Molecular diversity in the *Simulium damnosum* complex (Diptera: Simuliidae) in Cameroon

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Abstract: Der *Simulium damnosum*-Komplex ist eines der erfolgreichsten afrikanischen Taxa von Kriebelmücken. Diese Mücken übertragen Parasiten von Tier und Mensch, vor allem die Erreger der Onchozerkose (Flussblindheit). Um die Vielfalt der in Kamerun vorkommenden Arten dieses Komplexes zu erfassen, haben wir mit DNA-basierten Techniken und morphometrischen Methoden die phylogenetische Beziehung des *Simulium damnosum*-Komplexes an 26 Orten analysiert. Die Sequenzierung der Cox1 und ITS2-Genregionen zeigte überraschenderweise das Vorkommen von *S. sanctipauli* deren bekannte Verbreitung bislang auf Regionen westlich von Nigeria beschränkt war. Im Gegensatz dazu ist *S. squamosum*, die ursprünglich aus dem Sanaga beschrieben wurde, in vielen Gewässern und an neuen Standorten (Menchum-Tal, Mawong-Fluss und Benoue) mit mehreren Unterarten gut vertreten. Die in aus Westafrika beschriebene Zytospezies *S. yahense* wurde in unserer Studie nicht identifiziert. Eine noch undefinierte Gruppe aus der Zentralregion Kameruns (Bafia, Makouopsap, Bayomen und Konkwalla), und auch die Gruppe II aus dem Ntem-Tal im Regenwald Südkameruns nahe der Grenze zu Gabun könnten auf das Vorkommen von noch nicht beschriebenen Arten hinweisen und sollten Gegenstand weiterer Untersuchungen sein. In dieser Studie haben wir keine Individuen der *S. mengense*-Zytospecies gefunden, was jedoch sehr wahrscheinlich an der beschränkten Zahl von untersuchten Orten und Proben liegt. Insgesamt weisen die molekularen und morphologischen Resultate darauf hin, dass mindestens 4 Kladen (*S. squamosum*; *S. sanctipauli*; Klade I und Klade II) von Mitgliedern des *S. damnosum*-Komplexes vorkommen, die je nach geografischer Situation jeweils eine hohe Formenvielfalt aufweisen. Die exakte Identifizierung der jeweils vorkommenden Arten könnte bei der Planung geeigneter Vektorkontrollstrategien helfen. Die Vektorkontrolle wäre ein vielversprechendes Mittel zur Bekämpfung der Onchozerkose (Flussblindheit) in Koendemiegebieten von *Loa loa* und *Onchocerca volvulus*, wo die sonst übliche Massenbehandlung mit Ivermektin nicht indiziert ist.

Key words: *Simulium*, Cameroon, black flies, *Simulium damnosum* complex, *Simulium sanctipauli*

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Introduction

Black flies are present worldwide, there are 31 genera containing 2,348 species (2,331 living and 17 fossil species) (Adler 2022). In Africa, 124 black fly species were described, mostly in the Ethiopian region (McCrae 2008). In Cameroon, 55 species are morphologically described (Adler 2022) and are not yet all classified under various genera (Fain & Elsen 1973). Larvae and pupae are widespread in fast flowing water and tributaries. The females of most species are feeding blood on vertebrates, but only those of the *S. damnosum* complex are important vectors of *Onchocerca volvulus*, the causative agent of river blindness (Renz, Barthlmess & Eisenbeiss 1987) Besides of *O. volvulus*, blackflies transmit *Onchocerca ochengi,* the causative agent of bovine onchocercosis, respectively, *Leucocytozoon* to birds in Asia and North America (Akiba 1970) and numerous other filarial parasites of wild and domestic animals (*Onchocerca dukei*, *O. ramachandrini*, *O. lupi*, *Lappnema* spec.) (Li & al. 1998). The *Simulium damnosum* complex is the most important group and is largely distributed in all regions of Cameroon and little has yet been done by modern molecular genetic techniques. This study aims to apply DNA-based techniques to investigate the diversity of *S. damnosum* flies to better plan any vector control. In the present study, we used morphometry of pupal gills; antenna shape and of adult flies, we examined the Cox1 (Ruiz-Arrondo & al. 2018) with approximately 650 bp and the ITS2 (Kononov & al. 2016) region of about 400 bp to barcode and analyse the phylogenetic relationship of *Simulium damnosum* complex from Cameroon.

Materials and Methods

Sampling and Identification

Pupae of simuliids were collected from all available substrates (primarily trailing vegetation and stones) and adult female see flies were caught on humans in 29 localities from 2018 and 2022. (Fig.1 for sampling sites, Table 1 for the coordinates). Pupae were removed from substrates, cleaned using a fine brush and forceps and immediately preserved in 70 to 96% ethanol. Pupae were identified under a Wild M5 dissection and a Zeiss Axiophot compound microscope using standard keys as described by Freeman & de Meillon (1953). The adult females were classed according to their morphology (cf. Table 2). For this, the length, colour and shapes of the antenna were observed, in combination with the colour of post-cranial setae and the number of pale and dark hairs in the wing-tufts https://www.riverblindness.eu/onchocerciasis/simulium-vectors/adult-simulium-morphology/.

Molecular Procedures

The Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) was used to extract total genomic DNA from individual identified samples following the manufacturer's instructions. The mitochondrial protein coding gene (Cox1) which is approximately 650 bp long was amplified by Polymerase Chain Reaction (PCR) using the Lep primers forward (5'-ATTCAACCAATCATAAAGATATTGG-3') and reverse (5'-TAAACTTCTGGATGTCCAAAAAATCA-3') (Hajibabaei & al. 2006). For the internal transcribed Spacer 2 (ITS2) which is about 400 bp long, we used the forward primer (5′-TGTGAACTGCAGGACACAT-3′) and reverse (5′-ATGCTTAAATTTAGGGGGT-3′) (Kononov & al. 2016). All PCR reactions were performed in a final volume of 25 µl which comprised 2 µl of genomic DNA; 5 µl of Promega 5×DNAgo Buffer; 2 mM of $MgCl_2$; 0.25 mM of each dNTPs; 50 pmol for the forward and reverse primers and 1 U of Promega Taq Polymerase (Promega) (Kim & al. 2017). Amplifications were performed in a Master Cycler (Eppendorf Master Cycler). For the Lep primer PCR consisted of an initial denaturation (95 °C, 2 min) followed by 35 cycles (denaturation at 95 °C for 30s; annealing step at 51 °C for 30s and an extension at 72 °C for 60s) and a final extension at 72 °C for 5 min (Kim & al. 2017). For the ITS2, PCR consisted of an initial denaturation at 94 °C for 2 min, followed by 35 cycles (denaturation at 94°C for 40s; annealing step at 51 °C for 60s; extension at 72 °C for 60s) and a final extension at 72 °C, for 5 min (Prakash & al. 2006). The amplified genes were checked by electrophoresis using 1.5% agarose gel. Finally, PCR products were sent for commercial sequencing facilities (Macrogen, Netherland). Clear sequences with good chromatograms will be deposited in the GenBank. Sequence data were aligned using ClustalW on MEGA 7.0.26 with default parameter. Maximum likelihood analysis was performed in MEGA v7.0.14 (Krueger & Hennings 2006) with 1000 bootstraps as well as with calculation of pairwise p-distances.

Results

Sampling sites

Of 26 sampling sites in Cameroon, 16 (61.5 %), sites had larvae, pupae, or adult flies of members of the *Simulium damnosum* complex (Figure 1). Adult flies were morphologically classified into 3 groups (see Table 2): *S. mengense*, *S. yahense/squamosum* and *S. damnosum/sirbanum*.

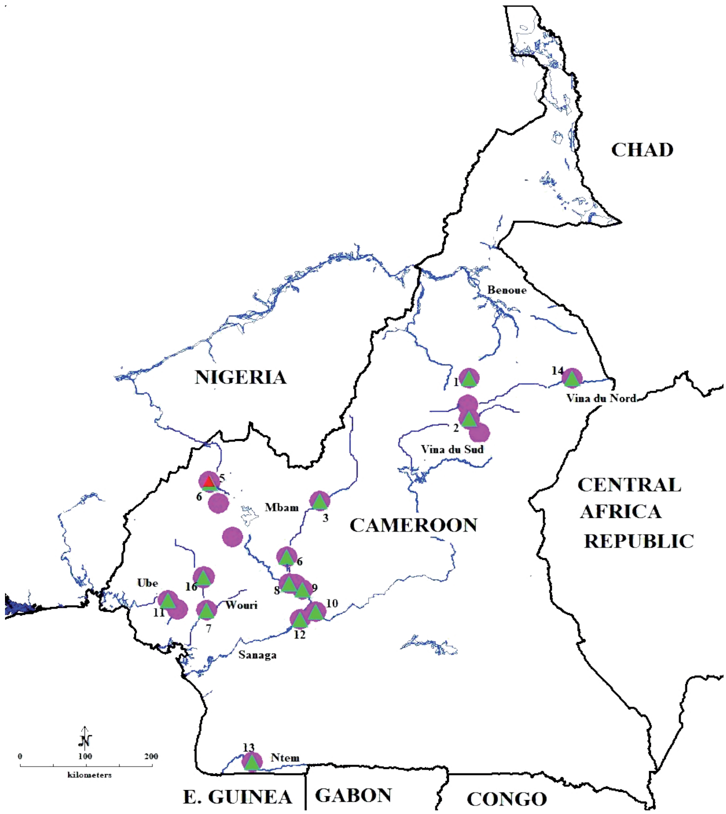

Fig. 1. Map of Cameroon showing sites at which larvae, pupae and adult female flies of the *Simulium damnosum* complex were collected (marked in green). Blue represent Cameroon water lines.

Table 1: Sampling sites coordinates

SN	Localities	Latitude	Longitude	Site	Date	Collector	Results
1	Benoue	7.7818	13.5888	Near Karna Manga	5 Sept 2014	Albert & David	pupae
2	Vina du Sud near Galim	7.2100	13.5862	Galim Pont	18 Jun 2020	David Ekale	Fem. ad. flies
3	Mbam near Bafia	6.046	11.538	Ferry	05.03.2022	Pierre Kamtsap	Fem. ad. Flies
4	Menchum near Befang	6.3070	10.0169	Menchum Falls	02 Aug 2018	Pierre Kamtsap	Fem. ad. Flies
5	Mawong river near Befang	6.3242	10.0037	Mawong	17 Nov 2017	Pierre Kamtsap	Pupae
6	Mbam	4.878	11.115	Near Bayomen	2022	Nana Djeuga	Fem. ad. Flies
7	Yabassi	4.496	9.971	Konkwalla	2022	Nana Djeuga	Fem. ad. Flies
8	Mbam	5.2551	11.0771	Near Makouossop	2022	Nana Djeuga	Fem. ad. Flies
9	Sanaga near Ebebda	4.3664	11.2676	Bridge	04 Mar 2022	Pierre Kamtsap	Fem. ad. Flies
10	Yabassi	4.79	11.29	Ndopoo	2022	Nana Djeuga	Fem. ad. Flies
11	Ube	4.70	9.35	Near Kumba		Peter Enyong	Fem. ad. Flies
12	Sanaga near Saa	4.4752	11.4735	Bridge	04 Mar 2022	Pierre Kamtsap	Fem. ad. Flies
13	Ntem valley	2.3394	10.6027	Near Ma'an	07 Nov 2020	Pierre Kamtsap	Fem. ad. Flies
14	Vina du Nord	7.7870	15.0073	Soramboum	13 Oct 2016	David Ekale	Fem. ad. Flies
15	Nkongsamba	4.9627	9.9342	Near Regional Hospital	29 Dec 2021	Pierre Kamtsap	Pupae

Table 2. Morphological classification of *S. damnosum* complex

Antennae	*S. mengense*	*S. yahense / squamosum*	*S. damnosum/ sirbanum*
Form			
Description	Very seldom any compression of the segments	segments 4 (and) 5 distinctly compressed	Segments 4 and 5 always compressed, often other segments also
Colour	All segments dark	First 2(3) segments pale	First 4 segments normally pale
Antenna /thorax ratio	1.80 – 2.04 (median 1.96)	1.96 – 2.25 (median 2.15)	2.05 – 2.40 (median 2.20 – 2.30)
Wing-tufts color	All dark	*S. yahense* most dark *S. squamosum* dark, mixed or pale	Almost all pale
Postcranial hairs color	Almost all hairs very dark	Typically many dark or grey, but often most hairs pale. Sometimes protruding	Typically, all pale, lying flat on the head. Few dark. *S. sirbanum* might be distinguished from *S. damnosum* by shorter antennae and paler hairs
Geographic distribution	Rain-forest - but also savanna (Meredith, Lamizanat, pers. communication)	Rain-forest, Guinea and Sudan savanna. Populations in the rain-forest are distinct, but interbreeding in the savanna	*S. damnosum*: rain-forest and savanna *S. sirbanum*: Sudan-savanna (dry season)

<u>Sequences analysis</u>

Sequences with good chromatograms were selected and included in the analysis. The internal transcribed spacer 2 (ITS2) barcode tree (Figure 2. (b)) shows the presence of species belonging to two of the morphological classification (*S. yahense/squamosum and S. damnosum/sirbanum*) and formed four clades. Samples from the deep forest along the Ntem valley in the South Region of Cameroon are in a very clear distinct clade. Similarly, the mitochondrial DNA barcode tree (Figure 2. (a)) also forms 4 clades and for similarities (due to lack of information in the literature), the morphologically identified groups are minimally specified.

Discussion

Integrated studies are necessary for the resolution of taxonomic problems in Simuliidae and ideally involve morphological and molecular analysis. All stages are difficult to distinguish on the basis of their morphology and even still, these data sets are not always in agreement or equal in their ability to resolve similar species (Adler & al. 2019). The molecular identification of our collected samples indicated for the first time the presence of *S. sanctipauli* which is known to occur in West Africa (Mafuyai & al. 1996). In contrast, *S. squamosum* described (AY695925) from the Vina du Sud falls near Galim is well present in this area and at others new sites (Menchum valley, Mawong river, and Benoue) of Cameroon. The cytospecies forming the *S. yahense* clade had been described in Nigeria, Benin, and Cameroon (Mafuyai & al. 1996) but were not identified in our study. The undefined clade collected in the Center Region of Cameroon (Bafia; Makouopsap; Bayomen; and Konkwalla) and also the clade II from the Ntem valley in the rain forest of Southern Cameroon close to the border to Gabon could indicate the occurrence of a not yet described species and should be subject of further studies. In this study, we did not find any *S. mengense* group but we guess that this could be if more sites were sampled. Taking as a whole, molecular information indicates to date 4 clades (*S. squamosum*; *S. sanctipauli*; Clade I and Clade II) of members of the *S. damnosum* complex, each containing a high diversity of forms depending on the geographical situation and genetic behaviour.

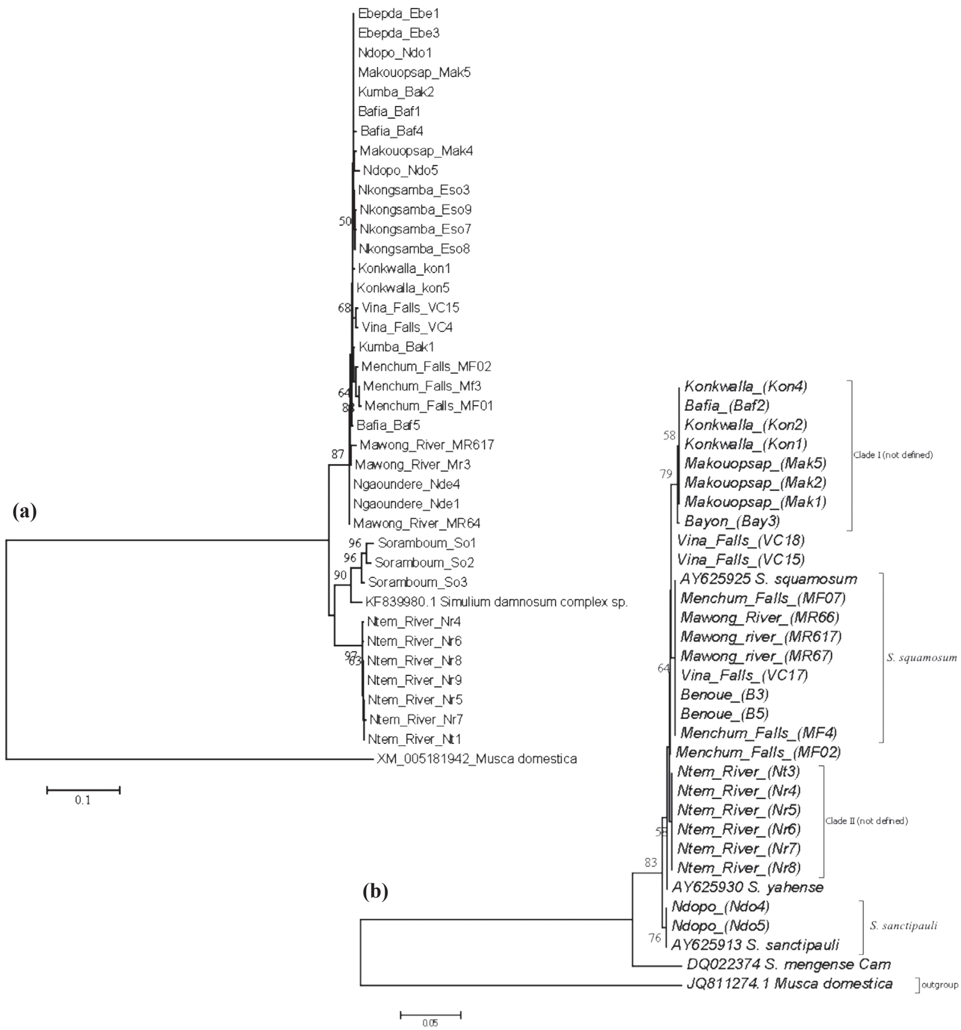

Fig. 2. Molecular Phylogenetic analysis by Maximum Likelihood method Cox1 (a); ITS2 (b)
Initial tree (s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 39 (Cox1) and 32 (ITS2) nucleotides sequences. There were a total of 673 positions (Cox1) and 514 (ITS2) in the final dataset.

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