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Cytotaxonomic identification of *Simulium damnosum* complex (Diptera: Simuliidae) in Oji River area of Enugu state, Nigeria

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Abstract
Simulium damnosum complex is the major vector of onchocerciasis in Africa. Very little is known about the *S. damnosum* complex sibling species in Nigeria. Cytotaxonomic studies were therefore carried out to determine the distribution of these cytospecies in Oji River Area of Enugu State, Nigeria. Larval samples were collected from eleven breeding sites. The larvae were fixed in Carnoy's solution and chromosome preparations made in the laboratory. The cytospecies identified include *S. squamosum* (45.02%), *S. yahense* (30.58%), *S. damnosum* s.s. (21.65%) and *S. sirbanum* (2.75%). Their relative distributions were as follows: Oji Mile 2 (26.80%), Oji Ehuhe (19.59%), Oji Adu (17.18%), Oji Alum (22.34%) and Mmamu (14.09%). *S. damnosum* complex larval abundance was higher in the dry season (51.89%) compared to the rainy season (48.11%). This study has revealed the breeding of forest and savannah dwelling groups of *S. damnosum* s.l. involved in onchocerciasis transmission in the study area.

Keywords: Oji River; Onchocerciasis; Cytotaxonomy; *Simulium squamosum; Simulium damnosum*; *Simulium yahense*; *Simulium sirbanum;* Cytospecies distribution

Introduction
Onchocerciasis, also known as river blindness, is a vector-borne disease caused by the filarial worm *Onchocerca volvulus*. The disease is endemic in Central and South America and the Yemen, but 99% occurs in sub Saharan Africa, where it causes blindness and skin disease [1]. About 37 million people in tropical Africa and 140,000 others in Latin America are infected with *O. volvulus* [1, 2], mostly those living in poor rural communities. Thirty-six (36) countries are affected, 30 of them are in Africa, South of the Sahara, in a wide zone that lies from Senegal to Ethiopia. The endemic area extends from south of the equator to Angola in the west and Tanzania in the east. Localized foci exist in Sudan and Yemen [3]. In many endemic countries including Nigeria, onchocerciasis constitutes a major public health and socio-economic problem. It is a disabling disease that causes significant morbidity, psychosocial problems and reduced work, especially reduced agricultural productivity in populations affected by the disease [4]. The programs attempting to control onchocerciasis all currently rely primarily on community-wide treatment of the endemic populations with Ivermectin.
In the major *O. volvulus* endemic areas of West Africa, the vectors are blackflies of the *Simulium damnosum* Theobald species complex [1]. Black flies are not easily identified because they are species complexes composed of two or more morphologically similar species. Species complexes pose challenges for taxonomic and parasitological studies as well as control efforts [5]. Cytotaxonomic study using the banding patterns of the polytene chromosomes has long been used in the taxonomy of blackflies [6]. This involves identification using the natural banding pattern of the polytene chromosomes (giant chromosomes occurring in the salivary glands and other tissues of many Diptera) of the salivary gland of the larvae of Simuliids, observed under the microscope to reveal the existence of structural rearrangements otherwise known as inversions [7, 8]. Early studies in West Africa revealed nine cytoforms [9-11], with the various sibling species showing differences in their geographical distribution and in their roles as vectors of onchocerciasis [12]. Many subsequent studies have broadened the range of known differences between the sibling species, which affect their importance in the transmission of onchocerciasis [10-12]. Early findings have shown that there were geographical

variations among West African populations of *S. damnosum* s.l. and that these were related to savannah and forest environment [13, 14]. Several studies have shown that the savannah-dwelling vectors of onchocerciasis transmit the blinding form of onchocerciasis while the forest-dwelling vectors transmit the non-blinding form of the disease known as onchodermatitis [15-17].

The current strategy for controlling onchocerciasis in the mesoendemic Oji Local Government Area [18] is based on annual chemotherapeutic treatment of the communities with Ivermectin (Mectizan®), a microfilaricide, as there is no vector control. Oji River, which is the major water body in the area, supports intense breeding of blackflies but little is known about the *S. damnosum* complex sibling species involved in human onchocerciasis transmission in the area. An understanding of the taxonomy of the vector sibling species is important for understanding disease epidemiology and for the rational design of disease control programmes. Moreover, following the recent shift from onchocerciasis control to onchocerciasis elimination by the end of 2015, the strategy developed by the WHO-APOC for verification of elimination includes entomological evaluation with regular collection of cytotaxonomic data in all foci [19]. Moreover, developing an effective elimination strategy relies upon understanding of the bionomic differences of the sibling species of *S. damnosum* complex and accurately mapping the extent of endemic foci. Therefore, the present work is aimed at identifying *S. damnosum* s.l sibling species breeding in Oji River LGA, which is important in describing the entomological profile of human onchocerciasis transmission, imperative for successful elimination of human onchocerciasis in the area.

Materials and Methods

Study area

Oji River Local Government Area of Enugu state is a semi-urban area located in the forest zone of southeastern Nigeria. The area has a landmass of approximately 403km² and a population of 126,587 at the 2006 census. The geographical coordinates are 06o16'N and 07o16'E and it has an altitude of 140m. Oji River Local Government Area is made up of 6 communities namely Oji urban (Oji town), Achi, Ugwuoba, Inyi, Awlaw and Akpugo-eze.

Oji has two seasons: wet (rainy) and dry seasons. The wet season lasts approximately from April till October and is characterized by high humidity and strong rainfalls. The average annual rainfall is around 2000 mm. Heavy rainfall occurs between June and July, with an average of 3600 mm in July. The dry season is usually hotter than the rainy period and lasts from November till March. The lowest rainfall of about 1600 mm normally occurs in February. This period is also characterized by dry and dusty harmattan winds in January and February. The mean temperature range is usually between 26.8oC to 32.5oC over the year and an average relative humidity of 84%.

The major water body in the area is the perennial, well aerated and fast-flowing Oji River, a tributary of the Anambra River, which itself is a major tributary of the lower Niger River. Many rivers and streams traversing the Udi Hill escarpments flow into Oji River. Luxuriant growth of vegetation along the riverbanks and several geological formations in the riverbeds create rapids, providing suitable breeding sites for the *Simulium* flies in the area. The water is usually turbid with increased turbidity during the rainy season due to allochthonous inputs. The river is dammed at the Oji town to serve the Oji River Power Station and this also increased the speed of flow of the river.

Cytotaxonomic identification

Blackfly larvae were collected from eleven (11) riverine breeding sites. Collections were carried out from December 2011 to November 2012. Samples were randomly collected from multiple substrates and fixed in Carnoy's solution (95% ethanol and glacial acetic acid mixture of 3:1 (v/v)) in universal sample bottles. The samples were labeled and transported to the laboratory in cold chain for examination. Stained preparations of the polytene chromosomes from the larval silk glands (salivary glands) of *S. damnosum* s.l. were made on microscope slides, and they were scored for species-diagnostic inversions according to standard methods [20-22].

Data Analysis

Data analysis was performed on GraphPad Prism version 6. Difference among groups was analyzed by Two-Way analysis of variance (2-way ANOVA). Statistical significance was set at $p < 0.05$

Results

The 11 riverine breeding sites sampled in the study area include Oji Mile 2, Oji Wonderful and Oji Power Station in Oji Urban community, Oji Ehuhe, Oji Adu, Oji Enuguakwu and Oji Ahani in Achi community, Oji Alum, Mmamu, Achi-iyi in Inyi community and Ozom in Ugwuoba community. Of these 11 sites, only five (5) sites were found positive for *S. damnosum* s.l. larvae. The five (5) sites are Oji Mile 2, Oji Ehuhe, Oji Adu, Oji Alum and Mmamu.

Table 1: Distribution and abundance of *S. damnosum* complex cytospecies in the sites positive for *S. damnosum* complex larvae

SITES	CYTOSPECIES				TOTAL
	S. squamosum **n (%)**	***S. yahense*** **n (%)**	***S. damnosum*** **s.s n (%)**	***S. sirbanum*** **n (%)**	**n (%)**
Oji Mile 2 (OM)	39 (29.77)	13 (14.61)	21 (33.33)	5 (62.50)	78 (26.80)
Oji Ehuhe	19 (14.50)	26 (29.21)	9 (14.29)	3 (39.50)	57 (19.59)
(OE) Oji Adu	28 (21.37)	16 (17.98)	6 (9.52)	0 (0.00)	50 (17.18)
(OA) Oji Alum	26 (19.85)	20 (22.47)	19 (30.16)	0 (0.00)	65 (22.34)
(OAl) Mmamu	19 (14.50)	14 (15.73)	8 (12.70)	0 (0.00)	41 (14.09)
(MM)					
Total	131 (45.02)	89 (30.58)	63 (21.65)	8 (2.75)	291 (100)

Table 1 shows that a total of two hundred and ninety-one (291) *S. damnosum* complex larvae were successfully scored and identified during the twelve months study period. The cytospecies identified include *S. squamosum, S. yahense, S. damnosum* s.s. and *S. sirbanum.* The relative abundance of larvae identified from the five positive sites mentioned above is 78 (26.80%), 57 (19.59%), 50 (17.18%), 65 (22.34%) and 41(14.09%) respectively. Generally, *S. squamosum* was the most abundant and constituted 131 (45.02%) of the total collections followed by *S. yahense* 89 (30.58%) and *S. damnosum* s.s. 63 (21.65%). *S. sirbanum* 8 (2.75%) was the least abundant and was only present in Oji Mile 2 and Oji Ehuhe. There was statistically significant difference in the distribution of larvae present in the study by cytospecies ($p = 0.0002$). The difference in abundance of *S. damnosum* complex in the five sites was not statistically significant ($p = 0.2551$).

Table 2: Seasonal/monthly *S. damnosum* larval collections in different sites in Oji River Local Government Area

SITE	DRY SEASON					DRY SEASON TOTAL n (%)	WET SEASON							WET SEASON TOTAL n (%)
	NOV	DEC	JAN	FEB	MAR		APR	MAY	JUN	JUL	AUG	SEP	OCT	
OM	12	14	5	4	9	44 (29.14)	9	10	7	1	2	3	2	34 (24.29)
OE	7	8	3	2	4	24 (15.89)	9	10	6	1	2	3	2	33 (23.57)
OA	7	8	2	3	5	25 (16.56)	6	8	5	2	0	2	2	25 (17.86)
OAl	10	12	4	3	7	36 (23.84)	7	9	6	1	2	2	2	29 (20.71)
MM	6	7	3	2	4	22 (14.57)	5	5	3	0	1	1	4	19 (13.57)
TOTAL	42	49	17	14	29	151 (51.89)	36	42	27	5	7	11	12	140 (48.11)

Key- OM: Oji Mile 2, OE: Oji Ehuhe, OA: Oji Adu, OAl: Oji Alum, MM: Mmamu

Table 2 and Figure 1 show the seasonal and monthly *S. damnosum* complex larval collections. *S. damnosum* complex larvae were present during both wet and dry seasons and in all the months covered in the present study. However, majority of the *S. damnosum* complex larvae were collected during the dry season (51.89%) compared to 48.11% in the wet season. Comparison of the dry and wet season collection shows that there were no significant differences in the distribution by season ($p = 0.7730$). *S. damnosum* complex larvae were most abundant in the month of December followed by November and May, which had equal larval abundance. The lowest number of larvae was collected in the month of July. The monthly abundance of *S. damnosum* complex larvae at the different sites was significantly different ($p = 0.0249$).

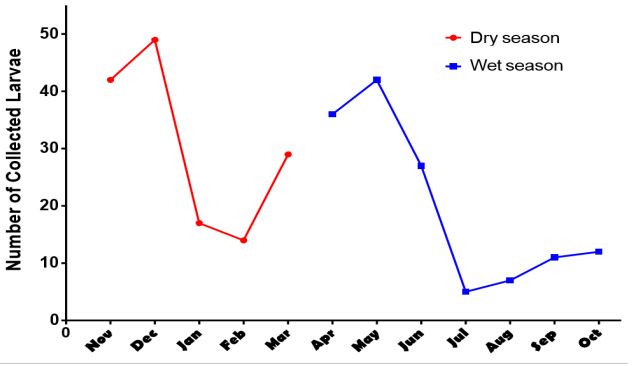

Fig 1: Monthly/Seasonal distribution of *S. damnosum* complex larvae

Table 3: Seasonal/monthly distribution of *S. damnosum* complex cytospecies

Cytospecies	DRY SEASON						WET SEASON							
	Nov	Dec	Jan	Feb	Mar	Dry Season Total N (%)	Apr	May	Jun	Jul	Aug	Sep	Oct	Wet Season Total N (%)
S. squamosum	19	28	12	6	9	74 (49.01)	11	18	5	2	3	8	10	57 (40.71)
S. yahense	18	14	0	5	10	47 (31.13)	8	10	15	3	4	2	0	42 (30.00)
S. damnosum s.s.	5	7	0	0	10	22 (14.57)	17	14	7	0	0	1	2	41 (29.29)
S. sirbanum	0	0	5	3	0	8 (5.30)	0	0	0	0	0	0	0	0 (0.00)
Total	42	49	17	14	29	151 (51.89)	36	42	27	5	7	11	12	140 (48.11

Table 3 and Fig 2 show the seasonal and monthly distribution of *S. damnosum* complex cytospecies in Oji River Local Government Area. *S. squamosum* was prevalent throughout all the months of the survey. *S. yahense* prevailed in all the months except January and October. Similarly, *S. damnosum* s.s. were also observed in all the months except January, February, July and August. *S. sirbanum* was found only in January and February. *S. squamosum, S. yahense* and *S. damnosum* s.s. prevailed during both dry and wet seasons, while *S. sirbanum* prevailed only during the dry season. Numerically, *S. squamosum* was the most abundant species all year round in both wet (40.71%) and dry season (49.01%).

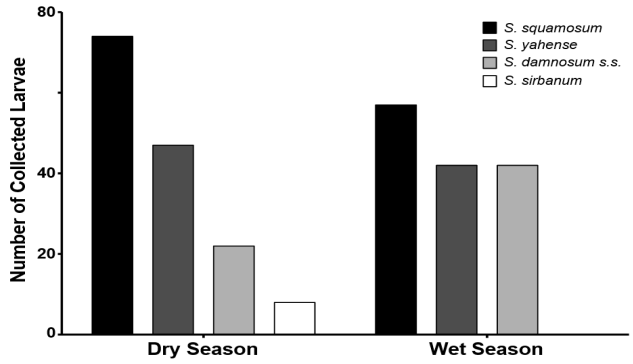

Fig 2: Seasonal distribution of *S. damnosum* complex cytospecies

Discussion

This study has further confirmed that some water bodies in Oji River Local Government Area support the breeding of *S. damnosum* species complex. There was a high number of breeding sites (forty-five percent) that were with *S. damnosum* complex larvae, which is an indication of intense breeding of *S. damnosum* complex in the area as well as preponderance of their breeding sites. The absence of *S. damnosum* complex in some of the likely habitats may be as a result of differences in the microhabitat conditions of these individual sites. For instance, during the study it was observed that at some of these negative sites like Oji Wonderful and Ozom, there was pollution of the water bodies with domestic wastes, and human and animal activities like defecation and washing of cassava and clothes were carried out in the water bodies. In these polluted waters, there may be an increase in ammonia concentration and a decline in dissolved oxygen, thereby reducing productivity and biodiversity. Also, at some of the negative sites, the fast flowing parts had very sparse substrates available for the adult female blackfly to oviposit. Moreover, some of the available breeding substrates were covered with algae which prevented attachment of eggs, and subsequently the larvae and pupal stages and this observation is in agreement with previous studies [23, 24].

This study has revealed that four cytospecies of *S. damnosum* complex breed in Oji River Local Government Area. They include *S. squamosum, S. yahense, S. damnosum* s.s. and *S. sirbanum.* The breeding of these cytospecies of *S. damnosum*

complex have also been reported by researchers from other onchocerciasis foci in Nigeria [10, 17, 25-34]. *S. squamosum* was the most abundant cytospecies in the study area. This is in agreement with observations made in West Africa [11], in Cameroun and Nigeria [30], and in south-eastern Nigeria [31, 32], who identified *S. squamosum* as being the predominant species at most breeding sites sampled. However, it contrasted with findings from River Eshi in Uzo-Uwani Local Government Area, Enugu State, Nigeria [33], where *S. sirbanum* was reported as the most abundant cytospecies. *S. squamosum* has been known to be very widespread across Nigeria and has been identified from mountain, rainforest and Guinea savanna zones [17]. *S. squamosum* is considered to be an efficient and important vector of *O. volvulus* in West Africa and has been known to have a patchy distribution in both West and Central Africa [30].

The variation in the pathology of onchocerciasis disease from one ecological zone to another has been attributed to the different sibling species of *S. damnosum* s.l transmitting different strains of *O. volvulus*. The presence of *S. damnosum* s.s. and *S. sirbanum* which are known to be a savanna species [35] in the study area, reveals the gradual migration of the typical savanna species into the forest area of Nigeria. It is most likely they came from the northern part of the country which predominately has a savanna vegetation type. Their existence in Oji LGA may be due to massive anthropogenic deforestation in this area for farming, logging and other agricultural activities [36]. Some studies have reported the impact of deforestation on the abundance and distribution of vectors of diseases [37, 38]. This has important epidemiological implications because it is possible that savanna onchocerciasis could get into this forest bioclimatic zone through the savanna vectors, bringing along with them, the blinding strain of *O. volvulus* [31, 32, 39, 40]. This may eventually lead to a change in the disease epidemiological pattern in Oji River area. *Simulium damnosum* s.s. and *S. sirbanum* are known to be strongly migratory species [41].

There were seasonal variations in the abundance and distribution of *S. damnosum* complex cytospecies. About fifty-two percent (52%) of the cytospecies were collected during the dry season. This concurred with previous results [31, 32] where higher numbers of *S. damnosum* complex were recorded in the dry season. The resultant high density of larvae during the dry season may be due to stability in the water level and rapid increase of breeding sites, low density of predators and the establishment of those species with high reproductive potential. In the present study, the month of December recorded the highest number of *S. damnosum* complex cytospecies while July when heavy rainfall occurs had the least number of larvae. The increased water level in July may have covered the available attachment substrates and also dislodged the aquatic stages of the flies from these substrates. Oviposition becomes affected due to the limited substrates that are will become available for the deposition of eggs and attachment of hatching out larvae.

It was also noticed during the present study that *S. squamosum* was the most frequently encountered and predominant species in both dry and wet seasons followed by *S. yahense*. They were however more prevalent during the dry season. This concurred with the findings of some studies in Nigeria [17, 32] where higher population of both cytospecies were recorded during the dry season. Unlike the forest species, *S. damnosum* s.s. was more encountered and prevalent during the wet than in the dry season. This was also in agreement with the findings from other localities in Nigeria [17, 32]. It should also be noted that *S. sirbanum* was present only during the dry season. This observation may reflect the scarcity of dry season breeding sites in the north resulting in their migration to the southern part of the country.

Conclusions

This study reveals the establishment and breeding of savannah-dwelling group of *S. damnosum* s.l. in the study area, which is primarily a rainforest zone. Their presence will consequently result in changes in epidemiology, transmission and pathology of onchocerciasis. There is therefore a need for systematic monitoring of any trend of change in transmission and epidemiology of onchocerciasis in the study area. This knowledge is crucial to the understanding of current risk and potential recrudescence in the transmission of the disease.

Abbreviations

WHO: World Health Organization

APOC: African Programme for Onchocerciasis Control s.l.: sensu lato s.s: sensu stricto

Competing interests

The authors declare that they have no competing interests.

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