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Research Article
Genetic Structure of *Phlebotomus orientalis* (Diptera: Psychodidae) in Leishmaniasis Endemic Foci of Sudan

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Abstract

Background and Objective: Visceral leishmaniasis (VL) remains a major concern in many parts of Sudan. The disease is transmitted by *Phlebotomus orientalis.* The objective of this study was to determine genetic structure of *Phlebotomus orientalis* population from 5 geographical regions in Sudan. **Materials and Methods:** A total of 194 individual sand flies were collected from 5 geographic regions in Sudan. The field collected sand flies were analyzed by Random Amplified Polymorphic DNA (RAPD) using 30 primers. **Results:** Eight hundred and 65 bands from 4 RAPD primers were analyzed for genetic variation. A higher level of intrapopulational variability was detected in populations of *P. orientalis* from eastern Sudan compared to those populations from central and northern Sudan. Diagnostic bands were detected in populations of *P. orientalis* central Sudan. Hieratical clustering analysis showed clear clustering into 2 main populations with 1 population subdivided into 4 subpopulations. However, these populations did not show any correlation with their geographical origins. Furthermore, the low genetic differentiation among subpopulations was supported by fixation index (F_{ST}) estimated by analysis of molecular variance (AMOVA). **Conclusion:** It is concluded that the populations of *P. orientalis* from the selected areas in Sudan have a low genetic differentiation. However, assessment of genetic structure of *P. orientalis* populations is important for understanding the patterns of transmission of VL in different endemic areas.

Key words: Visceral leishmaniasis, sand flies, *Phlebotomus orientalis*, RAPD-PCR, population structure

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Human visceral leishmaniasis (VL) is a major public health problem affecting large rural population in Sudan[1,2]. The disease is endemic over a large area in eastern and in the formerly southern Sudan with severe epidemics, which resulted in high mortality and morbidity figures[2]. In Sudan, the annual incidence was estimated to be varied from 15,700-30,300 human VL cases during the years[2] 2005 and 2009.

Phlebotomus orientalis (Parrot) is the principal vector of *Leishmania donovani* the etiologic agent of VL in Sudan[3]. It has been incriminated as a vector of human VL, following the finding of specimens naturally infected with *L. donovani* parasites[3], associated with human cases of the disease. *Phlebotomus orientalis* has been found to prefer habitats of woodlands dominated by *Acacia seyal/Balanites aegyptiaca* which grow on black cracking cotton clay soil in the savannah region[4] and the villages which interfere such habitats[5]. Currently, *P. orientalis* was collected from a village habitat from a semi-desert area of northern and western Sudan[6,7].

In last few decades, sand fly vectors have been the focus of genetic characterization based on morphological, chromosomal, biochemical and molecular characters[8-12]. Previous studies demonstrated that molecular markers are a valuable tool in population genetic studies as well as the level of genetic variability than isozyme analysis or morphological characters[13]. However, the amount of genetic variation reported in populations of sand flies may depend on the sensitivity of the techniques used and the targeted genetic markers. Molecular studies on sand fly vectors have included internal transcribed spacer (ITS)[11,14], mDNA[14], RAPD[9,15,16] and microsatellite (MLMT)[10].

RAPD analysis introduced by Williams *et al.*[17] proved to be a powerful tool for systematic of species complexes and population studies even within well-established species[18] and unlike other polymorphism assay no prior sequence knowledge needed for the RAPD technique to be applied. The RAPD markers have been considered as a valuable technique which can be used to differentiate genetically and geographically isolated populations as well as to elucidate evolutionary pattern of a population of species that occur either through genetic selection as a result of different environmental factors or genetic drift[19]. Moreover, RAPD is known to be a useful tool to study the genetic structure of population since it can reveal polymorphisms in non-coding regions of the genome[20].

To date, limited information is available on genetic variation among *P. orientalis* the vector VL in Sudan. Therefore, the current study was to determine the genetic structure of *P. orientalis* within endemic regions of VL in Sudan. Such studies will increase our knowledge on the epidemiology and transmission of *Leishmania* parasites in an endemic area with the disease. Therefore, genetic structure of *P. orientalis* populations from 5 regions in Sudan was assessed using RAPD-PCR.

MATERIALS AND METHODS

Collection sites of sand flies: Sand flies were collected during the period 2009-2013 from 5 different areas in Sudan (Fig. 1). The collection sites represent 3 distinct geographic zones these were, savannah with high rainfall (3 sites in Gedarif State, eastern Sudan), savannah with low rainfall (woodland in White Nile State, central Sudan) and semi-desert region (Surogia village in Khartoum State, northern Sudan).

Eastern Sudan: The selected sites for collection of sand flies in eastern Sudan were Dinder National Park (DNP, 35°2′ E, 12°36′ N), Rahad River area (RH, 35°11′ E, 12°51′ N) and Atbara River area (ATB, 36°13′ E, 14°15′). These areas are located in an endemic area of VL[21]. The ecology of the area in eastern Sudan has been described by Elnaiem *et al.*[21]. The area is a part of the *Acacia seyal* belt extending from the Sudanese-Ethiopian border up to Upper Nile area in southern Sudan.

Central Sudan (Woodland of Kadaba village, White Nile State, WN): The village is located on western bank of the White Nile (32°14 E, 14°72 N), 100 km south of Khartoum. The area is located in a re-emerging focus of VL[22]. The area is flat and covered by an alluvium of silt clay soil of black cracking clay soil on the river bank and sandy soil in the villages. The area resembles a semi-desert region therefore, it is part of the arid climate with 3 distinct seasons, winter (November-February), summer (March-June) and autumn (July-October) with an estimated annual rainfall is 250 mm. The vegetation of the area was almost entirely composed of 2 types, the semi-desert vegetation which occupy the area around and at the western part of the villages and low rainfall savannah woodland extending from the White Nile course up to 7 km to the west. The semi-desert region from North to south was divided into an *Acacia tortilis-Maerua crassifolia* desert scrub, *A. mellifera* desert scrub in the sandy areas and semi-desert grassland (perennial grasses) on sand to the west of the villages. The low rainfall savannah woodland on clay soil is dominated mainly by *A. seyal* and *Balanites aegiptiaca* trees and less by *A. mellifera* with annual grasses cover the ground. However, there are sands soil interrupting the savannah

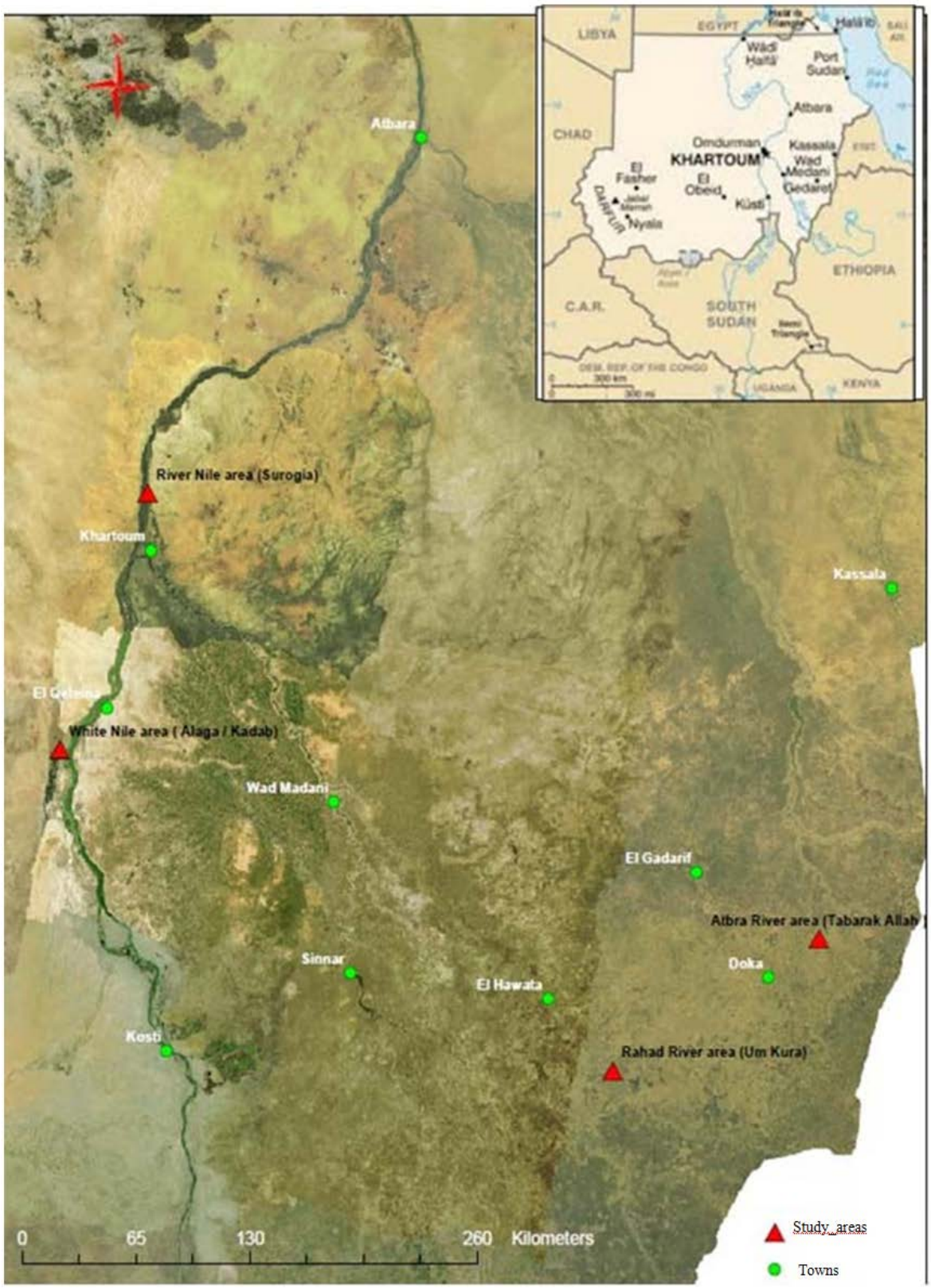

Fig. 1: A map showing *Phlebotomus orientalis* collection sites from different geographical regions in Sudan (https://www.google.com/maps/place/Sudan/)

woodland and is characterized *A. senegal* trees and annual grasses. The savannah woodland is widely destroyed by human activities such as cultivation and grazing. The majority of the animals in the village were domestic animals like sheep, cattle, goats and dogs. The people belong to the Hassaniya Arab tribes who live in the villages, however, some of these families spend the summer in woodland for cultivation, grazing and cheese industry.

Northern Sudan (Surogia village, SR): The village is located on the Eastern bank of the River Nile (32°14 E, 14°72 N) 35 km north of Khartoum. It lies in the endemic zone of cutaneous leishmaniasis mainly in the semi-desert area[6]. The ecology of the area was described by Hassan *et al.*[6].

Sand fly collection and identification: Five populations of *P. orientalis* were collected from 5 geographic areas in Sudan (Fig. 1). The collection sites represent 3 distinct geographic zones these are savannah with high rainfall (3 sites in Gedarif State, eastern Sudan), the savannah with low rainfall (woodland in White Nile State, central Sudan) and semi-desert region (Surogia village in Khartoum State, northern Sudan).

Sand flies were captured at by CDC miniature light traps from the collection sites. The traps were set at outdoor sites between 19:30-06:00 h, for 7 consecutive nights/region/survey. Three surveys were carried out in each collection sites.

Captured sand flies were preserved in 95% ethanol. Then the head and the last 2 segments of the females were carefully mounted in Berlese's medium on a glass slide whereas the rest of the body was individually transferred to eppendorf tubes with 95% ethanol. The slides were left for 12 h to air dry. The prepared specimens were identified using a binocular microscope at 40X. The female specimens were identified using a proper identification keys[23].

RAPD-PCR: Genomic DNA was extracted from single adult females of each *P. orientalis* from each population as described by De Queiroz Balbino *et al.*[8]. Briefly, the ethanol preserved females were placed individually into 1.5 µL eppendorf tube with 50 µL grinding buffer (0.1 mM NaCl, 0.1 M Tris HCl pH 8.0). The samples were ground into a fine powder using a glass pestle and then the homogenates were incubated at 95°C for 30 min. The supernatants were transferred to new tubes and then 200 µL of deionized H_2O was added. The DNA solutions were stored at 4°C for subsequent PCR analysis.

Thirty RAPD primers (Inqaba Biotec, South Africa) were used as genetic markers to screen DNA from *P. orientalis* from 5 geographic regions in Sudan. The amplification conditions were done as described by Williams *et al.*[17]. Amplification reaction was carried out in a final volume of 25 µL containing 3 µL DNA, 0.5µL dNTPs (200 µM of each dNTP final concentration), 2.5 µL 10X PCR buffer (50 mM KCl, 1.2 Triton-X100, 1.5 mM $MgCl_2$) (1x final concentration), 2.0µL each primer (15 pmole of each primer as a final concentration) and 0.2 µL *Taq* polymerase (1 U of *Taq*). The amplification conditions were 35 cycles with the following conditions: denature for 2 min at 94°C, annealing for 1 min at 35°C and extension for 2 min at 72°C. The final extension was held for 10 min at 72°C after the cycling was completed.

About 5 µL of the RAPD-PCR reaction products were mixed with 2µL of loading dye and loaded on to 0.8% agarose gel containing ethidium bromide. DNA marker 100-3000 bp was used as a molecular weight ladder to determine the RAPD band sizes. The gels were visualized and photographed using gel documentation system.

The reproducibility of the RAPD primers used was assessed by documenting the repeatability of RAPD 2-3 times using DNA of *P. orientalis* from different areas.

Statistical analysis: Data obtained from the *P. orientalis* by RAPD banding pattern were analyzed by comparing the number of bands within and between the populations of the 5 geographic regions. Bands were scored manually at each locus as 1 (present) or 0 (absent) across all polymorphic loci to create a binary matrix. A locus was considered polymorphic if the frequency of the most common allele did not exceed 95%. Only bright and reproducible bands were scored to generate the data matrix.

The genetic diversity within and among groups were calculated by the Dice coefficient using Genalex 6.5, genetic analysis in Excel software[24]. A phylogenetic tree was constructed using the neighbor-joining algorithm in XL-STAT 2015.1 software. Cophenetic analyzes was conducted to test the representativeness of the phenogram using XL-SATAT 2015.1 software. The distances (Euclidian) among the individuals representing the 5 populations were used to perform a principal coordinate analysis (PCA) on the molecular variation shown by the RAPD data to visualize the geometric relationships among *P. orientalis* populations using Genalex 6.5, genetic analysis in Excel software[24]. The geographic distances between the sample sites were calculated, so isolation by distance was examined by Mantel test (1000/mutation) using Genalex 6.5, genetic analysis in Excel software[24]. The genetic structure of the populations was estimated using the fixation index (F_{ST}). The F_{ST} value was calculated by analysis of molecular variance (AMOVA) arlequin software[25].

RESULTS

Morphological identification results: All mounted female sand fly specimens were morphologically identified as *P. orientalis* based on the shape and size of the pharynx and the shape, the number of the segments and the neck of the spermatheca.

RAPD profile: The DNA of 194 female sand flies identified as *P. orientalis* were amplified using 30 RAPD primers. Of the 30 primers tested, 4 RAPD (r469.1, r564.1, r751.2 and r807.1) produced clearly discernable and reproducible bands. The 4 primers produced 7-9 bands with sizes ranging from 100-3000 bp. Therefore, 90 profiles specific for each individual were analyzed: 25 from DNP, 19 from RH, 12 from ATB (Atbra River area), 17 from WN and 17 from SR. Of total bands, 81.8% were polymorphic (93, 65, 41, 71 and 64% in DNP, RH, WN, SR and ATB, respectively).

The number of RAPD markers and genotypes detected varied according to the geographic origin of sand fly collections (Table 1). The greatest number of fragments (273) was detected in the RH population, whereas the ATB population contained fewer amplification products (76). The frequencies of the bands varied in each population however, 500 bp DNA fragment generated by r564.1 wasn't detected in WN and 500 bp DNA fragment produced by r807.1 wasn't observed in ATB population. A 300 bp DNA fragment generated by primer r469.1 was observed in *P. orientalis* of all populations. Diagnostic allele at 3000 bp was also identified in 34 (68%) of WN population which was generated by r807.1.

Estimation of genetic diversity within and among *Phlebotomus orientalis* populations from 5 geographic regions in Sudan: The level of similarity obtained by the Dice's coefficient ranged from 0.444-1.00 in individuals of DNP, from 0.444-1.00 in individuals of RH, from 0.571-1.00 in individuals of ATB, 0.700-1.00 in individuals of WN and from 0.500-1.00 in individuals of SR. Moreover, the Dice's coefficients were calculated to determine the genetic distance between the populations showed a relatively high similarities between the populations of DNP and RH (0.803), DNP and SR (0.830) and ATB and SR (0.839) (Table 2).

The dendrogram generated from similarity/distance matrices of the individual specimens showed 2 main clades (Fig. 2). The 1st clade includes DNP, RH, ATB and SR populations whereas the 2nd clade includes WN population. The dendrogram obtained from the similarity/distance matrices of the population clustered also into main clades (Fig. 3). DNP, RH, ATB and SR populations clustered in the 1 clade which is subdivided into 3 small clades, DNP and RH populations clustered together whereas ATB and SR populations each clustered separately. WN population clustered in the second clade.

Principle Coordinate Analysis (PCA) was to determine the genetic relationships among *P. orientalis* populations. The scores showed that each population occurred in different axes, the exceptions were DNP and RH populations which were occupied the same axes. However, the total variance was 50.65% which indicated that a separate spatial distribution of each population and that agrees with that obtained by the NJ dendrogram in Fig. 2 and 3.

The correlation between genetic differences among the populations and geographic distance between the 5 populations of *P. orientalis* from Sudan were tested using a Mantel test, however, no association was observed between these populations (r = 0. 480).

Genetic structure of Phlebotomus orientalis populations: Genetic differentiations between the populations of

Table 1: Number of fragments and genotypes produced by the 4 RAPD primers for different populations of *Phlebotomus orientalis*

				Fragments/regions				
Primers	Sequences (3'→5')	Genotypes	Fragments	DNP	RH	ATB	WN	SR
r469.1	TCGCAACGTC	28	22	157	141	179	69	38
r564.1	GGGCACTCCG	30	20	112	162	84	83	24
r751.2	GCCTTCATCT	19	19	134	148	142	66	22
r807.1	GCCTCCTACT	32	29	135	232	312	87	28
Total		43	90					

DNP: Dinder national park, RH: Rahad river area, ATB: Atbara river area, SR: Surogia village, WN: White Nile area

Table 2: Geographic distance (km) (upper diagonal) and genetic distance (lower diagonal) as measured by Dice coefficient between the populations of *Phlebotomus orientalis* from different geographical regions in Sudan

Regions	DNP	RH	ATB	WN	SR
DNP		31.30	128.10	452.00	461.70
RH	0.803		96.40	391.10	451.50
ATB	0.753	0.595		345.80	382.30
WN	0.553	0.586	0.645		93.10
SR	0.830	0.758	0.839	0.505	

DNP: Dinder national park, RH: Rahad river area, ATB: Atbara river area, SR: Surogia village, WN: White Nile area

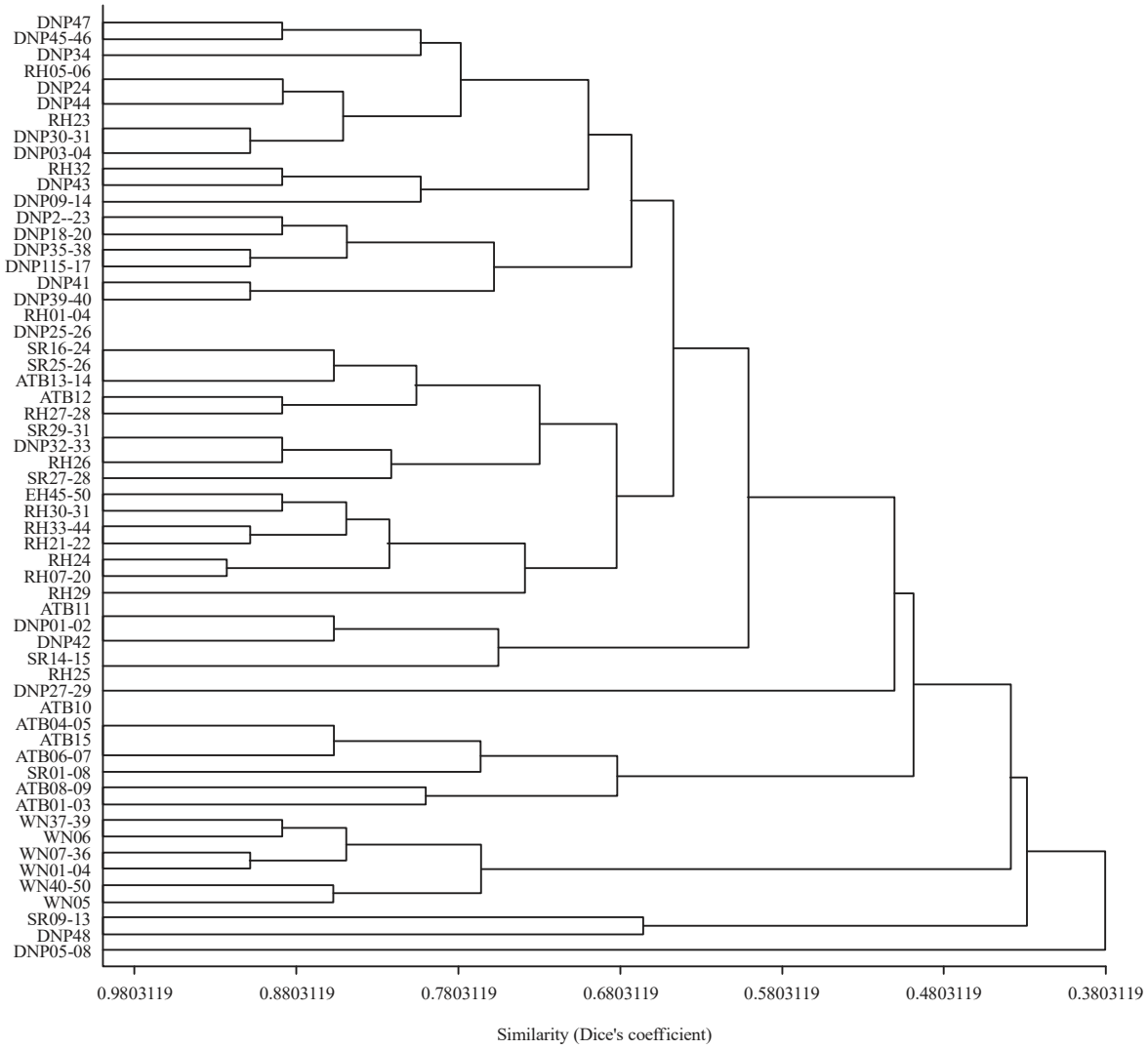

Fig. 2: Neighbor-joining dendrogram of Dice genetic distance (Nei, 1978) between *Phlebotomus orientalis* individuals from different geographical regions in Sudan

DNP: Dinder national park, RH: Rahad river area, ATB: Atbara river area, SR: Surogia village, WN: White Nile area

Table 3: Molecular variance and fixation index of the populations and the individuals of *Phlebotomus orientalis* obtained by AMOVA test

Source of variations	Degree of freedom	Sum of squares	Variance components	Variance (%)	Φ_{ST}
Among populations	1.00	16.93	0.001	1.60	0.041
Among groups within populations	3.00	19.74			
	0.02				
	4.30	0.042			
Among individuals within populations	189.00	1059.800			
	0.45				
	94.10	0.002			
Total	193.00	1096.500	0.50		

P. orientalis from different regions were evaluated using F_{ST} calculated by AMOVA test (Table 3). Patterns of genetic differentiation between populations of *P. orientalis* using measures of a fixation index F_{ST} showed a low genetic differentiation which indicates gene flow between these populations. AMOVA results also showed a high proportion of total variation within populations (94.1%) but a moderate proportion of variation would be attributed to either difference observed among groups within populations (4.3%) or among populations (1.6%).

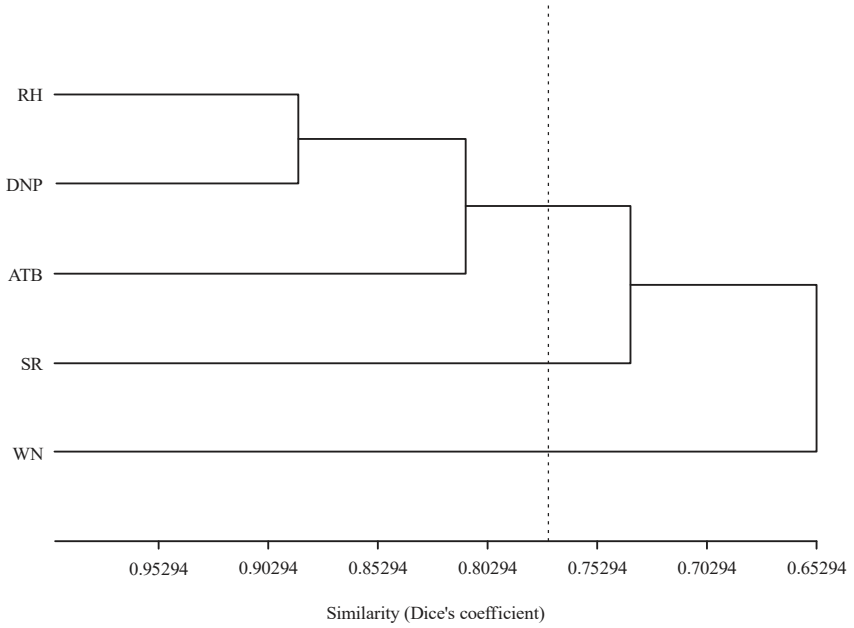

Fig. 3: Neighbor-joining dendrogram of Dice genetic distance (Nei, 1978) between *Phlebotomus orientalis* populations from different geographical regions in Sudan
DNP: Dinder national park, RH: Rahad river area, ATB: Atbara river area, SR: Surogia village, WN: White Nile area

DISCUSSION

Phlebotomus orientalis has a wide range of distribution in Sudan ranging from savannah areas in eastern parts[4] to semi-desert areas in northern part[6]. This wide range of geographical distribution in different ecological habitats may suggest the existence of structured populations due to isolation by distance. In Sudan, up to date no studies have been done, to the best of my knowledge, to elucidate the genetic structure of this vector. However, only a single study was carried out using molecular markers to differentiate between 2 closely related sand fly species in Sudan[26].

RAPD analysis has been frequently used to evaluate intraspecific variability and heterogeneity among sand flies[9,15]. This study appears to be the 1st report on genetic variation and population structure of a natural population of *P. orientalis* using RAPD-PCR in Sudan. A total of 194 specimens of 5 populations collected from savannah and semi-desert areas representing either current VL endemic area (eastern Sudan)[1,22] and CL endemic area (Surogia village)[6]. However, the RAPD pattern bands obtained in this study was clearly discernable and reproducible thus it reflects genetic variation in *P. orientalis*. This result is consistent with those obtained by the analysis of RAPD loci in other insect species[27,28]. Also, the results indicated that WN population is more homogenous than other populations which are further confirmed with the NJ analysis (Fig. 2). This result might indicate distinct evolutionary process due to an adaptation which is influenced by the unique environmental condition that prevails in the WN (i.e., the semi-desert and savannah with low rainfall).

The individual differences in the frequencies of bands, polymorphic bands between each population and the presence of species-specific bands suggest a degree of geographic differentiation among them. The differences in the RAPD-PCR banding pattern between the individuals of the same species often occur due to that some bands can be amplified in some individuals but not from other and that for dominant/recessive inheritance in diploid organisms[17]. No agreement on certain numbers of bands but a high number of polymorphic RAPD-PCR loci are recommended for the identification of subspecies or lineages of organisms[18] however, interpopulational variability has been suggested *Lu. whitmani* population in Brazil based on RAPD-PCR markers[16]. This finding is further supported by the specific-diagnostic RAPD markers which were detected among 68% (34/50) of the populations of WN. The specific-diagnostic bands generated by RAPD-PCR indicate existence of conserved regions within the species[29].

In this study, the NJ analysis and PCA coordination detected a high degree of genetic similarity among 4 geographic populations of *P. orientalis*. The NJ based on

Nei's[30] genetic distance (Fig. 2 and 3) showed a tendency of *P. orientalis* individuals to cluster according to their geographic origin with hierarchal splitting of the population into 2 main populations, where individuals of WN form the 1st population and the 2nd population is formed by the other 4 populations (DNP, RH, ATB and SR populations) which is further subdivided into 3 subpopulations (Fig. 3). Clustering of individuals in the same population might be a further evidence of the geographic separation of these populations. The genetic similarities between the populations of RH, DNP, ATB and SR detected in this study might be caused by genetic flow between theses populations. The 3 populations of eastern Sudan occupy the same climatic and environmental conditions of the savannah area with high rainfall which dominated by a woodland of A, seyal/B. aegyptiaca that grow on black clay soil[4]. Surprisingly, subpopulation representing individuals from SR was genetically closely related to eastern Sudan subpopulations (DNP, RH and ATB) although the geographic distance between them is large and environmental and ecological conditions differences. This finding might indicate that a recent speciation process took place between the SR population and eastern Sudan populations.

Genetic differentiation and substructuring in the population are caused by the poor dispersal capabilities[31] and the geographic isolation. However, F_{ST} analysis revealed moderate genetic differentiation and a moderate level of a geographic pattern in populations of *P. orientalis*. Despite, the genetic differentiation and substructuring, the geographic distance and genetic distance did not correlate significantly which indicates that geographic distance has no effect on the pattern of differentiation within this species. These findings might reflect isolation by a distance which indicates a distance-dependent gene flow which limits the genetic differences among natural populations[32]. Moreover, this might suggest that a recent speciation process of these populations caused by changes in both environmental and climatic factors such as deforestation, desertification, a change in rainfall[33]. Another reason that contributes to the population differentiation and substructuring of population might be differences in human colonization of the 2 areas. The eastern Sudan area has a relatively recent human settlement (50-60 years ago)[21] whereas, the central and northern area have an old history of human settlement (more than 250 years ago).

In Sudan, 3 Phylogenetic complexes were identified as agents of visceral leishmaniasis, *L. donovani*, *L. infantum* and *L. archibaldi*[34]. However, the 3 putative species isolated in Sudan was found to form 1 population by MLEE analysis[34]. Recently, RAPD analysis of *L. donovani* complex SGG-sensitive isolates gave 3 distinct genotypes[35]. However, further studies to infer the genetic diversity in *L. donovani* from different endemic areas in Sudan and its co-association with *P. orientalis* in these endemic areas or co-adaptations remains to lend itself for further exploration.

CONCLUSION

In conclusion, our results suggest the existence of a single species of *P. orientalis.* However, further investigation using more advance molecular markers such as MLMT and amplified sequence analysis of ITS regions and specific diagnostic bands are needed to provide answers towards the taxonomic situation and the level genetic variation of the populations of *P. orientalis* in Sudan. Moreover, studies on genetic variation in *Leishmania* parasites from these areas will help our understanding of the vector-parasite relationship.

SIGNIFICANCE STATEMENT

This study discovers the existence of a single species of visceral leishmaniasis vector in Sudan that can be beneficial for designing a proper strategy to control the disease in all endemic areas with disease in the country. This study will help the researcher to uncover the critical areas of speciation among sand fly vectors that many researchers were not able to explore. Thus, a new theory on sand fly vector species complex may be arrived at.

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