

## Research Article

# Discovery of *Phlebotomus* Species of Sand Flies in Mwea Irrigation Scheme, Kirinyaga County, Kenya: A Possible Leishmaniasis focus

Josyline Kaburi<sup>1,2\*</sup>, Philip Ngumbi<sup>1</sup>, Lucy Irungu<sup>2</sup>, Paul Ndegwa<sup>2</sup>, Johnstone Ingonga<sup>1</sup>, Bernard Osero<sup>1</sup>, and Christopher Anjili C<sup>1</sup>

<sup>1</sup>Centre for Biotechnology Research and Development, Kenya Medical Research Institute, Kenya

<sup>2</sup>School of Biological Sciences, University of Nairobi, Kenya

**\*Corresponding author**

Josyline Kaburi, Centre for Biotechnology Research and Development, Kenya Medical Research Institute, P.O. Box 54840-00200, Nairobi, Kenya, Tel: 254-020-2722541; Cell: 0722929617, Fax: 254-020-2720030; Email: jcirindi@kemri.org, jcirindi@yahoo.com

Submitted: 25 April 2018

Accepted: 22 May 2018

Published: 25 May 2018

ISSN: 2475-9430

Copyright

© 2018 Kaburi et al.

OPEN ACCESS

**Keywords**

• Sand flies; Mwea irrigation scheme; *Phlebotomus*, *Sergentomyia*

**Abstract**

**Background and objectives:** During our study that was evaluating control of mosquito and sand fly vectors of Mwea Irrigation Scheme, Kirinyaga County, Kenya, we came across a number of phlebotomine sand flies. A faunistic study of some Kenyan phlebotomine sand flies (Diptera: Psychodidae) was carried out to find out the species which were likely to be present in Mwea Irrigation Scheme.

**Methods:** Morphological and molecular vector species identification was done using taxonomic keys and cytochrome c oxidase subunit 1 (COI) gene amplification followed by Sanger sequencing and phylogenetic tree was constructed using the neighbor-joining method.

**Results:** A total of 251 sand flies were collected consisting of *Phlebotomus martini* Parrot 4.8% and *P. rodhaini* Parrot 0.8%. Majority of the sand flies collected were from *Sergentomyia* species (94.4%). High Resolution Melting (HRM) profiles of some male sand flies had same peak as that of *P. martini* controls and closely related in phylogenetic tree analysis.

**Conclusion:** The finding of these *Phlebotomus* species in Mwea is an indication that they can breed in non-arid/semi-arid urbanized areas and can be synanthropic unlike previously documented. PCR-High Resolution Melting profiles and phylogenetic analyses indicated that samples of sand flies were related to *P. martini* sequences (accession number JX105040).

**ABBREVIATIONS**

HRM: High Resolution Melting; VL: Visceral Leishmaniasis; CDC: Centre for Disease Control; KEMRI: Kenya Medical Research Institute; DNA: Deoxyribonucleic Acid; PCR: Polymerase Chain Reaction; CO1: Cytochrome Oxidase subunit1

**INTRODUCTION**

*Phlebotomus* sand fly species are the only proven vectors of visceral leishmaniasis (VL) but knowledge of their distribution and diversity in Kenya is partial. Fourteen sand fly species out of the known 47 reported in Kenya belong to the genus *Phlebotomus* Leow, whereas 33 are *Sergentomyia* Franca and Parrot species [1]. It was found out that a majority of *P. martini* occurs in Baringo (52.3%), Nakuru (8.6%), Marsabit (37.2%) [2], nothing has been documented on its occurrence in Mwea sub- Country.

Mwea irrigation scheme is an area that is predominantly malaria and schistosomiasis endemic and not known to harbor sand flies. It was intriguing to confirm whether the vectors trapped were *Phlebotomus* and fly species through morphological features and molecular techniques.

Presence of *P. Martini* in Mwea sub-County for the first time

could indicate a possible leishmaniasis disease focus. However, no cases of leishmaniasis transmission have been reported and this finding calls for more studies on vector surveillance and disease transmission in Mwea irrigation scheme.

**MATERIALS AND METHODS****Study site**

The studies were conducted in the Mwea rice irrigation scheme in Kirinyaga County, Kenya, 100 km northeast of Nairobi, Kenya. Mwea rice scheme covers an area of approximately 13,600 ha with a population of 150,000 people in 2,500 households [3]. Mwea sub-County is situated on the east of Mount Kenya at an altitude of 1100 to 1350 metres above sea level. The irrigation scheme is also a settlement scheme that produces 75-90% of the rice that is consumed in Kenya (National Irrigation Board Report). Water from the two major rivers, Nyamindi and Thiba is used to irrigate the rice-fields in a network of primary and secondary feeder canals. The villages are discrete units made up of homesteads/compounds with varying numbers of households. The majority of families keep domestic animals such as cattle, goats, sheep, donkeys and chicken among others [4]. The most common activities of the people of Mwea are connected with

rice growing and are the main income sources for most people. The homesteads keep small numbers of livestock which include cattle, goats, sheep, pigs, donkeys and chicken. Most homesteads store the rice stalks (chuff) in their compounds as these make good fodder for their animals.

### Morphological identification of sand flies

In this study, in order to trap sand flies, miniature SSM CDC Light traps (John W. Hock, Gainesville, Florida) were positioned in outside 30 homesteads on a daily basis at 18:00hrs and retrieved at 06:00hrs the following morning for two consecutive days. The sand flies were aspirated, counted, recorded on excel data sheets. They were put in small vials with desiccant pebbles to provide dry condition and transported to KEMRI laboratory where they were dissected and mounted on micro-slides using chloral hydrate gum according to the method of Minter (1963). They were allowed to dry for one day at room temperature before being identified under the compound microscope using identification keys of Abonnenc [5].

### Molecular identification of sand flies

DNA was extracted from the remaining parts of the specimens using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's recommendation. The insect was macerated first and briefly 180µl of buffer ATL was added to 1.5 ml micro centrifuge tubes containing each specimen and the tissues disrupted using micro pestles before addition of 20µl of proteinase K. The mixture was vortexed and incubated at 56°C for 1 hour and 30 minutes followed by vortexing for 15 seconds, addition of 200µl of buffer AL, mixing by vortexing, addition of 200µl of 96%-100% ethanol and vortexing again. The mixture from the previous step was pipetted into the DNeasy mini spin column placed in 2ml collection tube, centrifuged at 8000rpm for 1 minute and the flow-through and the collection tube discarded. The DNeasy mini spin was transferred into a new 2ml collection tube, 500µl of buffer AW1 added, centrifuged at 8000rpm for 1 minute and the flow-through discarded as in the previous step. The DNeasy mini spin column was transferred into a new 2ml collection tube followed by addition of 500µl of buffer AW2, centrifugation at 14000rpm during the pre-elution step for DNA and animal tissues (according to the protocol), for 3 minutes to dry the membrane and discarding the flow-through and the collection tube. The DNeasy mini spin column was placed in a clean 1.5ml microcentrifuge tube and 100µl of buffer AE pipette directly onto the DNeasy membrane. This was incubated at room temperature for 1 minute followed by centrifugation at 8000rpm to elute the DNA.

Molecular identification was done by polymerase chain reaction (PCR) of the mitochondrial cytochrome oxidase subunit 1 (COI) genomic marker. The COI gene was amplified using DNA barcoding primers; forward LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and reverse, HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Kumar, Srinivasan & Jambulingam, 2012). In a final volume of 15µl, the following was added: 7.5µl of 2x My Taq mix, 0.75µl of primer, 2µl of DNA extracted from the specimens (template) and 4 µl of PCR grade water. For the concentrations, we used ready reagents as follows: My Taq mix is a ready to use (2x) in concentration,

primer stock 100Um; <http://www.bioline.com/uk/downloads/dl/file/id/2741/mytaqmixproductmanual.pdf>. Thermal cycling conditions included an initial denaturation step of 15 minutes at 95°C followed by 40 cycles of 95°C for 1 minute (denaturation), 52°C for 30 seconds (annealing) and 72°C for 40 seconds (extension) and a final extension of 72°C for 10 minutes.

Further, the region was amplified using Uniminibar JV primers; forward primer, Uniminibar JV F (5'-ACAAATCATAARGATATTGGAAC-3') and reverse primer Uniminibar JV R (5'-AAAATTATAATAAAWGCATGAGC-3'), and the established 173bp PCR products resolved using high resolution melt (HRM) analysis (Ajamma *et al.*, 2016) in Rotar gene Q HRM real time PCR thermal cycler (QIAGEN, Hannover, Germany). The PCR mixture consisted of 2µl of 5x Hot FirepolEvagreen HRM mix (Solis BioDyne, Tartu, Estonia), 0.25µl of each primer, 1µl of DNA template and PCR grade water in a final volume of 10µl. The thermal cycling conditions involved an initial denaturation at 95°C for 15 minutes followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 45 seconds and extension at 72°C for 45 seconds and a final extension at 72°C for 10 minutes. Without stopping the reaction, the PCR products were denatured at 95°C for 1 minute, held at 40°C for another minute and melted by gradually raising the temperature from 65°C to 95°C by 0.1°C in each step, waiting for 90 seconds of pre-melt conditioning on first step and 2 seconds for each step afterwards. In both the amplifications, PCR grade water was used as the negative control while positive controls included DNA from known (molecularly identified) sand fly species. Sand fly species identification was done by comparing the HRM profiles generated to those of the reference control species. Representative samples of identified sand fly species with unique HRM curves were identified by purification, of about 650bp PCR products established in the LCO/HCO amplification above, using ExoSAP-IT (USB Corporation, Cleveland, OH) and submitted for sequencing using the Sanger method. To confirm the identity of PCR-HRM differentiated sand flies, the generated DNA sequences were edited using Geneious and queried against the Genbank database using the Basic Local Alignment Search. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test was shown next to the branches. The evolutionary distances were computed using the Poisson correction method and in the units of the number of amino acids. The evolutionary analyses were conducted in MEGA7 ([www.megasoftware.net](http://www.megasoftware.net))

## RESULTS

A total of 251 sand flies comprising of two genera (*Phlebotomus* and *Sergentomyia*) were caught and identified to species level. The collection comprised of two *Phlebotomus* species and six *Sergentomyia* species as shown in Table 1. The two *Phlebotomus* species that were caught were *P. martini*, (4.8%) and *P. rodhaini*, (0.8%) while *Sergentomyia* species were *S. schwetzi* Adler, Theodor and Parrot (6.0%), *S. bedfordi*

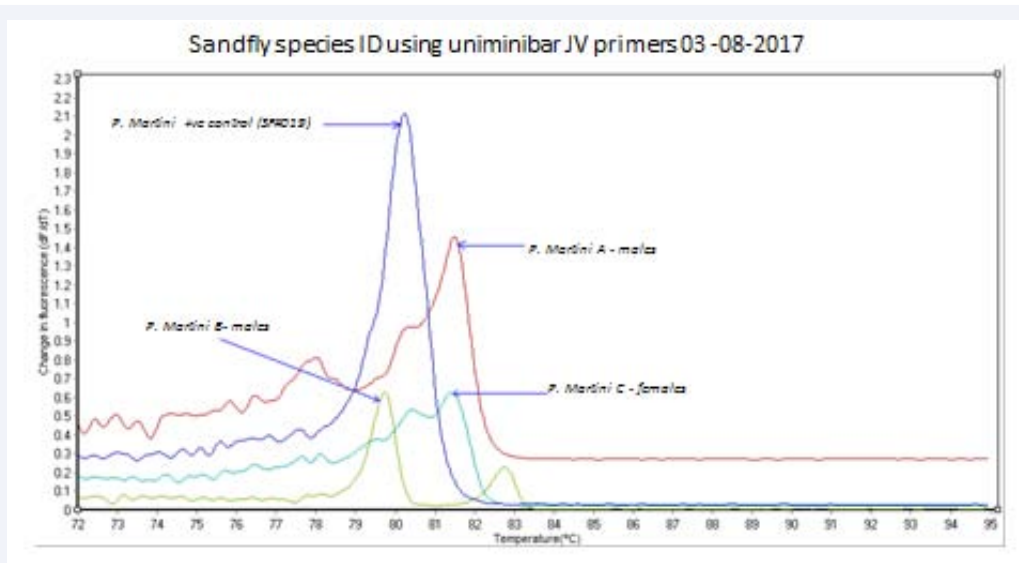


Figure 1 HRM profiles of sand fly samples A, B and C compared with P. martini positive control.

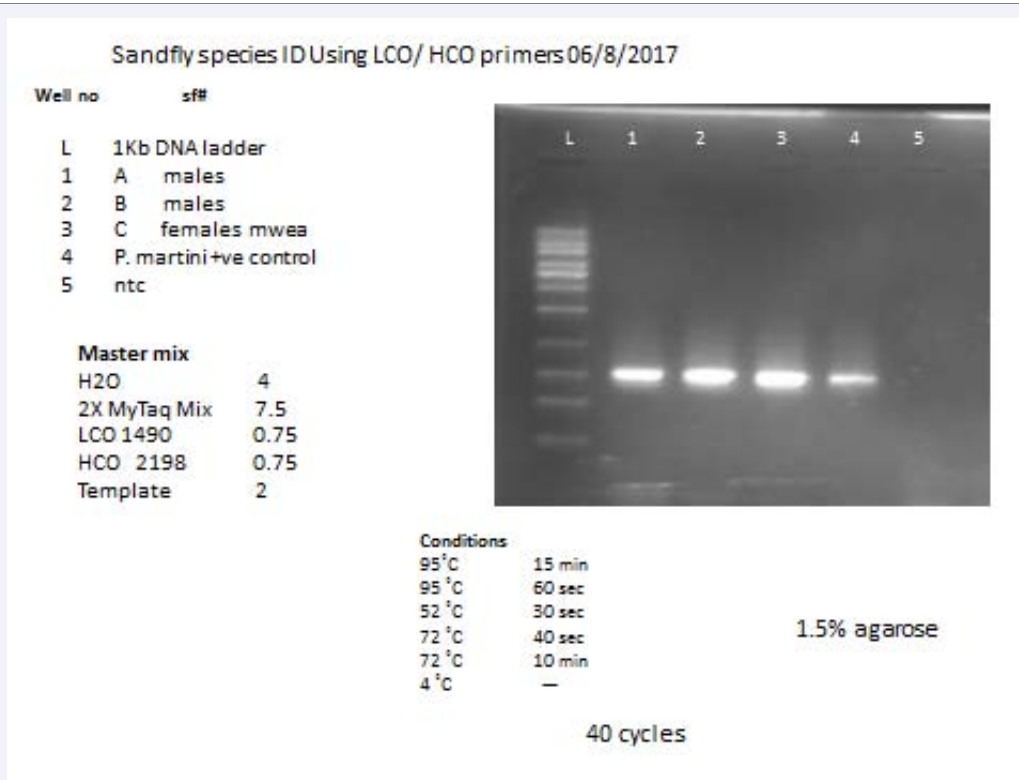
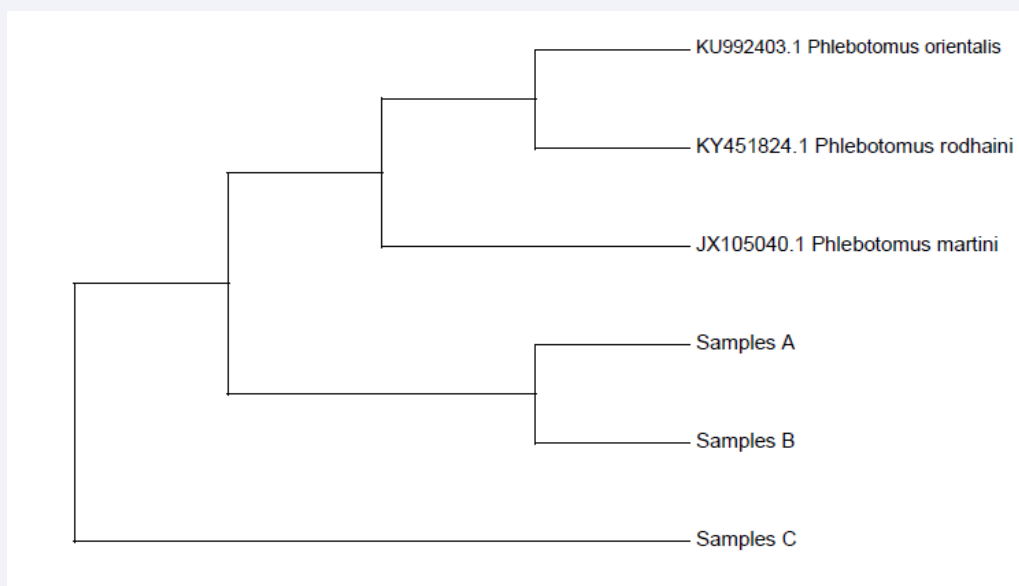


Figure 2 Electrophoretic analysis of PCR product (1kb).

Newstead, (3.2%), *S. squamipleuris* Newstead, (27.9%), *S. clydei* Sinton, (0.4%), *S. antennata* Newstead, (0.4%) and *S. inermis* Abonnenc (56.6%). Female sand flies were three times the number of males caught during the study. Out of the total number of sand flies caught, majority were *S. inermis*, Abonnenc followed by *S. squamipleuris*, Newstead. The results are summarized in the Table 1 and Figure 1. Sand fly species identification was done by comparing the High Resolution Melting (HRM) profiles generated

to those of the reference control species (Figure 2). HRM analysis is a post PCR technique that measures the rate of ssDNA to dsDNA with increasing temperature. It is applicable when detecting differences in the nucleotide composition of a specific real-time PCR product. PCR-HRM assay results demonstrated that sand fly samples B had a HRM profile peak similar to *P. martini* +ve control, whereas for C and A had profiles peak slightly outside that of *P. martini* +ve control, suggesting that B sand fly



**Figure 3** Ultrametric phylogenetic tree for *P. martini* and Mwea sand fly samples A, B, C.

Determination of the relationship of sand fly samples and *Phlebotomus martini* sand flies in Mwea site using phylogenetic tree. PCR-HRM assay results demonstrated that sand fly samples B had a HRM profile peak similar to *P. martini* +ve control, whereas for C and A had profiles peak slightly outside that of *P. martini* +ve control, suggesting that B sand fly samples were more related to *P. martini* +ve control and can be used to identify species. Multiple alignments of the sequences were done using CLUSTAL W and phylogenetic tree was constructed using The Neighbor-Joining method. This tree suggests that samples B for sandflies were genetically closely related to *P. martini* than samples A and C (Figure 3).

**Table 1:** Phlebotomine sand flies caught in households in Mwea Irrigation Scheme households.

Number	Sand fly species	Males	Females	Totals (%)
1	<i>P. martini</i>	8	4	12 (4.8)
2	<i>P. rodhaini</i>	1	1	2 (0.8)
3	<i>S. antenata</i>	1	0	1 (0.4)
4	<i>S. bedfordi</i>	4	4	8 (3.2)
5	<i>S. clydei</i>	0	1	1 (0.4)
6	<i>S. schwetzi</i>	3	12	15 (6.0)
7	<i>S. squamipleuris</i>	10	60	70 (27.9)
8	<i>S. inermis</i>	30	112	142 (56.7)
Total		57	194	251 (100)

**Abbreviations:** *P.* = *Phlebotomus*; *S.* = *Sergentomyia*

samples were more related to *P. martini* +ve control and can be used to identify species (Figure 2). Multiple alignments of the sequences were done using CLUSTAL W and phylogenetic tree was constructed using The Neighbor-Joining method. This tree suggests that samples B for sandflies were genetically closely related to *P. martini* than samples A and C (Figure 3).

## DISCUSSION

The trapping of sand flies in Mwea irrigation scheme was carried out during the dry and wet season between January 2014 and January 2015. Kirinyaga County is at the border line of the Kenya highlands and the semi-arid regions. The collection of 12 (twelve) *Phlebotomus martini* and 2 (two) *P. rodhaini* of both sexes in Mwea Irrigation Scheme was unexpected because there were no termite mounds which act as the breeding and resting

sites of the species, and moreover, the region is at the borderline between the highlands of Kenya and the semi-arid regions. The influence of climate variables on the distribution and activity of sand flies has been repeatedly reported [6-8]. In contrast with other reports, in the current study, climate variables did not affect the probability of finding *P. martini* and *P. rodhaini*, probably due to the short period of time when captures were performed and the homogenous geographical conditions of most trapping sites. Termite mounds have been incriminated as the ideal habitats for *P. martini*, *P. celiae*, and *P. vansomeranae*, in East Africa and their distribution is associated with the vectors of leishmaniasis, it now seems from the findings that the habitat of these, and *P. rodhaini* sand flies is dependent on additional environmental factors and can be found in areas lacking termite mounds [9]. The first record of natural infection of *Leishmania donovani* parasites

in *P. rodhaini* and any *Anopheles* sand flies was reported by Elnaïem et al., [10]. Despite its wide distribution in most leishmaniasis endemic foci, *P. rodhaini* is considered a rare species and therefore it was ignored as a probable vector of *Leishmania* parasites. The use of rodent baited traps was shown to increase the collection of *P. rodhaini* by more than 40 fold [11], supported the findings of Hoogstraal & Heyneman [11], who reported that *P. rodhaini* is more readily collected by rodent baited traps. This was not achieved in this study because only light traps were used. The presence of *P. martini* in Mwea Irrigation Scheme is a health issue since it is a vector of *L. donovani* in the endemic areas of Kenya.

## CONCLUSION

There are no termite mounds in the study site. This was taken to indicate that there are other breeding sites that are still unknown in Kenya for *P. martini*. Leishmaniasis should therefore be considered a risk not only for the habitual inhabitants of the scheme, but also for the large numbers of people visiting the area, in case of the introduction of the parasite, coinciding with the period of vector activity. Our account of a sand fly, *P. martini* with a proven or capacity to transmit *L. donovani* and *P. rodhaini*, a probable vector in the Mwea non-endemic area, together with the arrival of *L. donovani* infected persons and animals, would favor the possibility of autochthonous transmission in the area.

On the other hand, medical personnel who work in the Health facilities need to be sensitized on the kala-azar symptoms and diagnosis so as to differentiate malaria and kala-azar since the two diseases symptoms are similar.

## ACKNOWLEDGEMENTS

This study was conducted using funds from NACOSTI, and KEMRI Internal grant and hence, we are very grateful to KEMRI and NACOSTI for their support. We appreciate the assistance given to us in the field by local residents and the county administrative staff. This manuscript has been published with the authority of Director, KEMRI.

## REFERENCES

1. Anjili CO, Ngumb PM, Kaburi JC and Irungu LW. The phlebotomine sand fly fauna (Diptera: Psychodidae) of Kenya. *J Vector Borne Dis.* 2011; 48: 183-189.
2. Laura B, Damaris M, Philip N, Milkah M, Johnston I, Tobia L, et al. Sandfly Occurrence, Distribution and Diversity in leishmaniasis Endemic Areas in Kenya. *Health Sciences.* 2017; poster presentation.
3. Killick-Kendrick R. The biology and control of phlebotomine sand flies. *Clin Dermatol.* 1999; 17: 279-289.
4. Muriu SM, Ephantus JM, Josephat IS, Charles MM, Joseph MM, Benjamin GJ, et al. Host choice and multiple blood feeding behaviour of malaria vectors and other anophelines in Mwea rice scheme, Kenya. *Malar J.* 2008; 7: 43.
5. Mutero CM, Blank H, Konradsen F, van der Hoek W. Water management for controlling the breeding of Anopheles mosquitoes in rice irrigation schemes in Kenya. *Acta Tropica.* 2000; 76: 253-263.
6. Abonnenc E, Minter DM. Bilingual keys for the identification of the sandflies of the Ethiopian region. *Entomologie Medicale.* 1965; 5.
7. Aspöck H, Gerersdorfer T, Formayer H, Walochnik J. Sand flies and sandfly-borne infections of humans in Central Europe in the light of climate change. *Wien Klin Wochenschr.* 2008; 120: 24-29.
8. Barón SD, Morillas-Márquez F, Morales-Yuste M, Díaz-Sáez V, Gállego M, Molina R, et al. Predicting the risk of an endemic focus of *Leishmania tropica* becoming established in south-western Europe through the presence of its main vector, *Phlebotomus sergenti* Parrot, 1917. *Parasitology.* 2013; 140: 1413-1421.
9. Ngumbi PM, Kaburi JC, Anjili CO, Haas F. *Phlebotomus* (*Larrousius*) *orientalis* (Diptera: Psychodidae) as a probable second vector of visceral leishmaniasis in Kenya. *J Vector Borne Dis.* 2010; 58-60.
10. Elnaïem DE, Hassan HK, Osman OF, Maingon RD, Killick-Kendrick R, Ward RD. A possible role for *Phlebotomus* (*Anopheles*) *rodhaini* (Parrot, 1930) in transmission of *Leishmania donovani*. *Parasit Vectors.* 2011; 4: 238.
11. Hoogstraal J, Heyneman D. Leishmaniasis in the Sudan Republic. 30. Final epidemiologic report. *Am J Trop Med Hyg.* 1969; 18: 1091-1210.

### Cite this article

Kaburi J, Ngumbi P, Irungu L, Ndegwa P, Ingonga J, et al. (2018) Discovery of *Phlebotomus* Species of Sand Flies in Mwea Irrigation Scheme, Kirinyaga County, Kenya: A Possible Leishmaniasis focus. *Ann Clin Cytol Pathol* 4(3): 1103.