morphological data showed contrasting positions for certain species as they are important for illumining the evolutionary history of insects, including sand flies. Phylogenetic tree showed divergent intra-species clusters that corresponded to divergent evolutionary lineages, even though they were morphologically identical. Consistent with Depaquit *et al.* (2008), in the present study only slight intra-species variability between the *P. papatasi* individuals was noted. *P. bergeroti* ITS2 sequence intraspecific variability revealed both variable and constant regions, and these results are consistent with other reports (Di Muccio *et al.,* 2000; Latrofa *et al.,* 2011), where ITS2 region in *P. bergeroti* individuals was described as a mosaic form composed of constant and variable regions. However, *P. sergenti* individuals exhibited high variability at ITS2 region; they also exhibited high variability as compared with other individuals studied in different regions of the world (Depaquit *et al.,* 2002; Baron *et al.,* 2008). Similar results were obtained by Depaquit *et al.* (2008), who reported that ITS2 sequence differences between *P. papatasi* and *P. bergeroti* individuals varied in the range 3 to 5.5%.

For *S. antennta* and *S. clydei*, this is the first study that has analyzed DNA sequence at ITS2 region for each species; significantly high intra-species variability was observed. Genetic variability was highly significant among individuals in the genus *Sergentomyia*. Further, Latrofa *et al.* (2011) reported that the

Table 2 : Maximum pairwise distance (%) between inter and intra species of sandfly of Saudi Arabia

	P. papatasi	*P. bergeroti*	*P. sergenti*	*S. antennata*	*S. clydei*
P. papatasi	0.06	-	-	-	-
P. bergeroti	0.05	0.008	-	-	-
P. sergenti	0.196	0.208	0.207	-	-
S. antennata	0.84	0.829	0.866	0.972	-
S. clydei	0.337	0.4	0.387	0.75	0.431

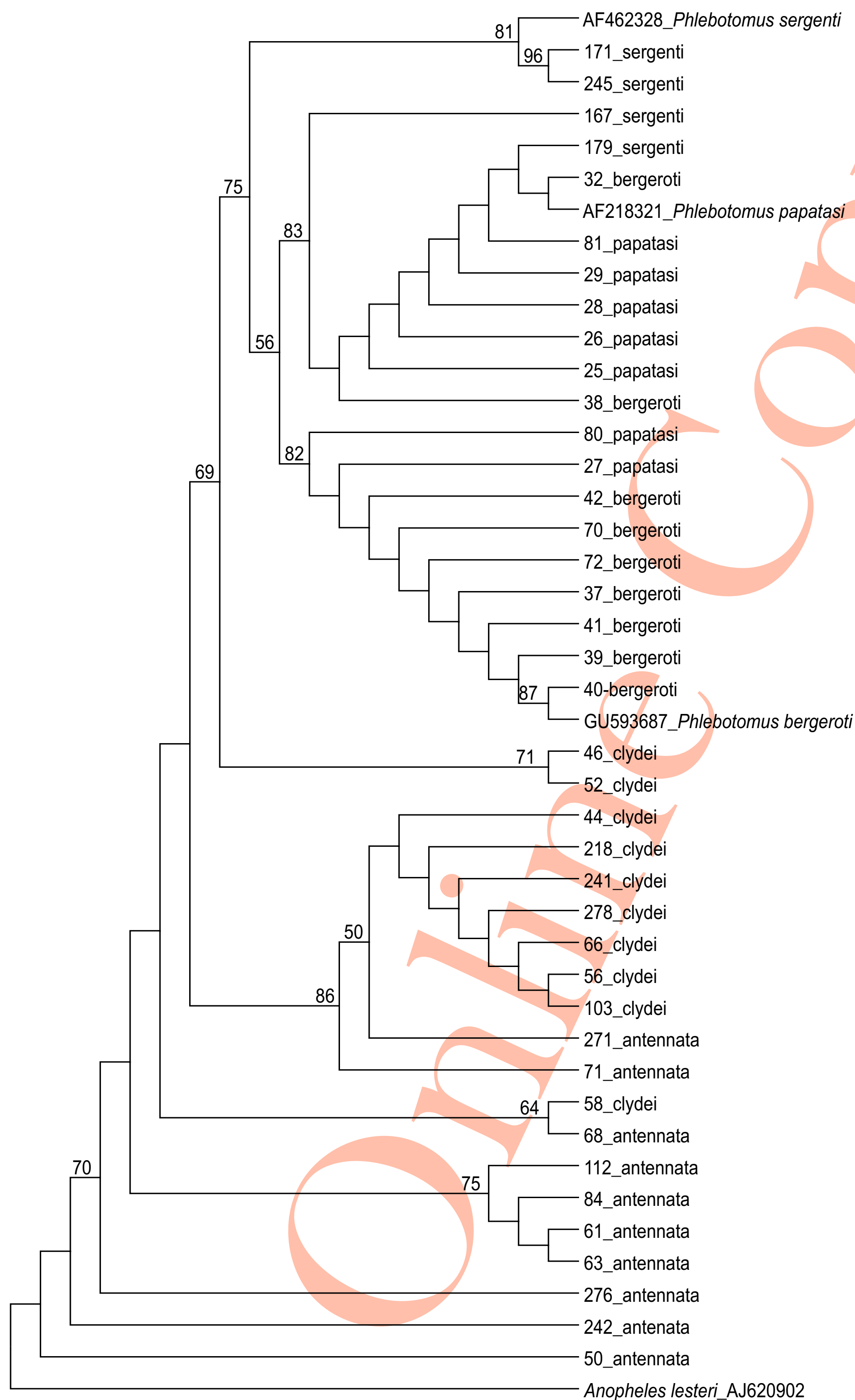

Fig. 2 : Phylogenetic tree of sandflies isolated from Saudi Arabia as inferred by NJ method showing molecular relation between different species based on ITS2 sequence. ITS2 sequence of *Anopheles lesteri* (accession number AJ620902) was used as an outgroup to root the tree

Online Copy

difference in ITS2 sequence of individuals related to *Sergentomyia* and *Phlebotomus* genera was 25.1%. However, the intra-specific variability ranged from 0.2 to 4.9%.

Together, these observations challenge a detailed analysis of sandflies distribution and may support further investigation into their role in transmitting infectious organisms. Other studies have successfully used these approaches to study molecular variations of certain disease-transmitting vectors, such as *Anopheles* (Kampen 2005a; Kampen 2005b)

In conclusion**,** below genera level genetic diversity among species was often pronounced. Positioning certain species close to other species indicated inconsistencies between molecular and morphological data.

Acknowledgments

This project was partly supported by the research center of Center for Female Scientific and Medical Colleges at King Saud University and King Abdulaziz City for Science and Technology (Project number 0028-10-TT). It was also approved by the Research Advisory Council (RAC) of King Faisal Specialist Hospital and Research Centre (Project # 2110015). We thank Ms. Nisha Viswan for her technical assistance. The support of the Research Center administration at King Faisal Specialist Hospital and Research Center is highly appreciated.

References

Balbino V. de.- Q., I.V., Coutinho-Abreu, I.V., Sonoda, M.A., Melo, P.P., de Andrade, J.A.F., de Castro, J.M., Rebˆelo, S.M.S., Carvalho, M. Ramalho-Ortig˜ao :Genetic structure of natural populations of the sand fly Lutzomyia longipalpis (Diptera: Psychodidae) from the Brazilian northeastern region. *Acta. Trop*., **98**, 15-24(2006).

Baron, S., J. Martin-Sanchez, M. Gallego, M. Morales-Yuste, S. Boussaa and F. Morillas-Marquez: Intraspecific variability (rDNA ITS and mtDNA Cyt b) of *Phlebotomus sergenti* in Spain and Morocco. *Acta Trop.,* **107**, 259-67(2008).

Choi, C.M. and E.A. Lerner: Leishmaniasis as an emerging infection. *J. Investig. Dermatol. Symp. Proc.,* **6**, 175-82(2001).

Depaquit, J., Ferté, H., Léger, N., Lefranc, F., Alves-Pires ,C., Hanafi ,H., Maroli, M., Morillas-Marquez, F., Rioux, J.A., Svobodova, M., Volf, P. : ITS 2 sequences heterogeneity in *Phlebotomus sergenti* and *Phlebotomus similis* (Diptera, Psychodidae): possible consequences in their ability to transmit *Leishmania tropica*. *Int. J. Parasitol.,* **32**, 1123-31(2002).

Depaquit, J., E. Lienard, A. Verzeaux-Griffon, H. Ferté ,A. Bounamous, J.C. Gantier, H.A. Hanafi, R.L. Jacobson, M. Maroli, V. Moin-Vaziri, F. Müller, Y. Ozbel, M. Svobodova, P. Volf and N. Léger: Molecular homogeneity in diverse geographical populations of *Phlebotomus papatasi* (Diptera, Psychodidae) inferred from ND4 mtDNA and ITS2 rDNA Epidemiological consequences. *Infect. Genet. Evol*., **8**, 159-70(2008).

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

Di Muccio, T., M. Marinucci, L. Frusteri, M. Maroli, B. Pesson and M. Gramiccia: Phylogenetic analysis of *Phlebotomus* species belonging to the subgenus *Larroussius* (Diptera, psychodidae) by ITS2 rDNA sequences. *Insect Biochem. Mol. Biol.*, **30**, 387-93 (2000).

El-Beshbishy, H.A., A. Al-Ali, H. Kh and A.A. El-Badry: Molecular characterization of *Leishmania* infection in sand flies from Al-madinah Al-munawarah province, western Saudi Arabia. *Experim. Parasitol*., **134**, 211–15 (2013).

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

Kakarsulemankhel, J. K.: Morphotaxonomy of Sergentomyia fallax (Parrot) (Diptera: Psychodidae) in Sindh and its evolutionary relationship with its closest allies. *Pak. J. Entomol*. **22,** 11-19(2007).

Kampen, H.: Integration of *Anopheles beklemishevi* (Diptera: Culicidae) in a PCR assay diagnostic for palaearctic *Anopheles maculipennis* sibling species. *Parasitol. Res*., **97**, 113-7(2005a).

Kampen, H.: The ITS2 ribosomal DNA of *Anopheles beklemishevi* and further remarks on the phylogenetic relationships within the *Anopheles maculipennis* group of species (Diptera: Culicidae). *Parasitol. Res*., **97**, 118-28(2005b).

Kuwahara ,K., H. Kato, E.A. Gomez, H. Uezato, T. Mimori, Y. Yamamoto and M.Y. Calvopiña: Genetic diversity of ribosomal RNA internal transcribed spacer sequences in *Lutzomyia* species from areas endemic for New World cutaneous leishmaniasis. *Acta. Trop.,* **112**, 131-6 (2009).

Latrofa, M.S., G. Annoscia, F. Dantas-Torres, D. Traversa and D. Otranto: Towards a rapid molecular identification of the common phlebotomine sand flies in the Mediterranean region. *Vet. Parasitol.,* **184**, 267-70 (2012).

Latrofa, M.S., F. Dantas-Torres, S. Weigl, V.D. Tarallo, A. Parisi, D. Traversa and D. Otranto: Multilocus molecular and phylogenetic analysis of phlebotomine sand flies (Diptera: Psychodidae) from Southern Italy. *Acta. Trop.,* **119**, 91-8 (2011) .

Mukhopadhyay, J., K. Ghosh and H.R. Braig: Identification of cutaneous Leishmaniasis vectors, *Phlebotomus papatasi* and *P. duboscqi* using random amplified polymorphic DNA. *Acta Trop.,* **76**, 277-83 (2000).

Munstermann, L.: Phlebotomine sand flies, the Psychodidae. In: Biology of Disease Vectors (Eds.: W.C. Marquardt WCB, J.E. Freier, et al.). 2nd Edn., Elsevier, San Diego, Calif, USA, pp. 141–151 (2005).

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.*, **28**, 2731-9(2011).

Thompson, J.D., D.G. Higgins, T.J. Gibson and W. Clustal: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*., **22**, 4673-80 (1994).

Wells, J.D. and J.R. Stevens: Application of DNA-based methods in forensic entomology. *Annu. Rev. Entomol*., **53**, 103-20(2008).

Online Copy