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Simulium vectors of the lower cross River Basin: A morphometric and cytotaxonomic identification

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Abstract
Human onchocerciasis in Nigeria is spread from person to person by infected adult female flies of the genus *Simulium*, a complex of verifiably real species. This study was designed to determine the specific vector species composition of the areas using the morphological and cytotaxonomic identification techniques. Adult female flies were collected from four sites using human landing collection method and identified morphologically. Larval samples were obtained from breeding sites in two of the four sites and identified cytotaxonomically. A total of 3,118 flies collected for morphological studies were identified as forest flies, thus: Agbokim, 582 (18.6 %), Aningeje, 1224 (39.2 %), Ekong Anaku, 978 (31.3 %), and Orimekpang 334 (10.7 %). A total of 243 larvae, 97 (39.9 %) from Aningeje and 146 (60.1 %) from Ekong Anaku were cytologically studied. Clear and distinctive chromosomal bands were obtained from 31 (12.7 %) flies, 11 (35.5 %) from Aningeje and 20 (64.5 %) from Ekong Anaku. Among the Aningeje samples, 1 (9.1 %) was identified as *S. damnosum* s.s., another 1 (9.1 %) was identified as *S. sirbanum*. The remaining 9 (81.8 %) of the samples were identified as *S. squamosum*. In Ekong Anaku, 3 (15.0 %) out of the 20 karyotyped larvae were *S. damnosum* s.s., 1 (5.0 %) was *S. sirbanum* and 14 (70.0 %) were *S. squamosum* while 2 (10.0 %) were of a fourth species, *S. yahense*. The results of this study showed only members of the forest species group were identified using morphological identification but cytotaxonomic studies indicated both forest and savannah species groups were present. The severe savannah and the mild forest strains of onchocerciasis could be endemic in these areas and requires improved control efforts.

Keywords: *Simulium damnosum*, black flies, larvae, cytotaxonomy, savanna, forest

Introduction
Onchocerciasis is a parasitic infection caused by the filarial nematode *Onchocerca volvulus.* The disease is transmitted by infected female adult flies of the *Simulium damnosum* complex whose breeding occur in fast flowing rivers and streams [1]. Black flies are of public health and socio-economic importance in Nigeria and West Africa [2 - 4]. The prevalence of onchocerciasis is directly related to the abundance and distribution of members of the *S. damnosum* complex [5, 6].
Nine sibling species of *Simulium damnosum* complex have been documented in West Africa. These species include *S. damnosum sensu stricto, S. sirbanum, S. dieguerense*, *S. konkourense*, *S. leonense, S. sanctipauli*, *S. soubrense, S. squamosum* and *S. yahense* [7, 8]. The first three species are known as savannah flies which transmit the savannah strain of *O. volvulus,* causing the blinding, severe form of onchocerciasis while the rest belong to the forest group and transmit the forest strain of the parasite which cause the milder form, the onchocercal skin disease [9, 10]. In Nigeria, 6 members of the *S. damnosum* complex are responsible for the transmission of *O. volvulus*. These include *S. damnosum sensu stricto, S. sirbanum, S. leonense, S. soubrense, S. squamosum* and *S. yahense* [9, 11]. *S. damnosum* s.s and *S. sirbanum* are both widespread in the northern savanna. *S. squamosum* is widespread in forest-savanna mosaic/forest areas of Nigeria including southern Adamawa, Jos Plateau areas. It is common in the South West and has also been identified in Oji River area of Enugu State, South Eastern Nigeria [10, 12 – 15]. These three species are the most widespread vectors in Nigeria [10]. *S. leonense, S. soubrense,* and *S. yahense.* Usually inhabit large rivers but may extend into Guinea savanna areas with smaller watercourses during the rainy seasons. *Simulium yahense* is mostly associated with the rain forests. However, in Eastern Nigeria, they have been found in different biotypes [16, 17]. They breeds in small watercourses The major efforts to control river blindness started with the establishment of the Onchocerciasis Control Programme in West Africa (OCP) in 1974. Synthetic larvicides were applied to *Simulium* breeding sites. The OCP succeeded in interrupting transmission in virtually all the core savannah areas of the seven initial OCP countries [18 – 20], shifting the largest numbers of infected persons to Nigeria, Cameroon, Ethiopia, Uganda, and the Congo.
Vector control have long been discontinued as a result of the negative impacts of the pesticides in the environment [21, 22], non-target organisms especially in the aquatic ecosystem, logistics among other reasons.

Current efforts towards elimination of onchocerciasis benefits form accurate vector species identification.
The larval stage of black flies has played key role in their cytotaxonomic identification whereas adults serve better in morphological identification. Their larvae possess giant polythene chromosomes that are used for cytotaxonomic identification. Micro-morphological features of the giant polytene chromosomes such as banding patterns have been used for species identification [23, 24]. Inversions in the banding sequence of one or more of the 3 chromosome complements of black flies have also been widely used for studying chromosomal polymorphisms among species [10, 17, 25-29]. However, morphological identification of the adults, the actual life stage transmitting the infective causative agent of onchocerciasis [30], remains an important field tool for the identification of black flies, especially, to species group level (forest and savanna groups). Morphological features in adult black flies have become subject to environmental, physiological, and a host of other complex factors and may now vary even among members of the same species exposed to divergent conditions. This downplays the importance and effectiveness of morphological characters in black fly identification. However, morphological characters can reliably tell savannah from forest black flies. A combination of cytological and morphological features provides reliable identification results.

Materials and Methods

Study sites

Adult flies were collected from Agbokim (Lat. 5.90381, Long. 8.90762) in Etung Local Government Area (LGA), where the major breeding site is the Agbokim waterfall; Aningeje (Lat. 5.1389761, Long. 8.50808) in Akamkpa LGA, where the Kwa fall forms the major breeding site, Ekong Anaku (Lat. 5.10654, Long. 8.66240833) in Akamkpa LGA, with the Ekong Anaku river as the major breeding site and in Orimekpang (Lat. 6.10132, Long. 8.834505) in Boki LGA along the Afi river basin. Suitable larval black fly samples were obtained in Aningeje and Ekong Anaku only.

Collection of adult black flies

Human landing method of catching black flies was adopted as described earlier in Walsh [31] and WHO [32]. Two trained fly collectors caught flies alternately per community each day between 7 am and 6 pm.

Morpholgical identification of members of the *S. damnosum* complex

Morphological identification of man biting female black flies were carried out in the field using a dissecting microscope, under a white stage. Adult flies were identified morphologically as savanna or forest species based on the colour of the antennae, the colour of the fore-coxa, colour of the scutella setae, colour of the arculus, colour of the wing tuft, and the colour of the 9th abdominal tergite setae [33, 34, 35]. The antennae were considered as pale when at least four (4) basal segments are completely golden yellow in colour, else it is dark. The colour of the fore coxa was scored as pale when it is golden yellow in contrast to the thoracic sternites and as dark when it has the same colour as the thoracic sternites. The scutellar hairs were scored on the basis of their colour, pale when golden yellow in colour but dark when same colour as the rest of the scutal plate. The arculus was scored as pale if creamy-white, having nearly same colour with the wing membranes, and intermediate when light brown to dark tan or darkish black in colour [35]. The stem vein setae (wing tufts) were scored on a scale of A–E (A = all pale; E = all dark; B = up to five dark hairs; D = up to five pale hairs; C = mixed (pale and dark hairs); and O = character missing according to Kurtak, *et al.* [36]. The ninth abdominal tergite setae were also scored in a similar way but considering all mixtures together (A = all pale; C = mixed; E = all dark; and O = character missing), as described by Wilson, *et al.* [35]. A combination of these characters was cautiously used in separating the savannah species from the forest. Flies with pale features were considered as savanna flies while those with dark features were forest species.

Collection and preservation of larval

Larval prospection was carried out in the dry months (November – April) in Aningeje and Ekong Anaku because breeding sites were inaccessible during the rainy season. Larval samples were obtained from fast flowing sections of rivers/streams serving as breeding sites, for cytotaxonomic identification of sibling species of *S. damnosum* s.l. They were stored in freshly prepared Carnoy's fixative solution (1 part glacial acetic acid in 3 parts ethyl alcohol).

Preparation and staining of larvae for cytotaxonomic identification

The sixth and seventh larval stages were selected for cytotaxonomic identification. Their abdomen were opened up along the mid-ventral line and stained overnight in Lacto-acetic Rrcein stain, followed by chromosome slide preparation.

Preparation of chromosome slides

Large, stained nuclear regions on the silk gland containing the giant polytene chromosomes were picked, spread using the thumb and counter stained.

Karyotyping and Cytospecies Identifications

Full karyotyping and cytospecies identifications were based on the criteria of Boakye [27], and Post, *et al.* [17].

Each of the three chromosome complements was read under a compound microscope to visualize the micromorphological features for each larva. Chromosomal inversions and banding patterns were used in the identification of each larva.

Results

Vector collection and breeding sites

Productive breeding sites of black flies were found in the study areas. Significant changes were observed in water levels, river flow rates, and preponderance of breeding sites during the different seasons of the year. There were increases in these parameters during the rainy season and a decrease during the dry season. Figures 1 show vector breeding sites at one of the studied areas.

Fig 1: A productive breeding site located on the Ekong Anaku.

Morphological Identification of Adult Black Flies

A total of 3,118 adult flies were morphologically identified, all showed dark features and were as such identified as members of the forest species group. Percentage contribution of each community to the total identified flies is as follows: Agbokim, 18.6 % (582), Aningeje, 39.2 % (1224), Ekong Anaku, 31.3 % (978), and Orimekpang 10.7 % (334). No savanna flies were identified. Occasionally, flies were caught, having a random mixture of dark and pale wing tufts, antennae or forecoxa. These were still classified as forest flies since the proportion of dark features were higher than the pale. Secondly, those flies also maintained the other features of forest flies (table 1).

Table 1: Summary of morphological fly identification from all four study communities

Month	Agbokim		Anigeje		Ekong Anaku		Orimekpang		
	Savanna species	Forest species	Savanna species	Forest species	Savanna species	Forest species	Savanna species	Forest species	Monthly total (%)
January	0	15	0	100	0	40	0	0	155 (5.0)
February	0	68	0	124	0	56	0	52	300 (9.6)
March	0	30	0	79	0	76	0	18	203 (6.5)
April	0	34	0	87	0	75	0	10	206 (6.6)
May	0	46	0	70	0	60	0	20	196 (6.3)

June	0	67	0	170	0	93	0	23	355 (11.4)
July	0	54	0	80	0	118	0	56	304 (9.7)
August	0	66	0	87	0	140	0	35	328 (10.5)
September	0	50	0	99	0	68	0	43	260 (8.3)
October	0	89	0	138	0	122	0	77	426 (13.7)
November	0	0	0	87	0	50	0	0	137 (4.4)
December	0	63	0	103	0	80	0	0	246 (7.9)
Community total (%)	0	582 (18.6)	0	1224 (39.2)	0	978 (31.3)	0	334 (10.7)	100
Grant Total	3118								

Cytotaxonomic Identification

A total of 243 larvae were dissected for the cytotaxonomic study, 97 from Aningeje and 146 from Ekong Anaku. However, only a total of 31 were clear and distinctive enough for chromosomal studies. Eleven (11) larvae were karyotyped from Aningeje while twenty (20) were karyotyped from Ekong Anaku. Some sympatric association were found between the forest and savanna black fly groups in both communities.

In Aningeje, out of the 11 karyotyped *Simulium* larvae, 1 larva was identified as *S. damnosum* s.s., another 1 larval specimen was identified as *S. sirbanum*. The remaining 9 samples were identified as *S. squamosum* (table 2). In Ekong Anaku, 3 out of the 20 karyotyped larvae were identified as *S. damnosum* s.s., 1 was identified as *S. sirbanum* and 14 were identified as *S. squamosum* while 2 samples were found to be of a fourth species, *S. yahense*. The following fixed and diagnostic inversions were used as basis for separating each species from the others: members of the *S. damnosum* s.s were identified by the presence of the diagnostic inversion IIL-C/C or IIL-C/C.8. Members of *S. sirbanum* were identified by the inversions IIL-C.8/C.8, IIL-C.8.3/C.8.3 or IIL-C.8/C.8.3. Flies belonging to the *S. squamosum* species were diagnosed by the presence of the inversion IIL-18, IS-23. Members of the *S. yahense* were identified by the presence of the inversion IIL-18/18.61. These inversions and some others found in the study are shown in figures 4.6 to 4.6.7. Some of the inversions recorded in this study included IS-23, IS-To, IS-2, IL-3/3.12, IIL-C/C, IIL-C/C.8.3, IIL-C.8/C.8, IIL-18/18.61, IIL-18, IIS-6, IIS-ST IIIL-22 among others (Figures 2 – 7).

Table 2: Summary of cytological identification of vector species

Parameters	Anigeje	Ekong Anaku	Total
Number examined	97	146	243
Number identified	11	20	31
Number identified as forest species	9	16	25
% of forest species found	82	80	-
Number identified as savanna species	2	4	6
% of savanna species found	18	20	-
Forest species identified	*S. squamosum*	*S. squamosum, S. yahense*	-
Savanna species identified	*S. damnosum s.s, S. sirbanum*	*S. damnosum s.s, S. sirbanum*	-

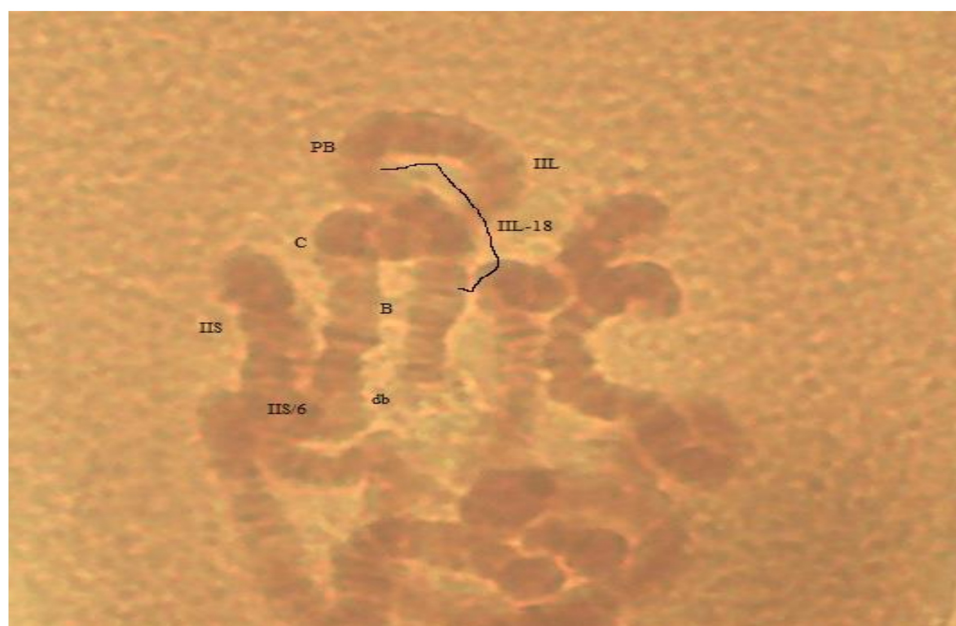

Fig 2: Complete chromosome complement of *S. damnosum* showing IIL-18 and IIS-6 homozygous inversions typical of *S. yahense* of the *squamosum* subcomplex. C = centromere,db = Double bubble, PB = Parabalbiani.

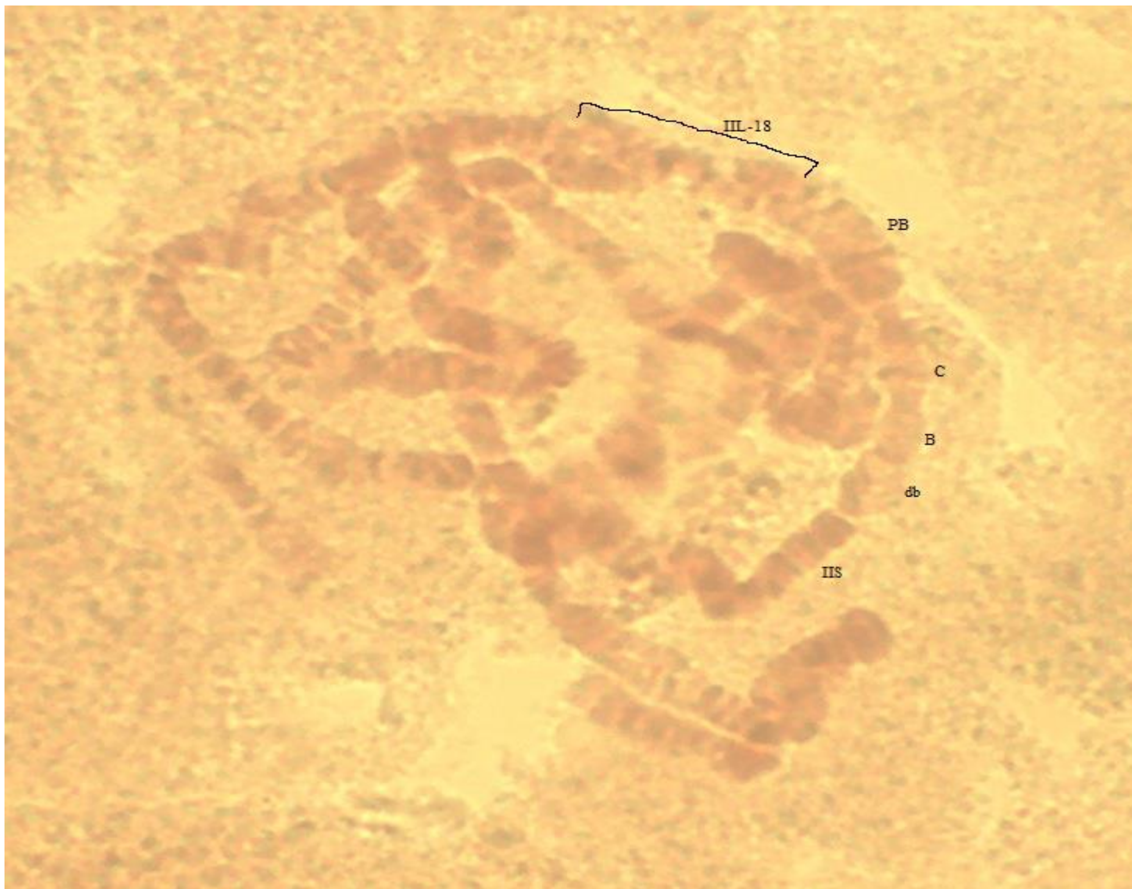

Fig 3: Complete chromosome complement of *S. damnosum* showing IIL-18 homozygous inversion typical of *S. squamosum.* C = centromere, db = Double bubble, PB = Parabalbiani.

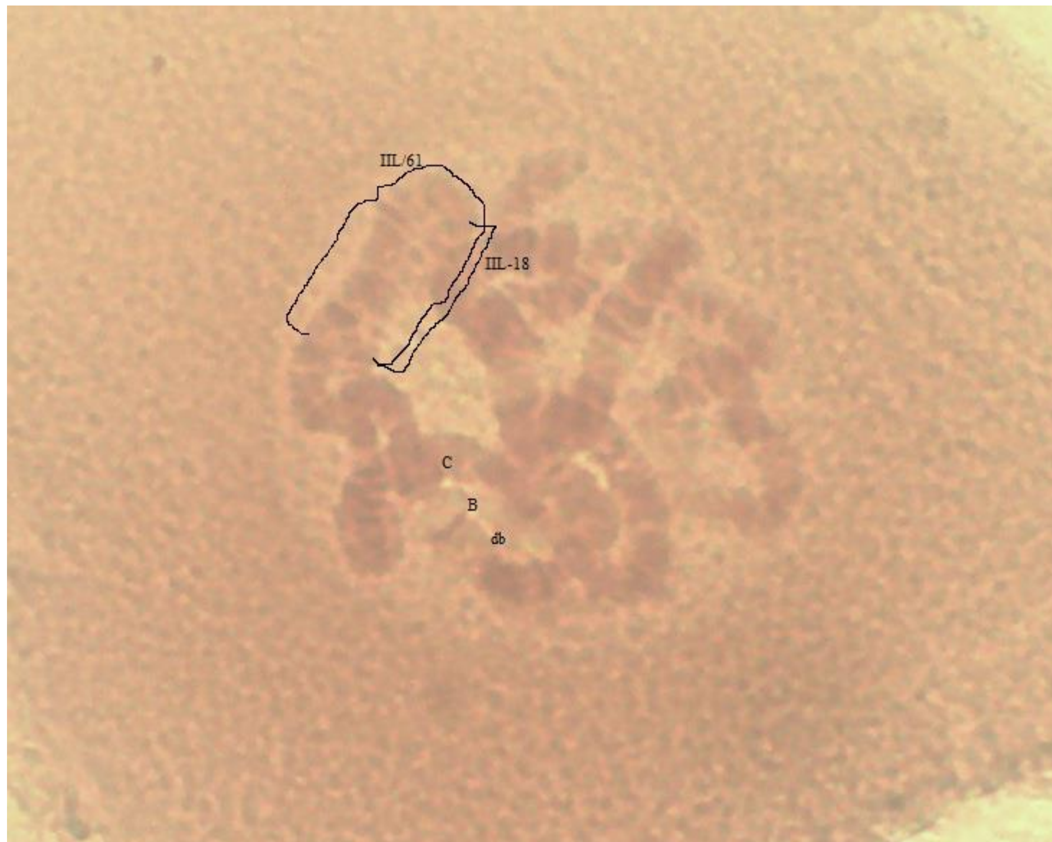

Fig 4: Complete chromosome complement of *S. damnosum* showing IIL-18 and IIL-61 heterozygous inversions found in *S. yahense* of the *squamosum* subcomplex. C = centromere, db = Double bubble, PB = Parabalbiani.

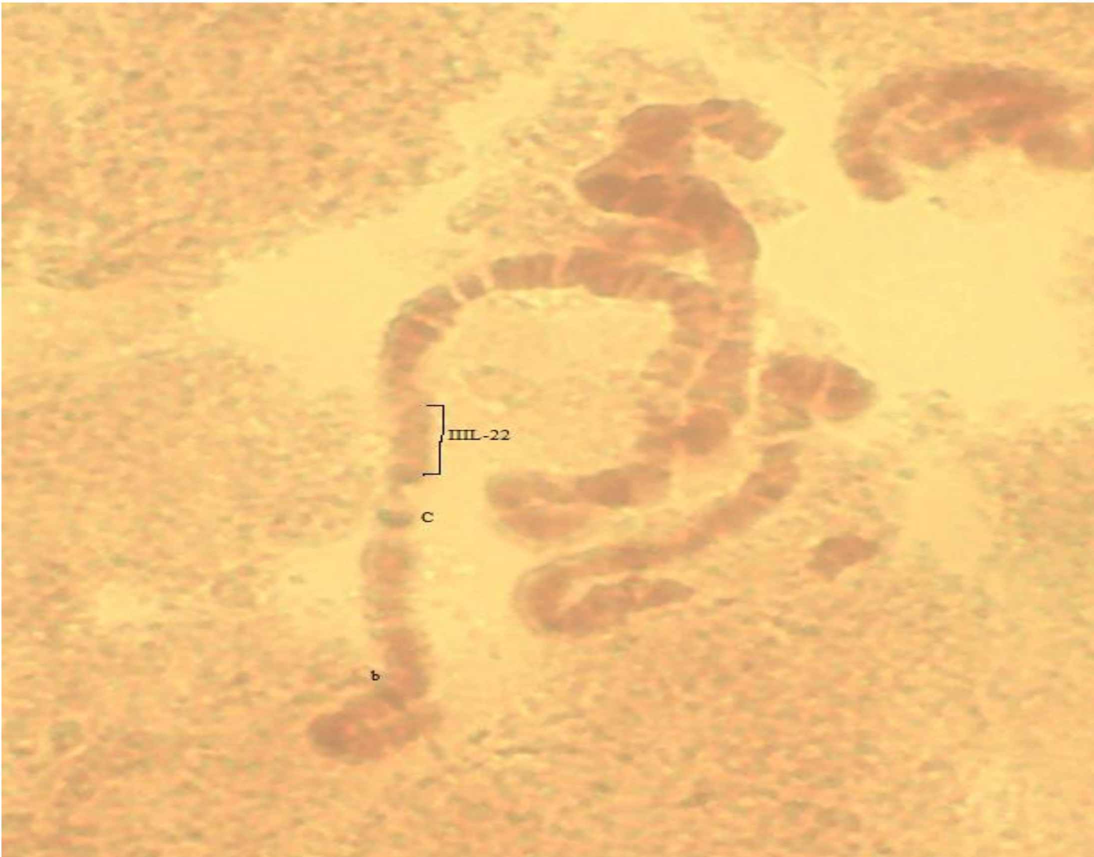

Fig 5: Chromosome III showing inversion IIIL-22, a floating inversion found in both *S. squamosum* and *S. yahense.* C = centromere, b = blister.

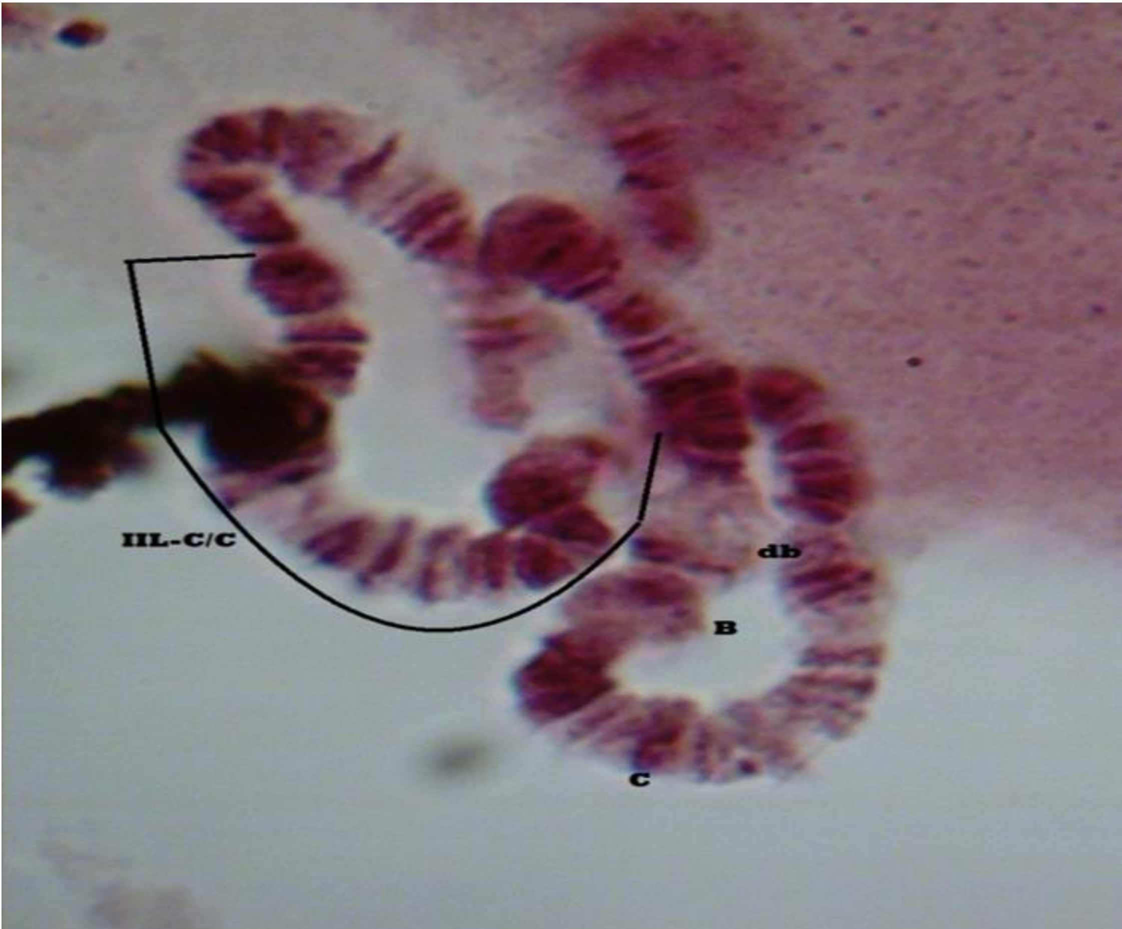

Fig 6: Chromosome II showing IIL-C/C homozygous inversion typical of *S. damnosum* s.s. C = centromere, B = balbiani ring, db = double bouble.

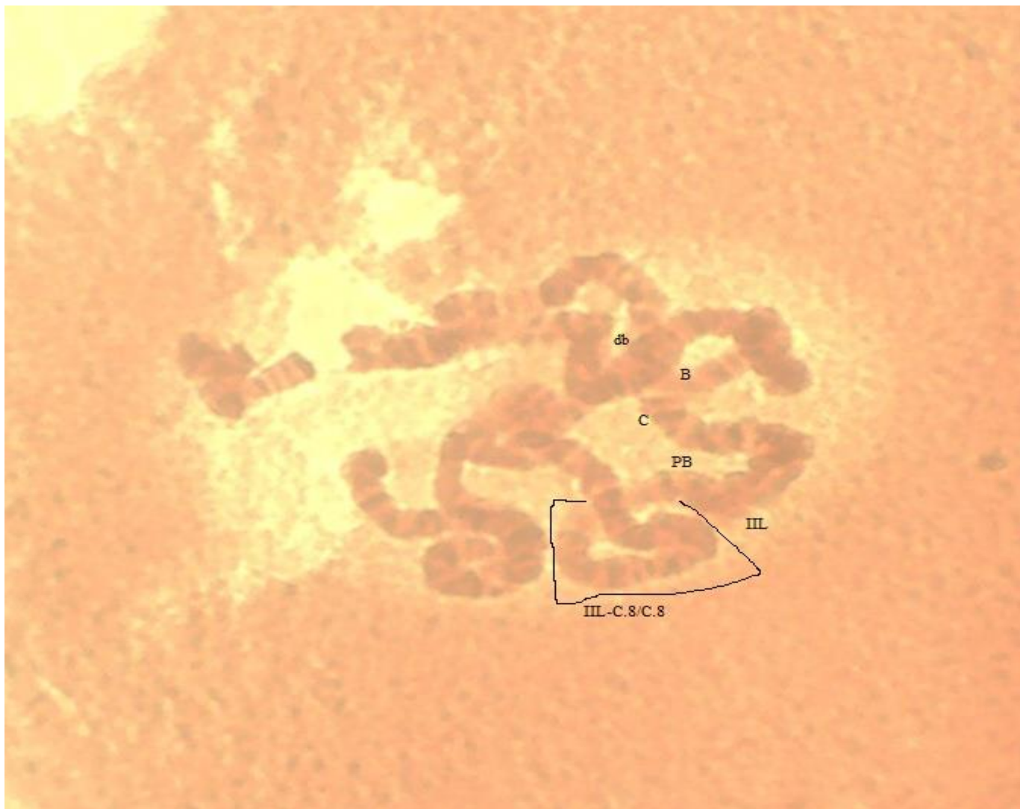

Fig 7: Chromosome II showing IIL-C.8 homozygous inversion typical of *S. sirbanum* of the *damnosum* subcomplex. C = centromere, db = Double bubble, PB = Parabalbiani.

Discussion

Cytotaxonomic identification of vectors of onchocerciasis in Aningeje and Ekong Anaku suggests that these communities could be endemic to both forms of onchocerciasis, namely: the forest/non-blinding form as well as the savanna/blinding form of onchocerciasis since both savanna and forest species of the vector were identified. Even though cytological identification was done for only a few larvae compared to the total number collected for this purpose, yet a combination of forest and savanna flies were identified.

Morphological identification, which was done on a larger number of flies could not identify a savanna fly. Instead, the result of morphological characters examined showed a mixture of both forest and savanna characteristic features in some flies, which were still classified as forest flies based on the proportion of savanna-forest characters, in line with published guides [27, 36, 37]. The current findings also supports reports of non-reliability of morphological characters in differentiating species of the black flies. Morphological characters are known to be highly variable and dependent on vector habitat as well as on environmental variables [14].

Suitable life stage of black fly larvae for cytotaxonomic studies were only obtained from two sites, namely: Anigeje and Ekong Anaku. There were either no identified breeding sites for *S. damnosum* complex in the other communities or these were not accessible at the time of prospection due to high levels of water or water current. Larval prospection was carried out in the dry season for safety reasons. Water levels were high and too risky to prospect in the rainy season. All larvae were collected during the dry season when water levels were low and breeding sites became easier to identify and reach.

Most samples were identified as *S. squamosum*, while few were identified either as *S. damnosum* s.s or *S. sirbanum* in Anigeje. In Ekong Anaku, *S. squamosum* also dominated, although *S. damnosum* s.s and *S. sirbanum* were also present. An additional fourth species, *S. yahense* was found from the result of cytotaxonomic investigations in Ekong Anaku.

The identification of savanna flies in these sites, known to be highly forested, is of serious epidemiological concern. It would have been possible to suggest that the savanna flies identified in this study could be migrant flies only if they were identified by morphological studies carried out on adult flies only. However, these flies were identified by cytotaxonomic investigation, conducted on larval samples obtained from the different breeding sites, indicating that these savanna flies are actually breeding in those locations. It appears that the savanna flies have become adapted to the forest conditions different from the conditions of the savanna areas for which they are known. The results of this study also suggest that our study areas which is in the tropical rain forest zone, could be suffering from the effects of climate changes due to deforestation and urbanization among others.

Therefore, it is important to carry out more cytological identifications especially with the dry season specimens to ascertain the frequency of occurrence of savanna species of black flies in these communities located in the rainforest belt. These findings could make a significant impact on the epidemiology of onchocerciasis in the

study areas, with the possible occurrence of both strains and forms of onchocerciasis. Savanna species of black flies are known to be efficient vectors of the severe blinding savanna strain of *Onchocerca volvulus* and inefficient vectors of the non-blinding forest strain, while the forest species on the other hand are known to be efficient vectors of the non-blinding forest strain of the parasite but not for the blinding savanna strain [37].

This study has revealed the presence of vectors of the blinding and non-blinding strains of onchocerciasis in the study areas. However, it is recommended that longitudinal cytotaxonomic studies be carried out to confirm these observations.

Conclusion

Onchocerciasis vectors are widely and actively abundant in the studied areas. Both forest and savannah species of black fly transmit the disease in the communities. This heightens concern that both mild and severe strain of onchocerciasis could be endemic to these rural communities of Cross River State and calls for modification of control/elimination strategies.

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Conflict of Interest

The authors declare that they have no conflicting interests.

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Ethics Statement

Not applicable

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