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Identification of three members of the *Simulium damnosum* (Diptera Simuliidae) group in South Western Nigeria

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Summary
Identification of the specific *Simulium* species at play in the transmission of *Onchocerca volvulus* is important because it helps to explain the epidemiology and clinical presentation of the disease and guides in monitoring of long term impact of ivermectin treatment on onchocerciasis transmission. The study therefore sought to use molecular markers for vector identification. A combination of Polymerase Chain Reaction (PCR) assay and Restriction fragment Length polymorphism (RFLP) was used to identify members of S*imulium damnosum* complex in forest area of south western Nigeria. The results revealed the presence of three members of the *Simulium damnosum* complex: *S. yahense, S. sirbanum* and *S. damnosum* ss. *S. yahense* was the predominant and major vector in the study sites. This provides baseline data for future monitoring vector of human onchocerciasis in the area.

Keywords: *Simulium damnosum, onchocerca volvulus, Nigeria, molecular identification*

Résumé
Identification des espèces spécifiques de *Simulium* transmettant l'Onchocercose *volvulus* est important parce qu'il aide à expliquer *l'*épidémiologie et la présentation clinique de la maladie et guide a contrôler l'impact a long terme du traitement de l'Ivermectine sur la transmission de l'onchocercose. Cette étude utilisait les marqueurs moléculaires pour l'identification du vecteur. Une combinaison de la réaction en chaîne Polymérase (PCR) et la digestion enzymatique polymorphique (RFLP) était utilisée pour identifier les membres du S*imulium damnosum* complexe dans la foret au sud Ouest du Nigeria. Les résultats révélaient la présence de trios sous types de *Simulium damnosum* complexe; *S. yahense, S. sirbanum* et *S. damnosum* ss. *S. yahense était le plus* prédominant et le vecteur majeur dans les sites étudiés. Ceci apporte une donnée de base sur le contrôle future du vecteur de l'onchocercose chez l'humain dans cette région.

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Introduction
Onchocerciasis caused by *Onchocerca volvulus* is transmitted by members of *Simulium damnosum* complex. The disease affects 18 million people worldwide, with 350,000 blind and 6 million cases of skin diseases. The global incidence of new cases of blindness is 40,000 annually, with 120 million people at risk and 1.09 million Disability Adjusted Life Years (DALYs) lost annually. Ninety nine percent of the infected people are found in tropical Africa and Nigeria account for one quarter of the global Infection [1-4].

The six main West African members of the *Simulium damnosum* complex have been grouped in three pairs namely *S. yahense/ S. squamosum, S. damnosum ss/ S. sirbanum*; and *S. sanctipauli/ S. soubrense*; based on polytene chromosome analysis [5-7]. Members of the first group are referred to as forest species while the other two groups are savannah species. Two different stains *Onchocerca volvulus* parasites are recognized; the forest and the savannah [8-10]. The stain differences are reflected in different pathogenicity and antigenic peculiarities. The savannah form mainly manifest as the blinding type and forest form as skin diseases with less blinding [11].

Chemotherapy is presently the main thrust of controlling the disease. Mass treatment with ivermectin is the major control intervention in Nigeria for more than a decade. Ivermectin is a microfilaricide with little or no effect on the adult parasites, while ivermectin treatment reduces the microfilarial burden, the adult parasites continue to reproduce and a source for new infection to the human population. Ivermectin is recommended for use annually in endemic communities for 15 continuous years, being estimated life span of adult worm to ensure good impact on transmission through elimination of all adults worms harboured by infected individual [12].

Different methods used in identifying the *Simulium damnosum* complex includes cytotaxonomy [5-6,13-18], adult morphometry [6,17-26] gas liquid chromatography of cuticular

hydrocarbon[29-30], isoenyme analysis [27-28] and more recently DNA assay [6, 16-18, 31-36]. Cytotaxonomic analysis of *S. damnosum* s.l larvae collected from 23 sites across 4 bioclimatic zones in Nigeria revealed the presence of 5 cytospecies: *S. damnosum s.s., S. sirbanum, S. squamosum, S. yahense, S. souberense* (including the Beffa forms)[6]. The biogeographical distribution of the sibling species was reported as mostly similar to that observed further west of Onchocerciasis control programme (OCP) area[15, 30].

Cloned and isolated three anonymous middle repetitive DNA probes showed different patterns of hybridization to different sub complexes within the West African *S. damnosum* complex [31]. Polymerase Chain Reaction (PCR) of Internal Transcribed Spacer (ITS) of the nuclear ribosomal DNA, to distinguish the sibling species of the *S. damnosum* s.s, *S. sirbanum* and *S. squamosum* has been demonstrated [34]. DNA sequence of portion of the 16s rRNA and NADH dehydrogenase sub unit 4(ND4) gene have been used to distinguish the sibling species of *S. damnosum* complex based on heteroduplex analysis of PCR products derived from ND4 gene [33]. Other workers studied the phylogenetic relationship of East and West African species of the *S. damnosum* complex by analyses of nuclear and mitochondrial DNA sequences [36].

Entomological surveys in the southern (forest) area of Sierra Leone between 1983 and 1987 revealed a limited distribution of the savanna flies. However later survey of the blackfly breeding sites by the Onchocerciasis Control Programme (OCP) showed that these savanna species (*S. damnosum sensus stricto* and *S. sirbanum)* were widespread in southern parts of Sierra Leone [37]. Furthermore cytotaxonomic identification of larvae of different members of *S. damnosum* complex collected from (forest) rivers of southern Ghana and south western Togo from 1975 to 1977 revealed that *S. damnosum ss, S. sirbanum* in the samples had been increasing progressively since 1975 [38]. Early warning on such change might be obtained by regular identification of the biting fly population. This is required to monitor the distribution and distinguish the sibling species of *S. damnosum* complex from the blinding and non blinding strains of *Onchocerca volvulus*. The study, which formed part of larger study that included the epidemiology of onchocerciasis and transmission dynamics of *S. damnosun* sl aim at identifying the principal vector (species) involved in local transmission using molecular based assay.

Materials and methods

Collection of Simulium flies

Adult blackfly were collected on human bait near River Aponmu Owena Ose and Ayede Ogbese all within Ondo State, Nigeria. The flies were preserved in 70% ethanol and kept at 4^{0}C [39]. Allopatric population samples were collected from: Agokim Falls, Cross Rivers State for *S. yahense*; Tiga Dam, Kano State for *S. sirbanum* and Sabon-Pegi, Niger State for *S. damnosum* s.s [15,30-40]. These samples were used as positive control. Twenty adult flies were assayed for each site (ie subjected to PCR AND RFLP).

DNA extraction and amplification

DNA was extracted from individual specimens[31]. PCR amplification was subsequently carried [32]. PCR reactions to amplify the NADH dehydrogenase subunit 4 (ND4) region were carried out in buffer containing 60mM Tris-HCl (pH 8.5), 15mM $(NH_4)_2SO_4$, 200μM of each DNTP, 400nM of each of the primers and 2.0mM $MgCl_2$. The primers used in the amplification of the ND4 gene fragments were 5' ATCAGTTGTTGCTCATATG 3' (ND4-1) and 5' TAGCAGAACACAATAAA 3' (ND4-2). For primary PCR, 1μl of genomic DNA template was amplified in 25μl of solution containing buffer B and 1 unit of AmpliTaq DNA polymerase. PCR reaction consisted of an initial denaturation step consisting of heating at 95^{0}C for 3 minutes and 30seconds. This was followed by 40 cycles consisting of denaturation at 95^{0}C for 31 seconds, annealing at 45^{0}C for 50 seconds and extention at 72^{0}C for 40 seconds. The reaction was completed by incubation at 72^{0}C for 7 minutes.

Agarose gel electrophoresis

Twenty microliter of the PCR products was separated in 2.5% agarose gel stained with 1.5% ethidium bromide at 100V with a constant current of 120mA for One hour and visualized with ultraviolet transilluminator (Vilber Lourmat, 772002 Marne-la-Vallee Cedex 1-France).

Restriction fragment length polymorphism

Identification of the species present at each study site involved a further test on each amplified PCR products using the RFLP- PCR assay. 25μl PCR product (purified DNA) was digested with HPa II. The digested product was separated in 2.5% agarose gel, stained with ethidium bromide and visualized under an ultraviolet trans-illuminator and subsequently photograph as described above.

Results

The PCR assay amplified the subunit 4 of NADH dehydrogenase (ND4) genes common to all *Simulium* species. The initial amplification indicated that each amplified sample belong to the *S. damnosum* complex (Figure 1). However 10%, 13%, and 20% DNA samples from Ipogun, Ose and Ayede-Ogbese respectively were negative for *S. damnosum* complex and not included in the subsequent analysis.

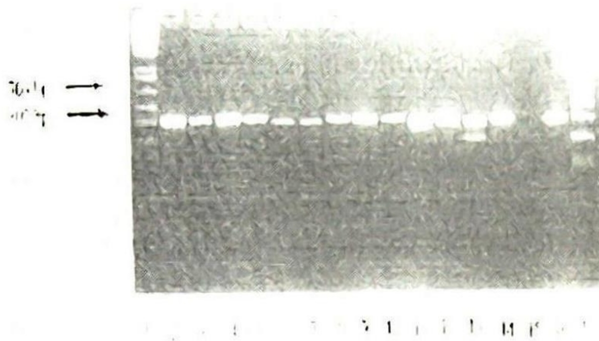

Fig. 1: DNA amplification of members of *Simulium damnosum* by Polymerase chain reaction (PCR)

Lane 1 and 17: 1Kb DNA ladder (standard)
Lane 2 and 3: test samples (Ipogun)
Lane 4 and 11: test samples (Ayede Ogbese)
Lane 7 and 12: test samples (Owena)
Lane 5 and 9 test samples (Ose)
Lane 6, 10, 13-14: positive control (S.damnosum s.s)
Lane 8: positive control (S. sirbanum)
Lane 16: positive control (S.yahense)
Lane 15: negative control (no DNA)

The amplified DNA were similar to the allopatric and test samples consequently each amplified product was subjected to RFLP-PCR for further analysis.

RFLP PCR identification

Restriction enzyme analysis of all amplified samples within the allopatric population revealed the presence of 3 species (Figure 2):

1. A 4 band restriction site corresponding to *S. yahense* allopatric population (lane 16).
2. A 3 band restriction site corresponding to *S. damnosum*, s.s. allopatric population (Lanes 6, 10, 13-14).
3. A 2 band restriction site corresponding to *S. sirbanum* allopatric population (lane 8 and 15).

Amplified test samples produced restriction fragments that correspond to *S. yahense*. None of the test samples correspond to *S. sirbanum* and *S. damnosum* ss.

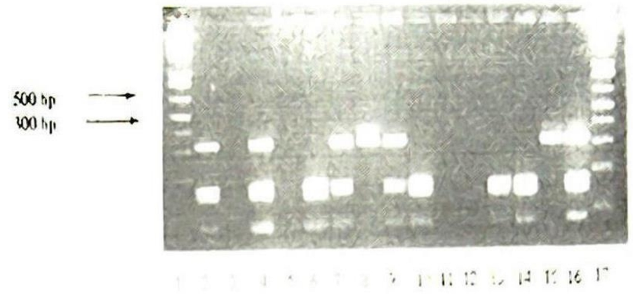

Fig. 2: Restriction enzyme (HpaII) digestion of PCR product of members of the Simulium damnosum complex

Lane 1 and 17: 1Kb DNA ladder (standard)
Lane 8 and 15: positive control (S. sirbanum)
Lane 16: positive control (S.yahense)
Lane 6, 10, 13-14: positive control (S. damnosum s.s)
Lane 2 and 3: test samples (Ipogun: S. yahense)
Lane 4 and 11: test samples (Ayede Ogbese: S. yahense)
Lane 7 and 12: test samples (Owena: S. yahense)
Lane 9: test samples (Ose: S. yahense)
Lane 5: negative control (no DNA)

Discussion

Different identification methods have been applied to distinguish various members of the *S. damnosum* complex [5-6,19,20]. In this study the combination of NADH-PCR and RFLP was useful in the identification of sibling species of *S. damnosum* complex. The RFLP-PCR revealed the presence of *S. yahense* as the principal vector at Ipogun (Aponmu), Owena, Ose and Ayede-Ogbese. The finding agrees with the cytotaxonomic investigation of other workers [6,15, 26] that *S. yahense* is one of the four forest species of the *Simulium damnosum* complex in Nigeria. Previous cytotaxonmomic studies [15, 26] reported the distribution of *S. yahense* only at 2 sites in South eastern Nigeria, although the author also reported *S. squamosum* as widespread species of in south western Nigeria. It is important that the specific species involved in local transmission be identified as local deforestation and possible invasion by

savanna vector sibling species might bring savannah stains of *O. volvulus* with them into forest zone and thus contribute to epidemiology of the disease in the area.

There is therefore the need to constantly monitor the geographical distribution of sibling species which will inform the control programme on vector species at play and help in formulating effective control measures. Cytotaxonomic identification of larvae of different members of *S. damnosum* complex collected from (forest) rivers of southern Ghana and south western Togo from 1975 to 1977 revealed that *S. damnosum ss*, *S. sirbanum* in the samples had been increasing progressively since 1975 [38]. Identification of the specific species of the vector at play in the transmission of the disease in a particular geographical area is important because it helps explain the epidemiology and clinical presentation of the disease and also guides monitoring of control progress.

The inability of the RFLP-PCR assay to identify 10% of the samples from three study sites is an area for further investigation. RFLP has the limitation of inability to distinguish *S. squamosum* from *S. yahense* as pointed by some workers [32, 36]. The application of other molecular biology technique such as the use of polymorphic DNA microsatelite markers could provide useful clues to understanding these important vectors.

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