Taxonomy and control of *Simulium* black flies, vectors of onchocerciasis in Cameroon

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von
MPH. Pierre Kamtsap
aus Mankon/Kamerun

Tübingen

2025

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:	25.07.2025
Dekan:	Prof. Dr. Thilo Stehle
1. Berichterstatter:	PD. Dr. Alfons Renz
2. Berichterstatter:	Prof. Dr. Oliver Betz

Dedication

To my beloved wife, **Paule Julie Ngouemeta Tse Epse Kamtsap**

Your unwavering love, patience, and strength have carried me through the most challenging moments of this journey.

Thank you for believing in me, even when I doubted myself.

This achievement is as much yours as it is mine.

Acknowledgments

I would like to express my sincere gratitude to my supervisors, PD Dr. Alfons Renz, Prof. Dr. Oliver Betz for their invaluable guidance, support, and mentorship throughout this research. Their expertise has been instrumental in shaping my work.

Special thanks to the academic staff and students of the Evolution and Ecology Research School Tübingen (EVEREST), and to Prof. Katharina Foerster for providing laboratory facilities. I am also grateful to PD Dr. Adrian Streit and Ms. Dorothee Harbecke at the Max Planck Institute for Biology in Tübingen for additional laboratory support.

I sincerely thank Dr. Archile Paguem (University of Buea) for his assistance in the molecular laboratory. I also acknowledge the Cameroon Ministry of Public Health for approving the research and particularly thank Mr. Noel Ngazoa Onana for facilitating the necessary authorizations.

My appreciation goes to Dr. Peter Enyong and Dr. Hugues Nana Djeunga for providing essential samples, as well as Mr. David Ekale and Mr. Jeremi Yembo from the Onchocerciasis Program Station in Ngaoundéré for their collaboration.

This work was supported by DFG-COBE: RE-1536/ff; the German Society for General and Applied Entomology (DGaaE); the Medical Research Council and CRID (Grant MR/P027873/); the Onchocerciasis Program Station in Ngaoundéré; and the state of Baden-Württemberg through the University of Tübingen (WS-KAMTSA-2021-02).

Heartfelt thanks to my children for their patience and understanding during my time away, and to my siblings, especially Marie Charlotte Takala, for their constant love and encouragement.

Finally, I am deeply grateful to my dear friends in general, Jimmy Selatsa Tchoffo, and Tigori Kobenang in particular for their unwavering moral support and friendship throughout my PhD journey.

Table of contents

List of abbreviations

Abbreviation	Meaning
%	Percent
1st	First
3rd	Third
APOC	African Programme for Onchocerciasis Control
APOD	Acute Papular Onchodermatitis
bp	Base Pairs
Bti	*Bacillus thuringiensis* var *israelensis*
BWS-plus	Baden-Württemberg Stiftung Plus
CDTI	Community Directed Treatment with Ivermectin
CI	Confidence Interval
Cox	Cytochrome Oxidase
Cox1	Cytochrome Oxidase Subunit 1
CPOD	Chronic Papular Onchodermatitis
CRID	Center for Research in Infectious Diseases
D	Day
DDT	Dichlorodiphenyltrichloroethane
DEC	Diethylcarbamazine
DFG	Deutsche Forschungsgemeinschaft
DGaaE	German Society for General and Applied Entomology
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ESPEN	Expanded Special Project for Elimination of Neglected Tropical Diseases
EVEREST	Evolution and Ecology Research School Tübingen
GPS	Global Positioning System
h	Hour
HDs	Health Districts
IgG	Immunoglobulin G
IQR	Interquartile Range
ITS2	Internal Transcribed Spacer 2
IUs	Implementation Units

KABP	Knowledge, Attitudes, Beliefs, and Practices
KOH	Potassium Hydroxide
L	Larva
LAMP	Loop Amplification
LC50 / LC90	Lethal Concentration 50% / 90%
LOD	Lichenified Onchodermatitis
ltr	Liter
MDA	Mass Drugs Administration
ME	Minimum Evolution
MEGA	Molecular Evolutionary Genetics Analysis
Mff	Microfilariae
min	Minutes
ML	Maximum Likelihood
Nber	Number
NTD	Neglected Tropical Disease
O	*Onchocerca*
OAE	Onchocerciasis-Associated Epilepsy
OCP	Onchocerciasis Control Programme in West Africa
OEAP	Onchocerciasis Elimination for the Americas
OSD	Onchocercal Skin Disease
Ov	*Onchocerca volvulus*
p	P-value
PC	Preventive Chemotherapy
PCR	Polymerase Chain Reaction
POC	Programme for Onchocerciasis Control
ppm	Parts Per Million
PTS	Post-Treatment Surveillance
RAPD	Random Amplified DNA Polymorphisms
RDT	Rapid Diagnostic Test
REMO	Rapid Epidemiological Mapping for Onchocerciasis
s.l.	Stricto lato
SAEs	Serious Adverse Events

WHO	World Health Organization
X2	Chi Square
yrs	Years

Summary

This doctoral thesis, titled "**Taxonomy and Control of *Simulium* Black Flies, Vectors of Onchocerciasis in Cameroon**," focuses on a neglected tropical disease that poses significant public health challenges in many regions of Cameroon. Onchocerciasis, commonly known as river blindness, is caused by the filarial worm *Onchocerca volvulus* and transmitted by black flies of the genus *Simulium*. Despite over two decades of mass ivermectin distribution, limited means of effective vector control has allowed the disease to persist in endemic communities.

The aim of the study was to identify and genetically analyse the diversity of *Simulium* species, and to assess the efficacy of commonly used insecticides, Temephos and *Bacillus thuringiensis* var. *israelensis* (*Bti*) to control the aquatic larval stages of the flies. Surveys conducted in four endemic communities (Mawong, Befang, Soramboum, Galim) revealed that while 90% of respondents perceived black flies as a nuisance, only 9.3% associated their bites with the risk of blindness. Over 1,100 *Simulium* larvae and pupae were collected and analysed using morphological characteristics and molecular markers (ITS2 and Cox1 genes). The study identified 19 *Simulium* species, including two previously undetermined ones, with *S. vorax* and *S. dentulosum* reported for the first time in Cameroon as potential vectors.

Larvicidal tests showed that black fly larvae in Cameroon were more susceptible to Temephos and *Bti* than European species, though continuous resistance monitoring is recommended. The study emphasizes the need for integrated vector control strategies, especially in areas where ivermectin cannot be used due to co-endemicity with *Loa loa*, which poses serious health risks.

Combining morphological and molecular tools significantly advances the understanding of black fly diversity and provides critical data for improving onchocerciasis control efforts in Cameroon and similar endemic regions.

Zusammenfassung

Diese Dissertation mit dem Titel **„Taxonomie und Bekämpfung der Kriebelmücken als Überträger der Onchozerkose in Kamerun“** widmet sich der Erforschung einer vernachlässigten Tropenkrankheit, die in vielen Regionen Kameruns eine erhebliche Gesundheitsbelastung darstellt. Die Onchozerkose („Flussblindheit“) wird durch den Fadenwurm *Onchocerca volvulus* verursacht und durch Kriebelmücken der Gattung *Simulium* übertragen. Trotz jahrzehntelanger Ivermectin-Massenbehandlungen bleibt die Krankheit in vielen Endemiegebieten bestehen – unter anderem aufgrund unzureichender Vektorkontrolle und fehlender Aufklärung.

Ziel der Arbeit war es, die aktuelle sozi-entomologische Lage zu analysieren, die Artenvielfalt der Vektoren zu erfassen, ihre genetische Diversität zu untersuchen und die Wirksamkeit gängiger Larvizide wie Temephos und *Bacillus thuringiensis* var. *israelensis* (*Bti*) zu bewerten.

In vier endemischen Dörfern (Mawong, Befang, Soramboum, Galim) wurden Befragungen und Larvenproben durchgeführt. Dabei zeigten 90 % der Befragten eine starke Belästigung durch Kriebelmücken, jedoch wussten nur 9,3 % um deren Zusammenhang mit Blindheit. Über 1.100 Larven und Puppen wurden aus nahegelegenen Fließgewässern entnommen und mittels morphologischer und molekularer Marker (ITS2, Cox1) untersucht. Insgesamt wurden 19 Arten identifiziert – darunter zwei bisher unbekannte sowie erstmals in Kamerun nachgewiesene potenzielle Vektoren wie *Simulium vorax* und *S. dentulosum*.

Larvizid-Tests bestätigten die Wirksamkeit von Temephos und Bti, offenbarten jedoch regionale Unterschiede in der Empfindlichkeit. Diese Ergebnisse betonen die Notwendigkeit standortspezifischer Vektorkontrollstrategien und gezielter Aufklärungsmaßnahmen – insbesondere in Regionen, in denen der Einsatz von Ivermectin aufgrund einer Ko-Endemie mit Loa loa eingeschränkt ist

Diese Arbeit liefert eine wichtige Grundlage zur Taxonomie der Kriebelmücken, zeigt praktikable Bekämpfungsstrategien auf und trägt damit zur Verbesserung der Gesundheitsversorgung in betroffenen Regionen bei.

Résumé

Cette thèse de Doctorat intitulée « **Taxonomie et contrôle des mouches noires du genre *Simulium*, vectrices de l'onchocercose au Cameroun** », porte sur une maladie tropicale négligée qui touche fortement certaines régions camerounaises. L'onchocercose, ou « cécité des rivières », est causée par le ver filaire *Onchocerca volvulus* et transmise par les piqûres de mouches noires (*Simulium* spp.). Bien que la distribution massive d'ivermectine ait été mise en œuvre depuis plus de deux décennies, les mesures de lutte vectorielle restent limitées, ce qui a entraîné la persistance de la maladie dans plusieurs zones endémiques.

Cette recherche visait à actualiser les données épidémiologiques sur l'onchocercose, à identifier et analyser la diversité génétique des espèces de mouches noires, et à évaluer l'efficacité de larvicides tels que le téméphos et *Bacillus thuringiensis var. israelensis* (*Bti*). Des enquêtes ont été menées dans quatre communautés endémiques (Mawong, Befang, Soramboum, Galim), révélant que si 90 % des participants considéraient les mouches comme nuisibles, seulement 9,3 % savaient qu'elles pouvaient causer la cécité. Plus de 1 100 larves et nymphes ont été collectées puis analysées morphologiquement et génétiquement (gènes ITS2 et Cox1). Au total, 19 espèces de *Simulium* ont été identifiées, dont deux (non identifiées) nouvelles pour la science, et *S. vorax* et *S. dentulosum*, confirmées pour la première fois comme vecteurs potentiels au Cameroun.

Les tests de sensibilité larvaire ont montré une efficacité des larvicides, avec une sensibilité plus élevée aux produits au Cameroun qu'en Europe. Toutefois, la surveillance de l'apparition d'une résistance est recommandée. L'étude souligne l'importance de la lutte vectorielle dans les zones où l'ivermectine est contre-indiquée, notamment en cas de co-endémicité avec *Loa loa*.

En combinant des approches morphologiques et moléculaires, ce travail apporte une contribution majeure à la compréhension des vecteurs de l'onchocercose et à l'élaboration de stratégies de contrôle plus efficaces et adaptées au contexte camerounais.

List of publications

Accepted/published publications

Included in this thesis

1. **Kamtsap, P.**, Paguem, A., & Renz, A.: **Molecular diversity in the *Simulium damnosum* complex (Diptera: Simuliidae) in Cameroon**. *Mitteilungen der Deutschen Gesellschaft für Allgemeine und Angewandte Entomologie*, 2024, **23**: 147-152.

2. **Kamtsap P.**, Paguem A., Nguemaïm Ngoufo F., Renz A.: **Morphological and Molecular Investigation of Non-*Simulium damnosum* Black Flies in Cameroon Using Nuclear ITS 2 and Mitochondrial Cox 1 Genes**. *Insects*, 2025; **16**(6):572-592, https://doi.org/10.3390/insects16060572

3. **Kamtsap P**., Nguemaïm Ngoufo F., Paguem A., Renz A.: **Knowledge and Practices of Four Onchocerciasis-Endemic Communities in Cameroon. Microorganisms**, 2025; 13(4):736-750, doi: 10.3390/microorganisms13040736

4. **Kamtsap P.**, Paguem A., Nguemaïm Ngoufo F., Njiokou F., Renz A.: **Testing the susceptibility of larval stages of *Simulium* to temephos and *Bacillus thuringiensis* var *israelensis* in Germany and Northern Cameroon**. *Medical and Veterinary Entomology*, 2022; **37**(2):286-299, doi:10.1111/mve.12630.

Not included in the thesis

1. Ndams I., Akafyi D., Nock I., Chechet G., Renz A., **Kamtsap P.**, Ibrahim M.A.M., Kelm S: **Emergence and genetic diversity of zoonotic *Onchocerca* species among human populations in Taraba State, Nigeria**. *German Journal of Microbiology* 2023, **3**:12-18, https://doi.org/10.51585/gjm.2023.2.0023.

2. Paguem A, Manchang K, **Kamtsap P**, Renz A, Schaper S, Dobler G, Bakkes DK, Chitimia-Dobler L. **Ticks and Rickettsiae Associated with Wild Animals Sold in Bush Meat Markets in Cameroon**. *Pathogens*, 2023, **12**(2):348-363. doi:10.3390/pathogens12020348

3. Paguem A., **Kamtsap P**., Manchang T.K., Yembo J., Achukwi M.D., Streit A., Renz A.: **Species identity and phylogeny of *Paramphistomoidea Fischoeder*, 1901 occurring in cattle and sheep in North Cameroon**. *Veterinary Parasitology*, 2023, **45**(13), https://doi.org/10.1016/j.vprsr.2023.100922.

Personal contribution

For the first four articles, included in this thesis, different data sets were used. I (Pierre Kamtsap) had a substantial role in the collection of samples, construction of semi-natural screen tests for larvicides in the laboratory and in the field, morphological and molecular analysis, bioinformatics analysis, data analysis, and drafting manuscripts.

Own contribution to the published paper 1

Kamtsap, P., Paguem, A., & Renz, A.: **Molecular diversity in the *Simulium damnosum* complex (Diptera: Simuliidae) in Cameroon**. *Mitteilungen der Deutschen Gesellschaft für Allgemeine und Angewandte Entomologie*, 2024, **23**: 147-152.

In this study, I actively participated in the collection of *Simulium* black fly specimens across multiple ecological zones in Cameroon, assisting in site selection based on historical black fly presence, water sources, and epidemiological relevance (**Fig. 1**). I collected larvae, pupae, and adult black flies from various substrates and documented GPS coordinates for all sampling sites to facilitate species distribution analysis. Using both morphological identification and molecular techniques, I contributed to the taxonomic classification of species within the *Simulium damnosum* complex. My molecular work involved extracting genomic DNA, optimizing PCR amplification for mitochondrial Cox1 and nuclear ITS2 gene regions, and ensuring high-quality sequencing through commercial facilities. I conducted phylogenetic analyses using ClustalW in MEGA 7.0.26 and Maximum Likelihood tree construction with 1,000 bootstrap replicates to determine evolutionary relationships. Additionally, I contributed significantly drafting the manuscript by authoring key sections, developing data visualizations, and assisting in revisions to ensure clarity and scientific rigor.

Figure 1. Ecological zone of black flies, water sources, and epidemiological relevance of one of the study areas along the Menchum Valley, Northwest Region, Cameroon.

Own contribution to the published paper 2:

Kamtsap P., Paguem A., Nguemaïm Ngoufo F., Renz A.: **Morphological and Molecular Investigation of Non-*Simulium damnosum* Black Flies in Cameroon Using Nuclear ITS 2 and Mitochondrial Cox 1 Genes**. *Insects*, 2025; **16**(6):572-592, https://doi.org/10.3390/insects16060572

This research significantly enhances the understanding of black fly (Simuliidae) biodiversity in Cameroon by integrating morphological and molecular approaches.

I played a key role in field collection (**Fig. 2**), helping to gather 1,184 black fly pupae from 13 locations across diverse ecological zones to ensure comprehensive species representation. Through morphological identification, I identified 19 species, including *2* yet undetermined species. I conducted DNA extractions and PCR amplification using nuclear (ITS2) and mitochondrial (Cox1) gene markers, enabling precise species identification. To validate cryptic species and establish genetic relationships, I performed sequence alignment and phylogenetic analysis. My work confirmed the presence of *Simulium vorax* and *Simulium dentulosum*, both known in others countries as onchocerciasis vectors, emphasizing the need for intensified vector surveillance. Additionally, I highlighted ecological influences on species distribution, noting the broad range of *S. cervicornutum* and the restricted distribution of *S. alcocki* and *S. kenyae*. To contribute to future research, I deposited 45 newly generated DNA

sequences in GenBank, enhancing molecular resources for black fly studies. By integrating morphological and molecular techniques, this study refines black fly identification methods and establishes a foundation for future vector monitoring and control strategies. Furthermore, it advances the understanding of non-*Simulium damnosum* species in disease transmission, providing critical insights for vector control initiatives and genetic research.

Figure 2. Collection of *Simulium* pupae along the Mawong River.

Own contribution to the published paper 3:

Kamtsap P., Nguemaïm Ngoufo F., Paguem A., Renz A.: **Knowledge and Practices of Four Onchocerciasis-Endemic Communities in Cameroon. Microorganisms**, 2025; 13(4):736-750, doi: 10.3390/microorganisms13040736

I played a central role in conceptualizing this study by formulating the key research questions concerning community knowledge and practices related to onchocerciasis. I also designed the research methodology, selected the study sites, and developed the survey instruments to ensure comprehensive and reliable data collection. During fieldwork, I administered structured questionnaires (**Fig. 3**), facilitated interviews across four onchocerciasis-endemic communities, and supervised the collection of black flies from vector breeding sites while guiding community sensitization efforts. My analytical contributions included performing chi-square tests in SigmaPlot 15.0 to examine demographic differences and variations in community knowledge, visualizing

data through heat maps and graphs, and interpreting results to inform public health interventions. I drafted the manuscript, contributing extensively to the introduction, methodology, and discussion sections by conducting a literature review on vector ecology and refining the discussion to align with recent epidemiological findings. Beyond research and analysis, I facilitated project coordination by managing communication between field researchers, local health authorities, and community leaders, securing ethical clearance, and overseeing logistics such as participant recruitment and field visit organization. Furthermore, I contributed to securing funding by assisting in grant applications, mobilizing resources, and facilitating access to laboratory materials essential for data analysis.

Figure 3. Study participant's recruitment through a structured questionnaire

Own contribution to the published paper 4:

Kamtsap P., Paguem A., Nguemaïm Ngoufo F., Njiokou F., Renz A.: **Testing the susceptibility of larval stages of *Simulium* to temephos and *Bacillus thuringiensis* var *israelensis* in Germany and Northern Cameroon**. *Medical and Veterinary Entomology*, 2022; **37**(2):286-299, doi:10.1111/mve.12630.

This study significantly advances the understanding of *Simulium* larvae's susceptibility to *Bacillus thuringiensis* var. *israelensis* (Bti) and temephos in both Germany and Cameroon. I was actively involved in designing and conducting experiments to assess the efficacy of these larvicides against *Simulium* species. This included conducting semi-natural field tests (gutter system) and laboratory-based bioassay experiments to

assess larvicide efficacy (**Fig. 4**). Representative *Simulium* larvae were collected from breeding sites in Germany and Northern Cameroon. In the laboratory, I determined LC50 and LC90 values for Bti and temephos through susceptibility testing, following established guidelines. Additionally, I performed morphological and molecular identification of *Simulium* species using nuclear (ITS2) and mitochondrial (Cox1) markers, verifying genetic diversity and species distribution. Data analysis revealed significant variations in larvicide susceptibility between Cameroonian and European *Simulium* species, highlighting potential pesticide resistance and the need for genetic resistance monitoring. My contributions extended to developing a practical methodology for tracking larvicide resistance in black flies, which has implications for improving vector control strategies. I also provided recommendations for onchocerciasis vector management, particularly in regions where *Simulium* species coexist with *Loa loa*. Additionally, I played a key role in manuscript preparation by drafting and interpreting findings in the context of vector control and public health, while also collaborating with research institutions and stakeholders to enhance data sharing and vector control mechanisms.

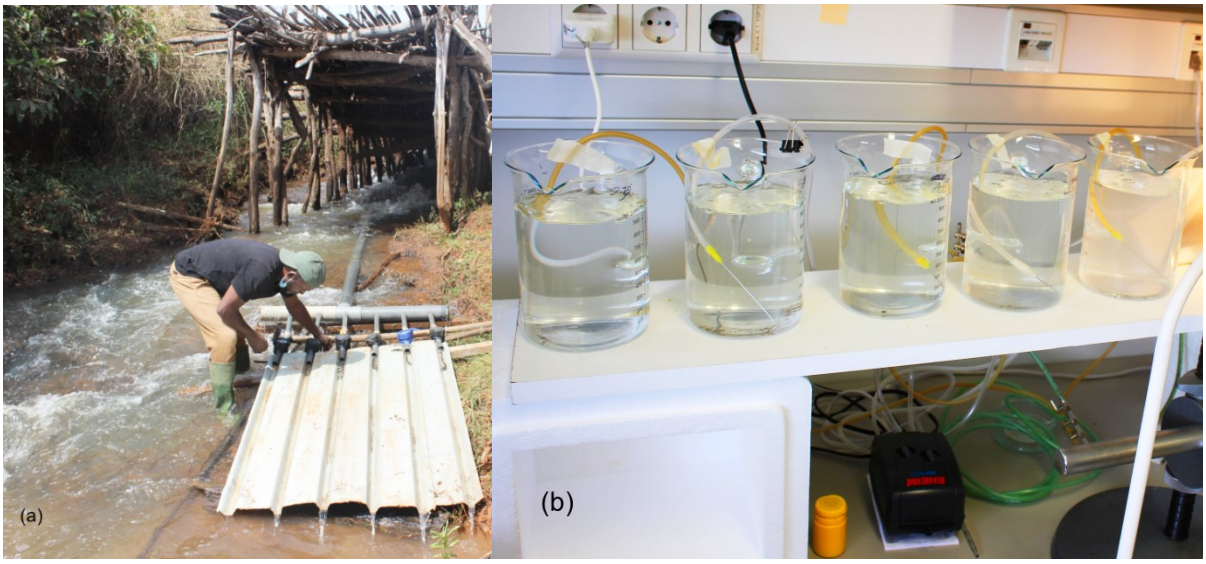


Figure 4. Semi-natural and laboratory experimental design for testing the susceptibility of larvae to larvicides.

This paper lays the groundwork for future research into *Simulium* susceptibility and resistance evolution, which will help guide control initiatives for black fly-borne diseases.

1. Introduction

Black flies (Simuliidae) are bloodsucking dipteran and have public health importance due to their transmission of *Onchocerca* filarial parasites to humans and animals (**Fig. 5**) [1, 2]. The evolutionary relationship between black flies and the parasite has given rise to highly adapted transmission systems, shaped by ecological constraints and selective pressures over time.

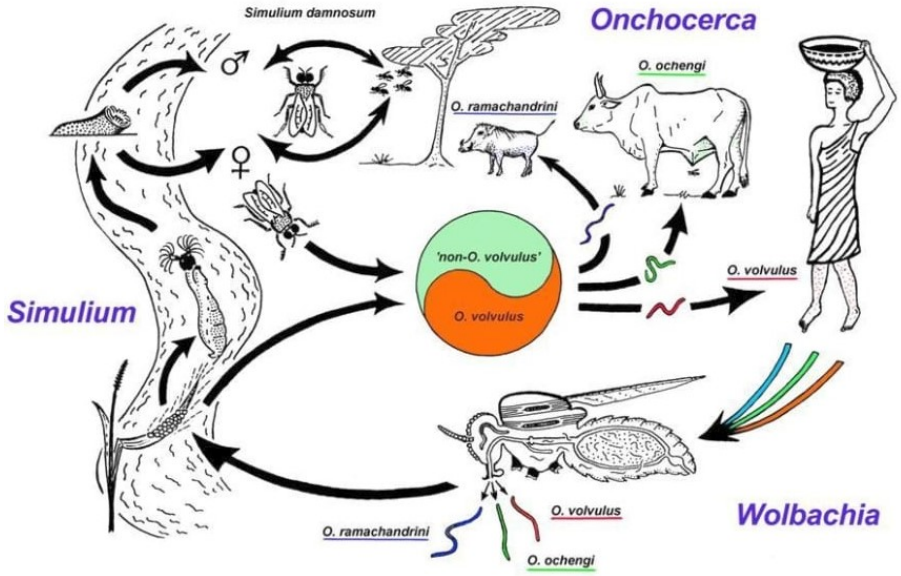


Figure 5. Cross-transmission cycles of human and animal *Onchocerca* spp. by *Simulium damnosum s.l*. in North Cameroon. Picture taken with the copyright permission of A. Renz.

Cameroon, a Central African country encompasses a variety of ecological zones – from tropical forest to savannah – supporting an exceptional diversity of black fly species. The country environmental and rivers systems have favoured local adaptation and vector distribution [3, 4].

The reproductive life cycle (**Fig. 5**) of *Simulium* spp. is closely related to fast flowing water. Black flies bred exclusively in fast-flowing rivers and streams, where the larvae filter and feed on the river plankton. The pupal cocoon is built under water, with respiratory gill of highly specific forms. The ecological conditions of these breeding sites, such as current velocity, substrate availability, and water chemistry, play a key role in determining their abundance and distribution and of course the intensity of the transmission.

Since the 1960s, studies in Cameroon have identified important breeding sites along rivers such as the Sanaga, Moungo and Mbam in the forest and Vina du Nord in the Sudan savannah, correlated with a high prevalence of onchocerciasis in surrounding communities [5, 6].

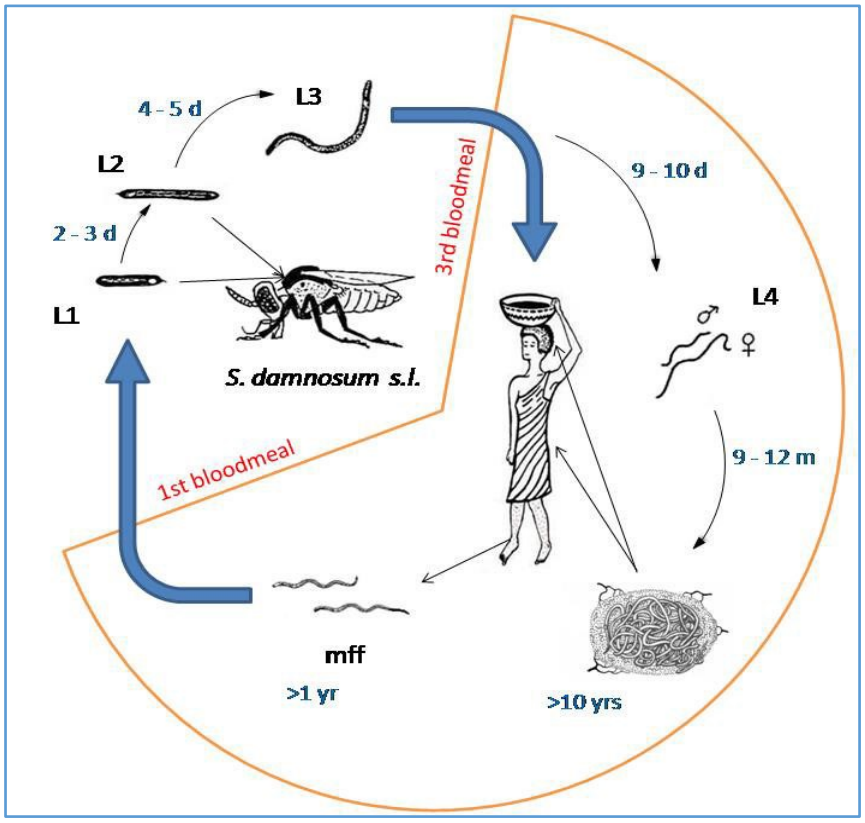


Figure 6. Life cycle of *Onchocerca volvulus*

Female and male worms (on average 1:1) lie surrounded by host connective tissue (onchocercoma or nodule) under the skin of an infected human, normally around the hips. One productive female gives birth to approx. 1,500 offspring per day. These microfilariae (mff) migrate to the skin predominantly in the leg region (lifetime > 1 year), where they are ingested by the vector (*S. damnosum s.l.*) in search of a blood meal. In the black fly, they penetrate the peritrophic membrane and encyst themselves in the flight muscle tissue to the so-called sausage stage (L1). After 2 to 3 days, they molt to the encysted L2 stage, and 4 to 5 days later to the infective larva (L3). During another blood meal, they enter a new host through the mouthpart during a blood meal, and develop via another molting stage (L4) to male and female adults after 9 to 12 months. Their life expectancy exceeds 10 years. Picture taken with copyright permission of A. Renz.

1.1. Vector diversity, distribution and transmission

The genus *Simulium* is present worldwide and consist of over 31 genera containing 2,348 species (2,331 living and 17 fossil species) [7]. In Africa, 124 black fly species have been described, mostly in the Ethiopian region, Sub Sahara region of West, Central and East Africa [8]. Intensive research has been carried out mainly on European black flies [7, 9]. In Cameroon, 55 species have been morphologically described [7] and only a few are as yet well classified [10]. These flies are usually small, black or grey, with short legs and antennae (**Table 1**).

Table 1. Morphological classification key of adult females of *S. damnosum s.l.* [11]

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 COLOUR	All dark	*S. yahense* (most dark) (dark, mixed or pale) *S. squamosum*	Almost all pale
POSTCRANIAL HAIRS COLOUR	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* can be distinguished from *S. damnosum s.str.* by their having shorter antennae and paler hairs
GEOGRAPHIC DISTRIBUTION	Rainforest (but also savannah (Meredith, Lamizanat, pers. comm.)	Rainforest, Guinea and Sudan savannah. Populations in the rain-forest are distinct, but interbreeding in the savannah	*S. damnosum*: rainforest and savannah *S. sirbanum*: Sudan savannah (dry season)

In Cameroon, ecological conditions ranging from dense forest to open savannah have driven local speciation and niche adaptation within this complex. For example, *S. yahense* is generally associated with forest regions, while *S. sirbanum* and *S. damnosum s. str*. predominate in savannah areas. *Simulium squamosum*, originally described by Enderlein in 1921, from a sample, collected in the river Sanaga in Cameroon, has recently been re-described and grouped into 4 taxa (*S. sq.* A, B, C, D) adapted to different bioclimatic zones. This illustrates the ecological divergence within

the group [5, 8]. The *Simulium* species exhibit reproductive isolation reinforced by ecological barriers such as altitude, current velocity, and vegetation cover, but can coexist in transition zones, resulting in competitive displacement. This evolutionary radiation has important implications for vector competence, as not all members of the complex transmit the parasites with the same efficiency. They are vectors of numerous known pathogens including (1) *Onchocerca volvulus* and *Onchocerca ochengi*, causative agents of human onchocerciasis (river blindness) and bovine onchocercosis [1, 2], respectively, (2) *Leucocytozoon* to birds in Asia and North America [12, 13] and (3) numerous other filarial parasites of wild and domestic animals (*Onchocerca dukei* from cattle and *O. ramachandrini* from warthogs in Cameroon *O. lupi* from dogs in Florida *O. flexuosa* from deers in Germany and *Lappnema* sp. from an yet unknown host in Northern Cameroon) [14-20]. *S. ochraceum*, *S. metallicum*, *S. exiguum* and *S. guianense* transmit *O. volvulus* in the Americas (**Fig. 5**) [21].

Furthermore, selective pressure forms parasite-host interactions, whilst climate change, and anthropogenic interventions (deforestation, dam construction) continue to shape the adaptive landscape of these vectors.

A wide range of cytological and molecular markers have been used for population studies [22, 23]. These include chromosomal inversions [24] allozymes [25] and random amplified DNA polymorphisms (RAPD) [26]. Furthermore, the sequencing of Cox1 genes [27, 28], nuclear genes [29] and microsatellite loci analyses [28] have been carried out.

Molecular phylogenetic, particularly mitochondrial DNA (Cox1 gene) and the nuclear markers (ITS2), has been instrumental in resolving these species complexes, allowing more precise identification and mapping of vector distribution [5, 29, 30]. These advances provide a basis for understanding the evolutionary ecology of vector-parasite relationships and for designing geographically targeted control interventions.

1.2. Health problems due to the bite of black flies

Black flies worldwide affect humans or animals health in several ways. Their saliva, injected during the bloodmeal (poolfeeder) results in severe systemic reactions characterized by headache, fever and nausea that lead to what it is called “black fly fever”. It does not last longer than 48h and occurs after a large number of bites [31-33]. As already mentioned, their bites, in some cases, transmit viral, protozoan and

others unknown pathogens, whilst the bites of some species are an intolerable nuisance, causing blood loss or rendering humans and animals vulnerable to various infections [34]. Infections with the filarial parasite *O. volvulus* cause manifestations such as dermatitis associated with microfilariae (**Fig. 7**) [35, 36] and irreversible eye lesions leading to blindness (also known as river blindness) [37, 38].

Figure 7. Leopard skin associated with microfilariae in Ma'an village along the Ntem valley in the South Region of Cameroon.

In Cameroon, human onchocerciasis is one of the 11 recognized neglected tropical diseases [39, 40] with community awareness estimated at 66.2% 66.2% [41]. Due to the disease burden and the selective breeding characteristics of the vector, parasitological and or entomological work had been carried out along selected river systems in some regions of Cameroon. These include: the North region [40, 41], West region [42], South West region [43, 44], and Central region [45].

The study locations were strategically selected for their ongoing onchocerciasis transmission, ecological diversity, and proximity to fast-flowing rivers—critical habitats for the larval development of *Simulium* species. The communities primarily engage in

farming, fishing, sand dredging, and cattle rearing, which increases exposure to black fly bites and potential transmission of *Onchocerca volvulus* [46-48].

The construction of the Dam near the Menchum falls, approximately 20 km south of Wum and 30 km north of Bafut (Latitude 6.2914° N, Longitude 10.0277° E) may create reservoirs of slow-moving or stagnant water, but more importantly, weirs, downstream rapids, and tailraces can generate fast-flowing habitats ideal for blackfly breeding (**Fig. 8**) [49]. Close watch on the vectors population along the rivers is important to determine the type of *Simulium damnosum* at a particular area, to predetermine the type of human onchocerciasis in the area and to rapidly assess fly invasion [50].

Figure 8. Menchum fall, the proposed site for a dam near Befang in Cameroon, Northwest Region.

In the Menchum valley at Befang in 2016, trainings and education of the population was organized and carried out by Alfons Renz and collaborators. We then planned to investigate the transmission of the disease along the Menchum River, a region known for its favourable breeding conditions for black flies (Fig. 8). Mass administration of ivermectin was the primary strategy for disease control since the late 1990s in

Cameroon [51], with the goal of reducing the parasite load in the population and ultimately interrupting transmission.

Entomological fieldwork was conducted in Menchum valley in 2016 to assess ongoing transmission and vector dynamics. Black flies were collected from multiple locations using human landings (**Fig. 9**) and subsequently identified morphologically and molecularly to determine species composition, particularly members of the *Simulium damnosum* complex. Entomological indicators such as bite rate and infection rate were calculated to estimate transmission risk. This fieldwork provides important data for evaluating the effectiveness of control efforts and guiding future interventions in the Menchum River basin.

Unfortunately, this laudable initiative was stopped by the occurrence of the social crisis, which has been plaguing this part of Cameroon since 2016. Such a crisis is a major handicap for the surveillance of this neglected tropical disease and sustainable planning of vector control. It also seems unlikely or even impossible since 2016 to carry out any entomological and epidemiological study. All these impossibilities further expose the population to potential transmission.

Figure 9. Black fly collection using human landing method

1.3. Implication for elimination monitoring

The integration of molecular diagnostic into vector surveillance has significantly strengthened the capacity of national programs to detect low-level transmission and verify elimination. In Cameroon, the NTD control program increasingly relies on a combined approach using entomological data to inform critical decisions, such as stopping mass drug administration, resuming vector control interventions, or intensifying surveillance in specific areas [52, 53].

2. Objectives and expected outcomes of my thesis

The overarching goal is to enhance our ecological and evolutionary understanding of *Simuliidae* (black flies), the vectors of onchocerciasis in Cameroon, in order to understand their vectorial role and to improve and adapt vector control strategies. By focusing on areas where historical control efforts have been limited, particularly forested regions, this study aims to generate critical data on species composition, genetic diversity, and ecological behaviour that can inform targeted and sustainable interventions.

A key focus is to overcome the limitations of morphology-based species identification, which often fails to detect cryptic or closely related species. Through the integration of molecular tools with ecological data, this research investigates species boundaries, phylogenetic relationships, and population structure of *Simulium* spp. This approach will clarify how evolutionary processes and environmental variation shape vector adaptation, distribution, and competence.

Particular attention is given to forested zones where *Loa loa* co-endemicity restricts the use of ivermectin due to severe adverse reactions. These neglected areas may harbour distinct black fly lineages with different ecological preferences and breeding behaviour compared to savannah populations, potentially requiring customized control strategies.

This work contributes to national and global health goals by addressing ecological gaps in vector control. The research is structured around four major components, each of which forms the basis of a peer-reviewed manuscript:

1. **Species delimitation and molecular taxonomy** — DNA-based identification of black flies to resolve species boundaries and describe genetic diversity.
2. **Phylogeographic structure across ecological zones** — Assessment of population connectivity and regional differentiation across Cameroon's bioclimatic gradients.
3. **Community knowledge and perception** — Analysis of local ecological knowledge and onchocerciasis-related practices in four endemic communities, following over 20 years of ivermectin distribution.
4. **Larvicide evaluation protocols** — Development of standardized laboratory test ('bioassey') and semi-field procedures (e.g., "système de gouttières") to assess the efficacy (LC_{50}/LC_{90}) of *temephos* and *Bacillus thuringiensis* var. *israelensis* (Bti) against black fly larvae.

Collectively, these components provide a comprehensive framework for understanding black fly ecology, supporting tailored vector control, and contributing to the sustainable elimination of onchocerciasis in Cameroon.

3. Results and discussion

Chapter 1: Morphological and molecular diversity of *Simulium damnosum s.l.*

Related publication 1

Kamtsap, P., Paguem, A., & Renz, A.: **Molecular diversity in the *Simulium damnosum* complex (Diptera: Simuliidae) in Cameroon**. *Mitteilungen der Deutschen Gesellschaft für Allgemeine und Angewandte Entomologie*, 2024, **23**: 147-152.

The *Simulium damnosum* complex comprises a group of closely related blackfly species that serve as the main vectors of *Onchocerca volvulus*, the parasitic nematode responsible for human onchocerciasis (river blindness) in Africa [54]. Biologically, the members of this complex are nearly indistinguishable based on external morphology, complicating their identification and the mapping of vector distribution [11]. Adult females are obligate blood feeders, and the need for a blood meal to initiate egg development makes them important from a disease transmission standpoint [54]. While morphometric data (thorax/antenna length), shape and colour of antenna and coloration of wing tuft hairs have been used in species descriptions, such traits have proven inconsistent for reliable taxonomy of single *S. damnosum s.l.* flies, further highlighting the biological complexity within the group [55, 56].

From an evolutionary standpoint, the *S. damnosum* complex demonstrates substantial genetic divergence despite morphological uniformity [57]. Molecular studies, particularly those based on mitochondrial DNA sequences such as the Cox1 gene, reveal high levels of genetic variation and support the existence of multiple cryptic species [58]. The cytotaxonomy of *Simulium damnosum sensu lato* (s.l.) is a pivotal scientific method that has greatly advanced our understanding of this taxonomically complex group of black flies, which serve as the primary vectors of human onchocerciasis in Africa. By examining the structure and arrangement of chromosomes, particularly polytene chromosomes from larval salivary glands, researchers have identified more than 50 morphologically indistinguishable sibling species within the *S. damnosum complex*. This technique enables the detection of fixed chromosomal inversions and distinct banding patterns that are characteristic of each species, providing a reliable basis for species-level identification where

morphological differentiation is inadequate [24, 59, 60]. Phylogenetic analyses indicate a strong geographical structuring of lineages, which is consistent with an allopatric speciation model where physical or ecological barriers—such as mountain ranges or shifts in river systems—have led to the separation and independent evolution of populations [29].

Ecologically, members of the *S. damnosum* complex are highly specialized to particular riverine environments. Their larvae require clean, well-oxygenated, and fast-flowing water for development, making them sensitive to environmental changes and useful indicators of aquatic ecosystem health. Different species and cytospecies within the complex occupy distinct ecological zones across the African continent, with some preferring forest environments and others thriving in savannah habitats [61, 62]. This ecological partitioning influences their distribution and the risk of onchocerciasis in human populations [63]. Seasonal rainfall patterns also affect their population dynamics, as increased river flow during rainy seasons creates more breeding sites, resulting in seasonal peaks of adult fly emergence and biting activity [64]. The biting behavior of females, which includes both the frequency and host preference, plays a critical role in disease transmission. Some species are more anthropophilic and aggressive in their biting, making them more effective vectors [65]. Thus, ecological factors such as habitat preference, climate, and human proximity directly shape the epidemiological significance of each species within the complex [58].

This complex exhibits considerable morphological, ecological, and genetic variability, making species identification challenging. This study aims to assess the molecular diversity of the *S. damnosum* complex in Cameroon, identify cryptic species using DNA sequencing of the Cox1 and ITS2 genes, compare genetic and morphological classifications, determine the geographic distribution of different taxa, and provide insights for vector control programs aimed at combating onchocerciasis.

The study was conducted between 2018 and 2022 across 26 sites in Cameroon, where black fly samples were collected for morphological and molecular analyses. Larvae and pupae were obtained from trailing vegetation and stones in fast-flowing rivers, while adult flies were captured biting humans at various locations. All samples were preserved in 70–96% ethanol for subsequent molecular analysis. Morphological identification was based on key taxonomic features, including antenna shape and

segment compression, antenna segment coloration, wing tuft coloration, postcranial hair color, and geographic distribution. Using these characteristics, specimens were classified into *Simulium mengense*, *S. yahense/squamosum*, and *S. damnosum/sirbanum*. For molecular analysis, DNA was extracted using the Wizard Genomic DNA Purification Kit, and two gene regions were targeted: Cox1 (~650 bp), a mitochondrial gene used for species barcoding, and ITS2 (~400 bp), a nuclear marker for phylogenetic analysis. PCR amplification was performed using Cox1 primers from Hajibabaei [27] and ITS2 primers from Kononov [66] in an Eppendorf Master Cycler. Phylogenetic analysis was conducted using ClustalW for sequence alignment and MEGA v7.0.14 for Maximum Likelihood (ML) tree construction, with bootstrap analysis (1,000 replicates) to assess branch support.

This study reports the presence of *Simulium sanctipauli* in Cameroon on the molecular level. The earliest documented identification of forest species in Cameroon appears to date from the mid-1970s. Vajime and Dunbar (1975) conducted a thorough cytotaxonomic study of the *Simulium damnosum* complex in West Africa, including Cameroon [24]. In their study, they recognized *S. sanctipauli* as a distinct sister species within the complex. Additionally, *Simulium squamosum*, originally described from the Sanaga River and the Vina du Nord and Vina du Sud rivers in the savannah, was found in new areas including the Menchum Valley, Mawong River, and Benoue River, indicating a broader ecological range and possible subspecies adaptation. *Simulium yahense*, expected based on past records from Nigeria, Benin, and Cameroon, was not detected — possibly due to sampling bias, seasonal variation, or past misidentification. Notably, the study uncovered two undescribed genetic clades of *Simulium damnosum s.l.*: Clade I in Central Cameroon (Bafia, Makouopsap, Bayomen, Konkwalla) and Clade II in Southern Cameroon (Ntem Valley near the Gabon border), suggesting the presence of previously unrecognized species within the *S. damnosum* complex. *Simulium mengense* and *S. sirbanum* was not found, likely due to limited sampling. Phylogenetic analysis revealed four major genetic clades — *S. squamosum*, *S. sanctipauli*, Clade I, and Clade II (**Fig. 10)** [67], which is critical for understanding local transmission dynamics and guiding onchocerciasis control strategies.

In conclusion, this study significantly enhances our understanding of the *Simulium damnosum* complex in Cameroon, revealing greater genetic and morphological diversity than previously recognized. The unexpected presence of *S.*

sanctipauli, the broader distribution of *S. squamosum*, and the discovery of two potentially new species underscore the importance of integrating molecular and morphological methods for accurate species identification. These findings have direct implications for onchocerciasis control, as the presence of cryptic species may influence transmission dynamics and the effectiveness of intervention strategies. Continued research is essential to confirm the taxonomic status of the newly identified clades, assess their role in disease transmission, and refine vector surveillance and control efforts.

Related publication 2

Kamtsap P., Paguem A., Nguemaïm Ngoufo F., Renz A.: **Morphological and Molecular Investigation of Non-*Simulium damnosum* Black Flies in Cameroon Using Nuclear ITS 2 and Mitochondrial Cox 1 Genes**. *Insects*, 2025; **16**(6):572-592, https://doi.org/10.3390/insects16060572.

This study investigates the ecological and evolutionary diversity of black flies (excluding *Simulium damnosum s.l.*) in Cameroon to improve species identification and assess their potential role in disease transmission, including onchocerciasis. While ecological and evolutionary studies in Cameroon have largely focused on the *S. damnosum* complex, the wider diversity of *non-Simulium damnosum* species remains poorly studied, in particular as concerns their molecular genetic markers. By integrating morphological characters with molecular data, this study uncovers cryptic lineages and sheds light on the evolutionary relationships and ecological distribution of black flies across diverse habitats. This integrative approach offers new perspectives on the vector ecology and evolutionary dynamics that shape blackfly populations in Cameroon.

Field collections yielded 1184 pupae from 14 distinct sites spanning diverse ecological zones in Cameroon. The sampling sites included rivers, tributaries, and streams with fast-flowing water where black fly larvae and pupae commonly thrive. Morphological identification was conducted based on pupal gill structure, which remains a key diagnostic feature. A total of 19 species were identified, including two unidentified species not previously recorded in Cameroon, marking a significant addition to the regional faunal inventory. Among the known species identified, *S. cervicornutum* was found to be the most widely distributed and abundant, detected at multiple sampling

sites, while species such as *S. alcocki* and *S. kenyae* exhibited restricted distributions**,** indicating possible ecological specialization or environmental sensitivity. Importantly, two known onchocerciasis vectors **-** *S. vorax* and *S. dentulosum* - were identified and confirmed in Cameroon using both morphological and molecular techniques (Fig. 10) [48].

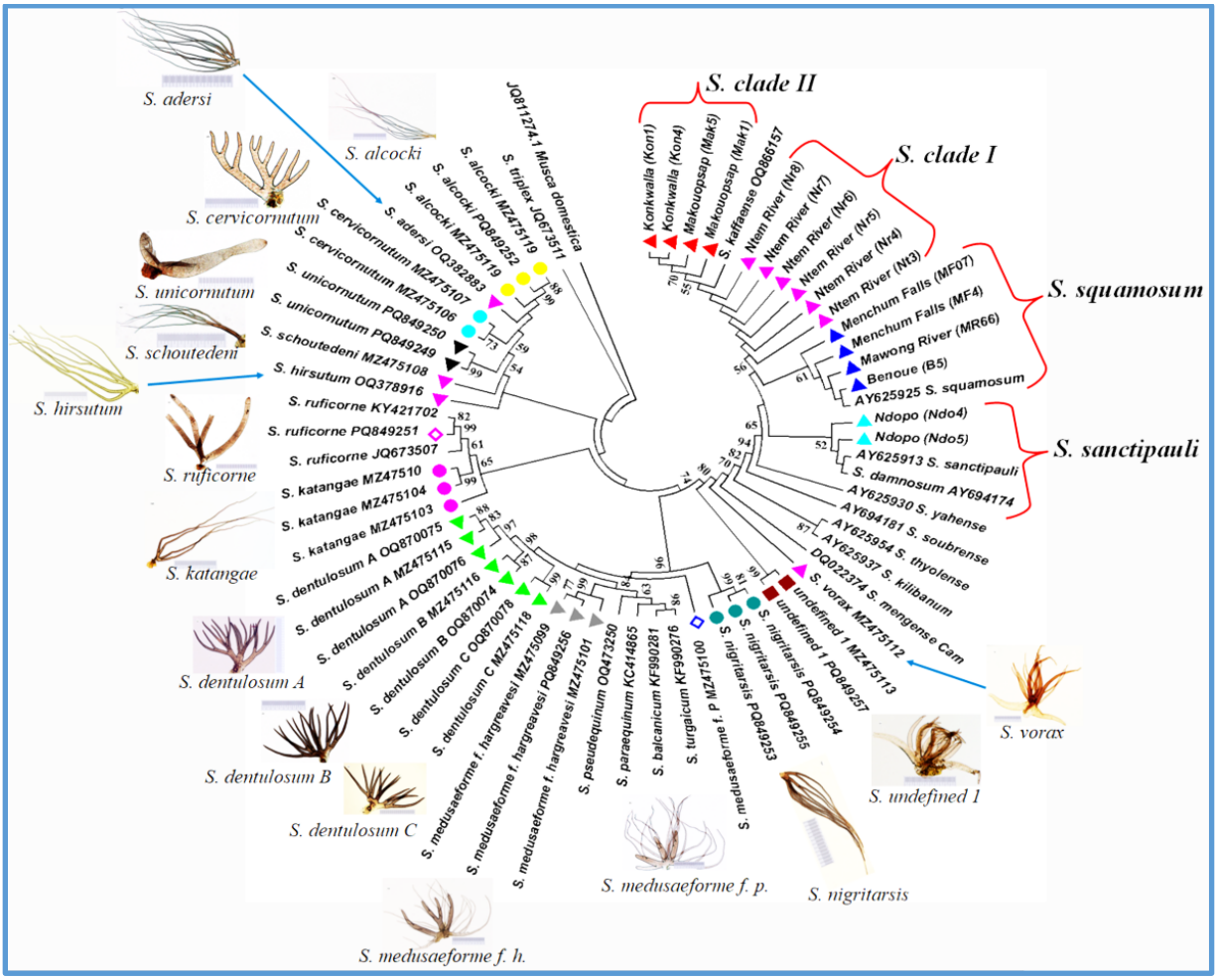


Figure 10. Evolutionary relationships of species based on ITS2 gene.

The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions. The analysis involved nucleotide sequences of 64 *Simulium* samples of our study (marked with deferent colours) and GenBank (not marked with colour). All ambiguous positions were removed for each sequence pair. There were a total of 685 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

To complement and refine species identification, the study employed molecular techniques targeting the nuclear ITS2 region and the mitochondrial Cox1 gene. These genetic markers were chosen for their established utility in species delimitation and population studies. DNA was successfully extracted from the collected specimens, and PCR amplification yielded clear bands with high-quality sequence data. Phylogenetic analyses using Neighbor-Joining trees revealed distinct species clusters, supporting morphological identifications and uncovering intra-species genetic variation. ITS2 analysis showed low to moderate divergence among species, suggesting recent divergence or close evolutionary relationships in most cases. Notably, *S. katangae* and *S. cervicornutum* exhibited high divergence values, indicating potential cryptic speciation or misclassification, and underscoring the need for further taxonomic and genomic studies. Meanwhile, *S. nigritarsis* exhibited no detectable divergence across sampled individuals, implying either recent divergence or a lack of genetic variability in the sampled population.

The mitochondrial Cox1 gene provided further resolution, revealing greater haplotype diversity and geographic structuring of populations, consistent with its higher mutation rate and suitability for population-level analysis. Clades were observed that reflected geographic clustering, suggesting that ecological barriers, breeding site isolation, or host preferences may limit gene flow and shape population structure (**Fig. 11**). Clades I (Ntem Valley) and II (central Cameroon) should be the subject of special studies because they are completely distant and different from the species described previously. These findings have direct implications for understanding vector competence, as population structuring may influence a species' ability to transmit pathogens.

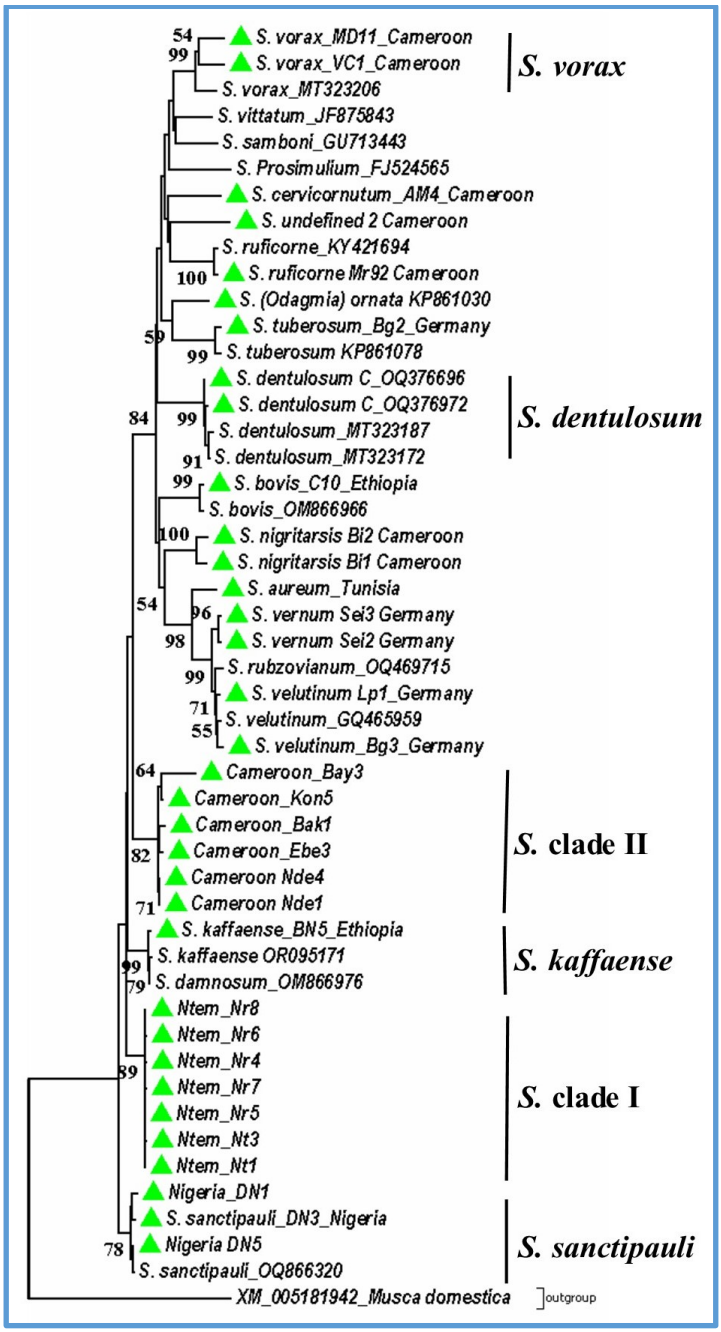


Figure 11. Molecular phylogenetic analysis by Neighbor-joining method using Cox1.

Species marked in green are from this study, while those not marked are from GenBank. Apart from the GenBank samples, collaborators contributed the following: the sample from Tunisia was collected and analysed by Rahel Schnell; Danlami Akafyi collected the sample from Nigeria; and Abebual Yilak collected the sample from Ethiopia. All other samples were collected and analysed by Pierre Kamtsap.

Beyond species identification, the study's ecological observations provide insights into species distributions and habitat associations. For instance, *S. cervicornutum*'s wide distribution may reflect ecological adaptability, while *S. vorax* and *S. medusaeforme*

forms (Pomeroy and hargreavesi) exhibited more niche specialization, limited to specific habitats. High diversity was observed in locations such as the Mawong River in North-West Cameroon, which hosted over 12 species, suggesting that certain habitats may serve as biodiversity hotspots for Simuliidae. Conversely, some sites exhibited near-monospecific populations, likely due to localized environmental conditions.

The identification of *S. vorax* and *S. dentulosum* in Cameroon is particularly significant, as these species have been implicated in onchocerciasis transmission in other African regions. Their confirmation as present and genetically validated in Cameroon raises the possibility that *non-damnosum* species could serve as disease vectors under favourable conditions, such as changes in land use, climate, or human activities that alter fly behaviour or habitat availability. This emphasizes the importance of molecular tools in public health entomology, allowing accurate surveillance and detection of potential vectors that may be overlooked using morphological methods alone [60, 68].

In conclusion, the integration of morphological and molecular data revealed a diversity of *non-Simulium damnosum* species in Cameroon, including the first molecular confirmation of several species and the discovery of two undefined species (**Fig.10**). The study highlights the critical role of molecular approaches in resolving species boundaries, detecting cryptic diversity, and informing vector control efforts**.** The findings also underscore the need for expanded surveillance and genomic studies, particularly in light of environmental changes that may influence vector dynamics. Future research should pursue whole-genome sequencing of *non-damnosum* species, ecological modelling, and expanded sampling to fully understand the taxonomy, ecology, and vector potential of Simuliidae in Cameroon and beyond, thereby supporting effective disease control and biodiversity conservation efforts.

Chapter 2. Improvement and update of the current epidemiological data on onchocerciasis in Cameroon

Related publication

Kamtsap P., Nguemaïm Ngoufo F., Paguem A., Renz A.: **Knowledge and Practices of Four Onchocerciasis-Endemic Communities in Cameroon. Microorganisms**, 2025; 13(4):736-750, doi: 10.3390/microorganisms13040736

Extended summary

Objective

The study investigates the knowledge and practices of four onchocerciasis-endemic communities in Cameroon, focusing on the understanding of the disease and its transmission to optimize control strategies.

Methods

A cross-sectional survey was conducted in four communities - Mawong, Menchum, Soramboum, and Galim (**Fig. 12**) to assess knowledge and perceptions of black flies and onchocerciasis. Data were collected from 452 participants aged 14 to 50 years using a structured questionnaire. The survey focused on key areas, including general knowledge of black flies and their association with onchocerciasis, awareness of black fly biting behaviours, breeding sites, and transmission risks, as well as perceptions of disease symptoms and prevention methods. Additionally, sociodemographic factors influencing knowledge levels were examined to identify potential gaps in awareness and understanding.

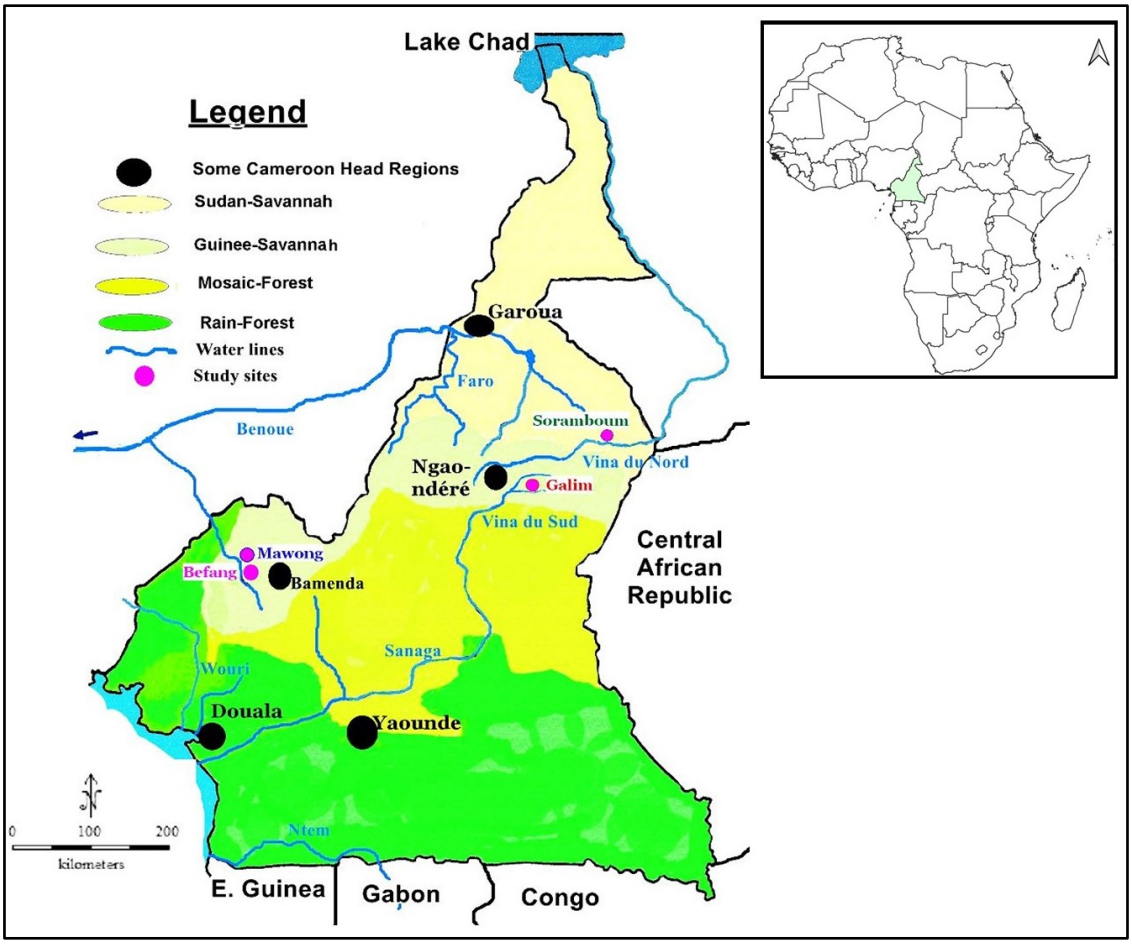


Figure 12. Map of Cameroon showing study sites represented by pink dots [46]

Key Findings (Figure 13)

Awareness of disease transmission by black flies remains limited, with 90% of participants recognizing them as a nuisance but only 9.3% aware that their bites could lead to blindness. Many incorrectly associated black fly bites with malaria, particularly in Mawong (64.7%) and Menchum (70%). Misconceptions extended to black fly behaviour, as only 34.1% correctly identified the evening as the primary biting period, while others believed they were active at any time (32.3%) or mainly in the morning (19%). Similarly, only 17% recognized fast-flowing water as the primary breeding site, with many assuming stagnant water or even tree holes were responsible. Transmission studies in Soramboum and Galim recorded annual biting rates of 16,000 and 13,500 flies per man-year in 2013 [69], respectively, yet dissection of 21,897 black flies revealed only a few infective larvae of *Onchocerca volvulus* in Soramboum and none in Galim, due to 26 and 20 years of annual ivermectin mass treatments respectively. Sociodemographic factors influenced knowledge, with illiteracy rates being highest in

Galim (55.9%) and Soramboum (31.8%), and lower awareness was observed among sand diggers, farmers, and younger participants under 20 years old. Preventive practices were inadequate, as while protective clothing was recognized as a defence, few respondents implemented additional measures, and awareness of vector control strategies such as larviciding and environmental management was low. Regional variations further shaped knowledge and practices, with Befang residents aware of dry-season black fly activity but misinformed about breeding sites, Galim participants correctly identifying fast-flowing water as a breeding ground, and Soramboum respondents demonstrating a balanced understanding of seasonal vector activity but limited knowledge of breeding behaviour. In contrast, many in Mawong incorrectly linked black flies to malaria, highlighting significant gaps in disease-specific knowledge.

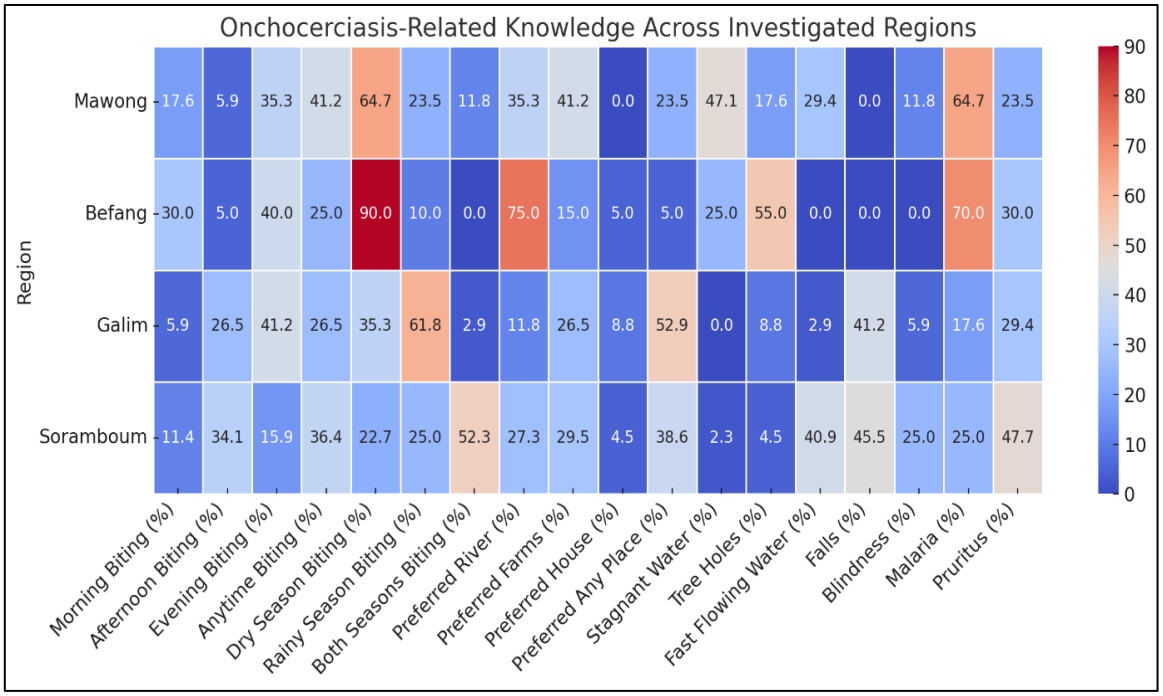


Figure 13. Onchocerciasis-related knowledge across investigated regions in Cameroon.

Colour intensity corresponds to knowledge level: dark red indicates high awareness (≥70%), orange to beige represents moderate awareness (40–69%), Light blue suggests low awareness (10–39%), and dark blue denotes very low or no awareness (≤9%) [46].

The heatmap displays the percentage of respondents in each region (Mawong, Befang, Galim, and Soramboum) who correctly identified aspects of black fly biting behavior, breeding site preferences, and disease symptoms. This visualization highlights regional differences in community knowledge, informing targeted health education and vector control strategies.

Partial conclusions, perspectives, limitations and recommendations

This study provides valuable insights into the current state of knowledge regarding onchocerciasis and black fly ecology in four Cameroonian communities. Although general awareness of black flies as a nuisance is high, detailed understanding of their role in transmitting *Onchocerca volvulus*, their reproductive behaviour, and seasonal activity remains limited. This mirrors findings from similar studies conducted in Cameroon, where community knowledge of black fly ecology and onchocerciasis transmission was found to be incomplete despite years of control programs [70].

Local ecological conditions, such as the presence of fast-flowing rivers in Galim and Soramboum, significantly influence black fly populations and disease transmission dynamics. However, misconceptions about breeding sites and biting behaviour persist, particularly in Mawong and Menchum, where black flies are incorrectly associated with malaria transmission. This is consistent with observations by Adeoye et al. [71, 72] who reported that many communities still misidentify black flies as malaria vectors.

A significant limitation of this study is its cross-sectional design, which restricts the ability to draw causal inferences between sociodemographic variables and levels of awareness. Longitudinal designs would better capture temporal trends in knowledge acquisition and the impact of ongoing public health interventions. Furthermore, reliance on self-reported data introduces potential biases, such as over reporting knowledge due to social desirability [73].

Given the regional variations in knowledge and perception, health education programs should be ecologically tailored. In regions like Galim and Soramboum—where some understanding of black fly breeding and seasonality exists—programs should include more advanced ecological content. In contrast, areas like Mawong and Menchum require foundational education emphasizing the link between black fly bites and onchocerciasis, as well as correcting myths around breeding sites and disease symptoms [70, 72].

Further ecological studies are needed to assess how black fly population dynamics relate to environmental factors such as water velocity, pollution levels, and vegetation types. Vector control measures like targeted larviciding in known breeding sites or environmental sanitation around rivers can be more efficient when communities are actively involved and trained in black fly identification.

Ultimately, empowering communities with both ecological and disease-specific knowledge will foster ownership of control efforts. This could include training on how environmental actions, such as removing vegetation along riverbanks or proper waste management, reduce breeding habitats. As highlighted in studies from other endemic regions, integrating local ecological knowledge into national onchocerciasis elimination strategies leads to more sustainable and community-driven interventions.

Chapter 3. Specification and standardization of procedures and guidelines for testing larvicides against *Simulium*.

It provides guidance on laboratory studies to implement small and large-scale field trials to determine the efficacy, field application rates, operational feasibility, and acceptability of *Simulium* larvicides by stakeholders.

Related publication

Kamtsap P., Paguem A., Nguemaïm Ngoufo F., Njiokou F., Renz A.: **Testing the susceptibility of larval stages of *Simulium* to temephos and *Bacillus thuringiensis* var *israelensis* in Germany and Northern Cameroon**. *Medical and Veterinary Entomology*, 2022; **37**(2):286-299, doi:10.1111/mve.

Extended summary

Introduction

Blackflies (*Simulium* spp.) are vectors of *Onchocerca volvulus*, the parasite responsible for onchocerciasis (river blindness). Before the widespread use of ivermectin, blackfly control relied on chemical and biological larvicides. Temephos, an organophosphate insecticide, was used in Africa for decades, but resistance was observed in certain populations. As an alternative, *Bacillus thuringiensis* var. *israelensis* (Bti) was introduced for its efficiency and environmental safety. This study evaluates the susceptibility of *Simulium* larvae in Germany and Northern Cameroon to these two larvicides.

Methodology

Black fly specimens were collected from two regions: Germany and Cameroon. In Germany, *Simulium (Odagmia) ornatum* and *Simulium latipes* (*Simulium vernum*) were sampled from the Weilerbach and Käsenbach near the University of Tübingen. In Cameroon, *S. damnosum s.l., S. medusaeforme* f. *hargreavesi, S. vorax*, and *S. cervicornutum* were collected from rivers around Ngaoundéré, particularly the Vina du Nord and Vina du Sud rivers and their tributaries.

To assess susceptibility of larvae to larvicides, two experimental approaches were employed:

1. Laboratory Bioassays ("bio-assay") – Fifty larvae were exposed to varying concentrations of temephos and *Bacillus thuringiensis israelensis* (*Bti*) for 10 minutes, with lethal concentration (LC50/LC90) values recorded after 3, 6, and 12 hours.
2. Semi-Natural Field Trials (“gutter system”) – Larvae attached to natural substrates were exposed to temephos and *Bti* for 10 minutes within a controlled water-flow system, and mortality rates were assessed at 3 and 6 hours.

Results

Laboratory Bioassays

German Species:

Temephos LC50: 3.1 ppm (3h), 0.14 ppm (6h)

Bti LC50: 7.8 ppm (6h), 1.7 ppm (12h)

Cameroonian Species:

Temephos LC50: 0.42 ppm (3h), 0.14 ppm (6h), 0.073 ppm (12h)

Field Trials (Système de Goutières)

The LC50 for temephos in semi-natural conditions was 0.84 ppm (3h)

Bti LC50: 5.52 ppm and 3.12 ppm respectively after 3 and 6h.

Partial conclusion and recommendations

The study demonstrated that *Simulium* larvae in Northern Cameroon and Germany remain susceptible to both temephos and *Bacillus thuringiensis* var. *israelensis* (*Bti*). However, larvae from Cameroon exhibited higher susceptibility to temephos in laboratory than their German counter parts. Laboratory bioassays and semi-natural field trials confirmed the efficacy of temephos at LC50 of 0.42 ppm (3h) and 0.14 ppm (6h).

The results of this study confirm the high sensitivity of *Simulium* larvae to *Bacillus thuringiensis* var. *israelensis* (*Bti*), corroborating earlier results by Rutschke [74]. The latter demonstrated that a dose of 1 ppm applied for 10 minutes in the Vina du Nord River in Cameroon resulted in larval mortality of more than 90% up to 10 km downstream, with effects detectable even after 20 hours. Similarly, our laboratory and semi-natural experiments corroborate the larvicidal efficacy of *Bti*, with LC_{50} values ranging from 1.7 ppm in German species after 6 hours to 2.6 L (32 ppm) – 4.6 L (1.47 ppm) of working solution required to reach LC_{50} in Cameroonian species after 3–6

hours of exposure [47]. Interestingly, our results indicate that *Simulium* species from Cameroon, including *S. damnosum s.l.*, are more susceptible to *Bti* than their European counterparts, a trend also observed in Rutschke's previous field studies. The methodological continuity between the two studies, including the 10-minute exposure protocol and the use of aerated bioassays, improves the reliability and reproducibility of *Bti* test systems. Furthermore, while Rutschke (1988) reported no evidence of resistance, Kamtsap et al. (2022) emphasize the need for molecular surveillance to prevent the development of resistance, particularly in light of historical reports of temephos resistance in West African *Simulium* populations [75]. Overall, *Bti* remains a cornerstone of vector control strategies in onchocerciasis-endemic areas due to its species-specific action, environmental safety, and operational flexibility, particularly in sites where mass drug administration with ivermectin is limited due to the co-endemicity of *Loa loa*.

4. General discussion

This research significantly advances the understanding of onchocerciasis epidemiology, black fly vector diversity, and larvicide susceptibility in Cameroon. The integration of community knowledge surveys, molecular phylogenetics, and vector control evaluations provides a comprehensive view of the challenges and opportunities in onchocerciasis control, especially in light of persistent transmission despite long-term ivermectin distribution.

Despite over 20 years of mass drug administration (MDA) with ivermectin, many Cameroonian communities remain inadequately informed about the disease and its transmission. As shown in Chapter 1, only 9.3% of participants recognized that black fly bites could lead to blindness, with misconceptions linking black flies to malaria particularly common in Mawong and Menchum [46]. These findings are consistent with earlier reports from other endemic African regions, where low awareness of vector-borne disease transmission has undermined control efforts [76]. The persistence of black fly populations, due to a lack of vector control, further exacerbates the risk of transmission, especially in areas where annual biting rates is high flies per person-year, as recorded in Soramboum and Galim [77]. These data underscore the need to integrate vector control into MDA programs and to develop targeted health education strategies, particularly in communities with high illiteracy rates, such as Galim (55.9%) [46].

The diversity of black fly species in Cameroon is greater than previously recognized [29]. Chapter 1 revealed 19 morphologically distinct species, including two undefined species, and provided the first molecular confirmation of *S. vorax* and *S. dentulosum* in Cameroon [48]. The presence of this non-*S. damnosum* species, previously implicated in onchocerciasis transmission in other African countries [78], raises concerns that alternative vector species may sustain transmission. The use of ITS2 and Cox1 gene sequencing facilitate the identification of cryptic species and population structure, with *S. cervicornutum* showing high divergence and widespread distribution, suggesting ecological adaptability [48]. This molecular approach aligns with findings from similar studies in Uganda and Ghana, which also reported genetically diverse *Simulium* species [29, 79].

Chapter 3 demonstrated that black fly larvae in Cameroon are susceptible to both temephos and *Bacillus thuringiensis var. israelensis* (Bti), with higher susceptibility levels than German populations [47]. The LC50 for temephos in Cameroon was as low as 0.073 ppm at 12 hours, while *Bti* achieved 50% mortality at 5.52 ppm and 3.12 ppm respectively after 3 and 6h [47]. These findings confirm that larviciding remains a viable control option, particularly in combination with MDA. However, the historical development of temephos resistance in West Africa [80] highlights the need for continuous resistance monitoring and ecologically sound larviciding strategies. The semi-natural field trials provided realistic efficacy data, underscoring the value of standardized testing protocols for larvicidal implementation at scale [47].

The molecular diversity study of the *Simulium damnosum* complex in Chapter 4 identified four major clades, including the record of *S. sanctipauli* on the molecular level in Cameroon and two undescribed clades [67]. This is significant because *S. sanctipauli* has been a key vector in West Africa and was previously thought to be absent in east of Nigeria [51]. Its presence in Cameroon may reflect recent migration, environmental changes, or historical misclassification. Accurate identification is essential for vector control planning, as cryptic species may vary in vector competence, biting behaviour, and susceptibility to interventions [7].

In summary, the combination of knowledge assessments, species identification, and larvicide susceptibility testing reveals a complex and evolving onchocerciasis transmission landscape in Cameroon. Integrated vector management, incorporating community education, larviciding, and molecular surveillance, is essential to achieve sustainable disease control. Future research should focus on whole-genome sequencing, ecological modeling, and expanded geographic surveillance to inform tailored intervention strategies that reflect local vector ecology and community needs.

In Cameroon in general and in the localities of Menchum in the northwest of the country, Soramboum in the North, in Galim, as well as along the Ntem River in the Ma’an village located in the Southern region in particular, the identification of black flies and their control were little or almost not documented, much less the knowledge of onchocerciasis of the populations living there.

The only way to deal with the irreversible effects of the disease in these localities is the mass distribution of ivermectin undertaken by the state and its partners. Consequently,

populations are somewhat left to their own devices since it is believed that the effects of onchocerciasis would be more tolerable than the side effects of the drug [81, 82]. Therefore, the main and best solution to deal with this neglected tropical disease in these forest areas with the presence of *Loa loa* would be a good implementation of vector control and the identification of the species circulating there.

The many studies carried out on the identification of black flies based on chromosomes over the decade's present considerable limitations. This study identifies a wide range of species using advanced, up-to-date methods, enabling precise determination of the concentration and timing required for effective vector control in this type of area. The implementation of suggested ideas and methods could help planning proper vector control strategies and could then be an excellent mean to fight against onchocerciases in co endemicity area with other filariae such as *Loa loa*.

References

1. Crosskey RW: **The natural history of blackflies**. Chichester: John Wiley & Sons; 1990, 722.
2. Wahl G, Achu-Kwi MD, Mbah D, Dawa O, Renz A: **Bovine onchocercosis in North Cameroon**. *Veterinary Parasitology,* 1994, **52**(3-4):297-311, https://doi.org/10.1016/0304-4017(94)90121-X.
3. Duke BOL, Moorehouse DE, Vince DS: **Studies on factors influencing the transmission of onchocerciasis in the Cameroon rain-forest**. *Bulletin of the World Health Organization,* 1966, **35**(5):567-646.
4. Barbazan P, Escaffre H, Mbentengam R, Boussinesq M: **Entomologic study on the transmission of onchocerciasis in a forest-savanna transition area of Cameroon**. *Bulletin de la Société de pathologie exotique,* 1998, **91**(2):178-182.
5. Hendy A, Krit M, Pfarr K, Laemmer C, De Witte J, Nwane P, Kamgno J, Nana-Djeunga HC, Boussinesq M, Dujardin JC *et al*: ***Onchocerca volvulus* transmission in the Mbam valley of Cameroon following 16 years of annual community-directed treatment with ivermectin, and the description of a new cytotype of *Simulium squamosum***. *Parasites and vectors* 2021, **14**(1):563-577, 10.1186/s13071-021-05072-y.
6. Mendoza Aldana J, Piechulek H, Maguire J: **Forest onchocerciasis in Cameroon: its distribution and implications for selection of communities for control programmes**. *Annals of Tropical Medicine and Parasitology* 1997, **91**(1):79-86, 10.1080/00034983.1997.11813114.
7. Adler PH: **World blackflies (Diptera:Simuliidae): A comprehensive revision of the taxonomy and geographical inventory.** 2024, 145.
8. McCRAE AWR: **Ecology and speciation in African Blackflies (Diptera:Simuliidae)**. *Biological Journal of the Linnean Society*, 2008, **1**(1-2):43-49, https://doi.org/10.1111/j.1095-8312.1969.tb01811.x.
9. Ruiz-Arrondo I, Hernandez-Triana LM, Ignjatovic-Cupina A, Nikolova N, Garza-Hernandez JA, Rodriguez-Perez MA, Oteo JA, Fooks AR, Lucientes Curdi J: **DNA barcoding of blackflies (Diptera: Simuliidae) as a tool for species identification and detection of hidden diversity in the eastern regions of Spain**. *Parasites and Vectors* 2018, **11**(1):463, https://doi.org/10.1186/s13071-018-3046-7.

10. Fain A, Elsen P: **Notes on *Simulium* of Eastern Cameroon**. *Journal of African Zoology and Botany*, 1973, **87**(34):519-554.
11. **Morphological Criteria for *Simulium damnosum*: Identification of adult females of the *S. demnosum* complex** [https://www.riverblindness.eu/wp-content/uploads/2010/05/RENZ-Togo-1982-Morphological-criteria-*Simulium-damnosum*.pdf], June 2025.
12. Lee, Y C. **Studies on the leucocytozoonosis of chickens**. *International Office of Epizootics,* 1968, **69**(1):215-220.
13. Li G, Lin H, Ong Y, Qiu Z, Zhu J, Zhong C: **Epidemiological surveys of leucocytozoon caulleryi infection in chicks in Guangdong, Shandong and Fujian, China**. *Chinese Journal of Veterinary Science and Technology*, 1998, **28**:17-19.
14. Wahl G: **Identification of a Common Filarial Larva in *Simulium damnosum* s.l. (Type D, Duke, 1967) as *Onchocerca ramachandrini* from the Wart Hog**. *The Journal of* Parasitology, 1996, **82**(3):520-524.
15. Wahl G, Bain O: **Development by injection in *Simulium damnosum* s.l. of two *Onchocerca* species from the wart hog to infective larvae resembling type D larvae (Duke, 1967)**, *Parasite*, 1995, **2**(1):55-62, 10.1051/parasite/1995021055.
16. Wahl G, Ekale D, Enyong P, Renz A: **The development of *Onchocerca dukei* and *O. ochengi* microfilariae to infective-stage larvae in *Simulium damnosum s.l.* and in members of the *S. medusaeforme* group, following intra-thoracic injection**. In: *Annals of Tropical Medicine and Parasitology*, 1991 **85**:329-337, 10.1080/00034983.1991.11812569.
17. Bain O, Wahl G, Renz A: ***Onchocerca ramachandrini* n. sp. from the warthog in Cameroon**. *Annales de Parasitologie Humaine et Comparée, 1993,* **68**(3):139-143, 10.1051/parasite/1993683139.
18. Bain O, Renz A: **Infective larvae of a new species of *Robertdollfusidae* (Adenophorea, Nematoda) in the gut of *Simulium damnosum* in Cameroon**. *Annales de Parasitologie Humaine et Comparée*, 1993, **68**(4):182-184, 10.1051/parasite/1993684182.
19. Wenk P, Loubier C: ***Onchocerca tarsicola* (Filarioidea: Nematoda) in the dwarf goat (*Capra hircus L.*)**. *Zeitschrift für Parasitenkund*, 1980, **62**(2):197-198, https://doi.org/10.1007/BF00927866.

20. Schulz-Key H: **Investigations on the filariae of cervids in southern Germany, 1, Nodule formation, sex determination and microfilariae release in *Onchocerca flexuosa* (Wedl, 1856) in red deer (*Cervus elaphus*)**. *Tropical Medicine and Parasitology*, 1975, 26(1):60-69.
21. Nwoke BEB: **Studies on the Field Epidemiology of Human Onchocerciasis on the Jos Plateau, Nigeria—VII. The Effects of Climatic Factors on the Diurnal Biting Behaviour of *Simulium damnosum* Theobald (Diptera: Simuliidae)**. *International Journal of Tropical Insect Science*, 1988, **9**(3):323-328, https://doi.org/10.1017/S1742758400006275.
22. Đuknić J, Jovanović VM, Popović N, Živić I, Raković M, Čerba D, Paunović M: **Phylogeography of *Simulium* bubgenus *Wilhelmia* (Diptera: Simuliidae)-Insights from Balkan populations**. *Journal of Medical Entomology*, 2019, **56**:967-978.
23. McCreadie JW, Adler PH: **Scale, time, space, and predictability: Species distributions of preimaginal black flies (Diptera: Simuliidae)**. *Oecologia*, 1998, **114**: 79-92, https://doi.org/10.1007/s004420050423.
24. Vajime CG, Dunbar RW: **Chromosomal identification of eight species of the subgenus Edwardsellum near and including *Simulium* (*Edwardsellum*) *damnosum Theobald* (Deptera: Simuliidae)**. *Tropical medicine and parasitology*, 1975, **26**(1):111-138.
25. Mafuyai H, Post R, Vajime C, Molyneux D: **Cytotaxonomic identifications of the *Simulium damnosum* complex (Diptera: Simuliidae) from Nigeria**. *Tropical Medicine and International Health*, 1996, **1**:779-785, https://doi.org/10.1111/j.1365-3156.1996.tb00110.x.
26. Duncan GA, Adler PH, Pruess KP, Powers TO: **Molecular differentiation of two sibling species of the black fly *Simulium vittatum* (Diptera: Simuliidae) based on random amplified polymorphic DNA**, 2004, *Genome*, **47**:373-379, 10.1139/g03-144.
27. Hajibabaei M, Janzen DH, Burns JM, Hallwachs W, Hebert PD: **DNA barcodes distinguish species of tropical Lepidoptera**. *Proceedings of the National Academy of Sciences of the United States of America, 2006,* **103**(4):968-971, 10.1073/pnas.0510466103.
28. Simard F, Fontenille D, Lehmann T, Girod R, Brutus L, Gopaul R, Dournon C, Collins FH: **High amounts of genetic differentiation between populations of**

the malaria vector *Anopheles arabiensis* from West Africa and eastern outer islands. In: *The American journal of tropical medecine and hygiene*, 1999, **60**(6):1000-1009, 10.4269/ajtmh.1999.60.1000.

29. Krueger A, Hennings IC: **Molecular phylogenetics of blackflies of the *Simulium damnosum* complex and cytophylogenetic implications**. *Molecular Phylogenetics and Evolution*, 2006, **39**(1):83-90, 10.1016/j.ympev.2005.11.007.
30. Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PD: **DNA barcoding Australia's fish species**. *Philosophical transactions of the Royal Society of London*, 2005, 360(1462): 1847-1857, 10.1098/rstb.2005.1716.
31. Sitarz M, Buczek AM, Buczek W, Buczek A, Bartosik K: **Risk of attacks by blackflies (Diptera: Simuliidae) and occurrence of severe skin symptoms in bitten patients along the eastern border of the european union**. *International journal of environmental research and public health*, 2022, **19**(13):7610-7628, 10.3390/ijerph19137610.
32. Opara KN, Fagbemi BO, Atting IA, Oyene UE, Okenu DM: **Status of forest onchocerciasis in the Lower Cross River Basin, Nigeria: change in clinical and parasitological indices after 6 years of ivermectin intervention**. *Public Health*, 2007, **121**(3):202-207, 10.1016/j.puhe.2006.09.010.
33. Tabatabaei F, Azarmi S, Abbaszadeh Afshar MJ, Yarizadeh H, Mohtasebi S: **Blackfly fever and dermatitis caused by *Simulium kiritshenkoi*: a human case report in Iran**. *BMC Infectious Diseases*, 2020, **20**(1):348-352, 10.1186/s12879-020-05070-y.
34. WHO: **Entomological manual for onchocerciasis elimination programmes**, 2023.
35. Renz A, Wenk P: **The distribution of the microfilariae of *Onchocerca volvulus* in the different body regions in relation to the attacking behaviour of *Simulium damnosum s.l.* in the Sudan savanna of northern Cameroon**. *Transactions of the Royal Society Tropical Medicine and Hygiene*, 1983, **77**(6):748-752, 0.1016/0035-9203(83)90278-x.
36. Youssefi Mr, Aminpour A, Arab F: **Dermatitis caused by *Simulium* (Blackflies) bite**. *Iranian Journal of Parasitology* 2008, **3**(3):48-53.
37. Anderson J, Fuglsang H, Hamilton PJS, dE C. Marshall TF: **Studies on onchocerciasis in the United Cameroon Republic II. Comparison of**

onchocerciasis in rain-forest and Sudan-savanna. *Transactions of The Royal Society of Tropical Medicine and Hygiene*, 1974, **68**(3):209-222, https://doi.org/10.1016/0035-9203(74)90117-5.

38. Duke BO, Anderson J, Fuglsang H: **The *Onchocerca volvulus* transmission potentials and associated patterns of onchocerciasis at four Cameroon Sudan-savanna villages**. *Tropical medicine and parasitology*, 1975, 26(2):143-154.
39. Kamga HL, Njc A, Anna N, Njimoh D: **A community survey on the knowledge of neglected tropical diseases in Cameroon**. *International Journal of Medicine and Biomedical Research*, 2012, **1**(2):131-140.
40. Katabarwa MN, Eyamba A, Chouaibou M, Enyong P, Kuété T, Yaya S, Yougouda A, Baldiagaï J, Madi K, Andze GO *et al*: **Does onchocerciasis transmission take place in hypoendemic areas? a study from the North Region of Cameroon**. *Tropical Medicine of International Health*, 2010, **15**(5):645-652, 10.1111/j.1365-3156.2010.02501.x.
41. Katabarwa MN, Eyamba A, Nwane P, Enyong P, Yaya S, Baldiagaï J, Madi TK, Yougouda A, Andze GO, Richards FO: **Seventeen years of annual distribution of ivermectin has not interrupted onchocerciasis transmission in North Region, Cameroon**. The *American Journal of Tropical Medicine and Hygiene*, 2011, **85**(6):1041-1049, 10.4269/ajtmh.2011.11-0333.
42. Katabarwa MN, Eyamba A, Nwane P, Enyong P, Kamgno J, Kuete T, Yaya S, Aboutou R, Mukenge L, Kafando C *et al*: **Fifteen years of annual mass treatment of onchocerciasis with ivermectin have not interrupted transmission in the West region of Cameroon**. *Journal of Parasitology Research*, 2013, 2013:420-928, 10.1155/2013/420928.
43. Mustapha M, Post RJ, Enyong P, Lines J: **A new cytotype of *Simulium squamosum* from south-west Cameroon**. *Medical and Veterinary Entomology,* 2004, **18**(3):296-300, 10.1111/j.0269-283X.2004.00501.x.
44. Somo RM, Enyong PA, Fobi G, Dinga JS, Lafleur C, Agnamey P, Ngosso A, Ngolle EM: **A study of onchocerciasis with severe skin and eye lesions in a hyperendemic zone in the forest of southwestern Cameroon: clinical, parasitologic, and entomologic findings**. The *American Journal of Tropical Medicine and Hygiene,,* 1993, **48**(1):14-19, 10.4269/ajtmh.1993.48.14.

45. Pion SDS, Clément MCA, Boussinesq M: **Impact of four years of large-scale ivermectin treatment with low therapeutic coverage on the transmission of *Onchocerca volvulus* in the Mbam valley focus, central Cameroon**. *Transactions of The Royal Society of Tropical Medicine and Hygiene,* 2004, **98**(9):520-528, 10.1016/j.trstmh.2003.11.010.
46. Kamtsap P, Nguemaïm Ngoufo F, Paguem A, Renz A: **Knowledge and Practices of Four Onchocerciasis-Endemic Communities in Cameroon**. *Microorganisms*, 2025, **13**(4):736-750, 10.3390/microorganisms13040736.
47. Kamtsap P, Paguem A, Nguemaïm Ngoufo F, Njioukou F, Renz Al: **Testing the susceptibility of larval stages of *Simulium* to temephos and *Bacillus thuringiensis* var. *israelensis* in Germany and Northern Cameroon**. *Medical and Veterinary Entomology*, 2023, **37**(2):286-299, 10.1111/mve.12630.
48. Kamtsap P, Paguem A, Nguemaïm Ngoufo F, Renz A: **Morphological and Molecular Investigation of Non-*Simulium damnosum* Black Flies in Cameroon Using Nuclear ITS 2 and Mitochondrial Cox 1 Genes**. *Insects*, 2025, **16**(6):572-597, https://doi.org/10.3390/insects16060572.
49. Cameroon Bi: **The Chinese company, CWE, concludes environmental impact study on Menchum Dam**; 2014.
50. Cheke RA, Hawkes FM, Carnaghi M: **Short- and Long-Range Dispersal by Members of the *Simulium damnosum* Complex (Diptera: Simuliidae), Vectors of Onchocerciasis: A Review**. *Insects*, 2024, **15**(8):606-619, 10.3390/insects15080606.
51. Amazigo UV, Leak SGA, Zoure HG, Okoronkwo C: **Community-directed distributors—the "foot soldiers" in the fight to control and eliminate neglected tropical diseases**. *PLOS Negleted Tropical Diseases,* 2021, **15**(3):979-986, https://doi.org/10.1371/journal.pntd.0009088.
52. World Health O: **Guidelines for stopping mass drug administration and verifying elimination of human onchocerciasis: Criteria and procedures**. *Geneva: World Health Organization*, 2016.
53. Adeleke, M. A., Opara, K. N., Mafuyai, H. B., Nwoke, B. E. B., Surakat, O. A., Akinde, S. B., Nwoke, M., Chikezie, F. M., Yaro, C. A., Mmaduabuchi, U., Igbe, M., Makata, E., Oyediran, F., Anyaike, C., Tongjura, J., Hawkes, F., & Iwalewa, Z. O.: **Improving onchocerciasis elimination surveillance: trials of odour baited Esperanza Window Traps to collect black fly vectors and real-time**

qPCR detection of *Onchocerca volvulus* in black fly pools. *Parasites and Vectors*, 2024, **17**(1):471-483, 10.1186/s13071-024-06554-5.

54. Crosskey RW: **The Natural History of Blackflies**. London, UK: British Museum (Natural History), 1990.
55. Ibeh O, Nwoke B, Adegoke J: **Morphological differentiation of vectors of onchocerciasis, *Simulium damnosum* complex, in south-east Nigeria**. *Nigerian Journal of Parasitology*, 2008, **29**(1)**,** 10.4314/njpar.v29i1.37905.
56. Post RJ, Onyenwe E, Somiari SA, Mafuyai HB, Crainey JL, Ubachukwu PO: **A guide to the *Simulium damnosum* complex (Diptera: Simuliidae) in Nigeria, with a cytotaxonomic key for the identification of the sibling species**. *Annals of tropical medicine and parasitology*, 2011, **105**(4):277-297, 10.1179/136485911X12987676649700.
57. Anderson JR, DeFoliart GR: **Feeding Behavior and Host Preferences of Some Black Flies (Diptera:Simuliidae) in Wisconsin**. *Annals of the Entomological Society of America*, 1961, **54**(5), 716–729, https://doi.org/10.1093/aesa/54.5.716.
58. Anong C.I.N1, Akinboade O.A1, Abiola J.O2, Uwalaka E.C: **Factors enhancing the survival and activities of the *Simulium damnosum***. Nature and science of sleep, 2015, **13**(4):102-105.
59. Dunbar RW: **Four sibling species included in *Simulium damnosum* Theobald (Diptera: Simuliidae) from Uganda**. *Nature*, 1966, **209**(5023):597-599.
60. Post RJ, Mustapha M, Krueger A: **Taxonomy and inventory of the cytospecies and cytotypes of the *Simulium damnosum* complex**. *Tropical medicine and international health,* 2007, **12**(11):1342-1353, 10.1111/j.1365-3156.2007.01921.x.
61. McCall PJ: **Oviposition aggregation pheromone in the Simulium damnosum complex**. *Medical and Veterinary Entomology,* 1995, **9**(2):101-108, DOI:10.1111/j.1365-2915.1995.tb00165.x
62. Ayisi F, Sedou N, Dieunang SK, Yaya F, Tchago EF, Ndellejong CE, Biholong B, Boakye DA: **A cross-sectional study of *Simulium damnosum sensu lato* breeding sites and species distribution in Sudan savanna, mixed savanna–forest and rainforest regions in Cameroon**. *Parasites and Vectors* 2022, **15**(1):382, https://doi.org/10.1186/s13071-022-05462-w.

63. Ezugbo-Nwobi IK, Eneanya CI: **Ecology and Biting Activity of *Simulium Damnosum* Complex in Nigeria:** *The Bioscientist*, 2013, **1**(1):39-46.
64. Cheke RA, Post RJ, Boakye D: **Seasonal variations and other changes in the geographical distributions of different cytospecies of the *Simulium damnosum* complex (Diptera: Simuliidae) in Togo and Benin**. *Elsevier*, 2023,**245**(2):106970-106978, https://doi.org/10.1016/j.actatropica.2023.106970.
65. Morales-Hojas R, Krueger A: **The species delimitation problem in the *Simulium damnosum* complex, blackfly vectors of onchocerciasis**. *Medical and vetenary entomology,* 2009, **23**(3):257-268, 10.1111/j.1365-2915.2009.00816.x.
66. Kononov A, Ustyantsev K, Wang B, Mastro VC, Fet V, Blinov A, Baranchikov Y: **Genetic diversity among eight *Dendrolimus* species in *Eurasia* (Lepidoptera: Lasiocampidae) inferred from mitochondrial COI and COII, and nuclear ITS2 markers**. *BMC genetic*, 2016, 17(3):157-167, 10.1186/s12863-016-0463-5.
67. Kamtsap P, Paguem A, Renz A: **Molecular diversity in the *Simulium damnosum* complex (Diptera: Simuliidae) in Cameroon**. *Mitteilungen der Deutschen Gesellschaft für allgemeine und angewandte Entomologie* 2024, **23**:147.
68. Post RJ, Laudisoit A, Mandro M, Lakwo T, Laemmer C, Pfarr K, Hoerauf A, Tortosa P, Gomard Y, Ukety T *et al*: **Identification of the onchocerciasis vector in the Kakoi-Koda focus of the Democratic Republic of Congo**. *PLOS Neglected Tropical Diseases,* 2022, **16**(11):27, https://doi.org/10.1371/journal.pntd.0010684..
69. Seidenfaden R, Fischer A, Bonow I, Ekale D, Tanya V, Renz A: **Combined benefits of annual mass treatment with ivermectin and cattle zooprophylaxis on the severity of human onchocerciasis in northern Cameroon**. *Tropical Medicine and International Health*, 2001, **6**(9):715-725, 10.1046/j.1365-3156.2001.00771.x.
70. Domche A, Nana-Djeunga HC, Yemeli LD, Nanga CL, Boussinesq M, Njiokou F, Pion S, Kamgno J: **Knowledge/perception and attitude/practices of populations of two first-line communities of the Centre Region of Cameroon regarding onchocerciasis and black fly nuisance and bio-**

ecology. *Parasites and vectors,* **14**(1): 2021: 546, 10.1186/s13071-021-05048-y.

71. Adeoye AO, Ashaye AO, Onakpoya OH: **Perception and attitude of people toward onchocerciasis (river blindness) in South Western Nigeria**. *Middle East African journal of ophthalmology*, 2010, **17**(4):310-314, 10.4103/0974-9233.71594.
72. Alonso L, Ortiz Z, Garcia B, Nguema R, Nguema J, Ncogo P, Gárate T, González-Escalada A, Benito A, Azcarraga P: **Knowledge, attitudes, and practices toward onchocerciasis among local population in Bioko Island, Equatorial Guinea**. In: *Annals of tropical medicine and parasitology* ,2017, **10**(5) 2017: 1228.
73. Markham LKB, Ruiter RC, Fernandez M, Kok G: **Planning health promotion programs: An intervention mapping approach, 2nd ed**. Hoboken, NJ, US: Jossey-Bass/Wiley, 2006.
74. Rutschke J: **Untersuchungen zur Bekämpfung der Simuliiden-Larven (Diptera) mit Bacillus thuringiensis H-14**. *Ph.D. Dissertation.* University of Tübingen; 1988.
75. Guillet P, Escaffre H, Ouedraogo M, Quillévéré D: **Evidence of resistance to temephos in the *Simulium damnosum* complex (*S. sanctipauli* and *S. soubrense*) in Ivory Coast**. *ORSTOM Notebooks, Medical Entomology and Parasitology Series,* 1980, **18**(4):84-92.
76. Worku H, Mola M, Alemu BD, Jemal SS, Ayiza A, Getachew S, Chauhan NM, Tulshiram Hajare S, Chandra Singh S, Kuddus M, Upadhye VJ: **Knowledge, attitude, and practice of community towards an onchocerciasis elimination program from South West Ethiopia**. *Journal of Tropical Medicine,* 2022, **2022**:11, **https://doi.org/10.1155/2022/1417804**.
77. Eisenbarth A, Achukwi MD, Renz A: **Ongoing transmission of *Onchocerca volvulus* after 25 Years of annual ivermectin mass treatments in the Vina du Nord river valley, in North Cameroon**. PLoS *Neglected tropical diseases,* 2016, **10**(2):16, 10.1371/journal.pntd.0004392.
78. Cheke RA, Basáñez MG, Perry M, White MT, Garms R, Obuobie E, Lamberton PH, Young S, Osei-Atweneboana MY, Intsiful J, Shen M, Boakye DA, Wilson MD: **Potential effects of warmer worms and vectors on onchocerciasis transmission in West Africa**. *Philosophical transactions of the royal society of*

London. Series B-biological sciences, 2015, **370**(1665):14, 10.1098/rstb.2013.0559.

79. Lamberton PH, Cheke RA, Walker M, Winskill P, Osei-Atweneboana MY, Tirados I, Tetteh-Kumah A, Boakye DA, Wilson MD, Post RJ et al: **Onchocerciasis transmission in Ghana: biting and parous rates of host-seeking sibling species of the *Simulium damnosum* complex**. *Parasites and vectors*, 2014, **7**(5):511-534, 10.1186/s13071-014-0511-9.
80. Kurtak D, Meyer R, Ocran M, Ouédraogo M, Renaud P, Sawadogo RO, Télé B: **Management of insecticide resistance in control of the *Simulium damnosum* complex by the Onchocerciasis Control Programme, West Africa: potential use of negative correlation between organophosphate resistance and pyrethroid susceptibility**. *Medical and veterinary entomology,* 1987, **1**(2):137-146, 10.1111/j.1365-2915.1987.tb00334.x.
81. Gardon J, Gardon-Wendel N, Demanga N, Kamgno J, Chippaux J-P, Boussinesq M: **Serious reactions after mass treatment of onchocerciasis with ivermectin in an area endemic for *Loa loa* infection**. *The Lancet* 1997, **350**(9070):18-22, 10.1016/S0140-6736(96)11094-1.
82. Kamgno J, Pion SD, Chesnais CB, Bakalar MH, D'Ambrosio MV, Mackenzie CD, Nana-Djeunga HC, Gounoue-Kamkumo R, Njitchouang GR, Nwane P *et al*: **A test-and-not-treat strategy for onchocerciasis in *Loa loa*-endemic areas**. *The new England journal of medicine* 2017, **377**(21):2044-2052, 10.1056/NEJMoa1705026.

Appendix:

a) Accepted publications

1. **Kamtsap, P.**, Paguem, A., & Renz, A.: **Molecular diversity in the *Simulium damnosum* complex (Diptera: Simuliidae) in Cameroon**. *Mitteilungen der Deutschen Gesellschaft für Allgemeine und Angewandte Entomologie*, 2024, **23**: 147-152.

2. **Kamtsap P.**, Paguem A., Nguemaïm Ngoufo F., Renz A.: **Morphological and Molecular Investigation of Non-*Simulium damnosum* Black Flies in Cameroon Using Nuclear ITS 2 and Mitochondrial Cox 1 Genes**. *Insects*, 2025; **16**(6):572-592, https://doi.org/10.3390/insects16060572

3. **Kamtsap P**., Nguemaïm Ngoufo F., Paguem A., Renz A.: **Knowledge and Practices of Four Onchocerciasis-Endemic Communities in Cameroon. Microorganisms**, 2025; 13(4):736-750, doi: 10.3390/microorganisms13040736

4. **Kamtsap P.**, Paguem A., Nguemaïm Ngoufo F., Njiokou F., Renz A.: **Testing the susceptibility of larval stages of *Simulium* to temephos and *Bacillus thuringiensis* var *israelensis* in Germany and Northern Cameroon**. *Medical and Veterinary Entomology*, 2022; **37**(2):286-299, doi:10.1111/mve.12630.

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

Pierre Kamtsap [1,3], Archile Paguem [2,3] & Alfons Renz [1,3]

[1] University of Tübingen, Institute for Evolution and Ecology, Comparative Zoology
[2] University of Buea, Faculty of Agriculture and Veterinary Medicine, Department of Veterinary
[3] Programme Onchocercoses, Field Station of the University of Tübingen, Ngaoundéré-Cameroon

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*

Pierre Kamtsap, University of Tübingen, Institute for Evolution and Ecology, Comparative Zoology, Auf der Morgenstelle 28, D-72076 Tübingen, Germany
E-Mail: kamtsapbuea200@gmail.com

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*.

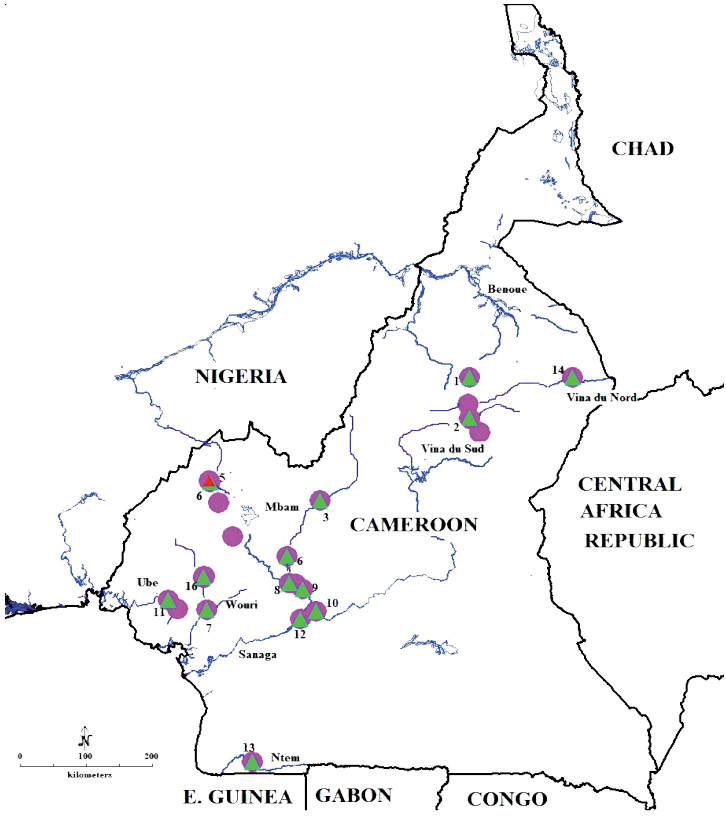


Fig. 1. Map of Cameroon showing sites at which larvae, pupae and adult female flies of the *Simulium damnosum* complex were collected (marked in green). Blue represent Cameroon water lines.

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)

Sequences analysis

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.

Acknowledgments
This study was supported by the Medical Research Council of the UK through the Global Challenges Research Fund and granted by the United Kingdom's Official Development Assistance (ODA) through the Centre for Research in Infectious Diseases (CRID), Grant/Award Number: MR/P027873/1; PK was supported by the Baden-Württemberg-Stipendium. We acknowledge David Ekale and Jeremi Yembo from the ProgOncho Laboratory in Ngaoundere-Cameroon for having been implicated in sample collections and Peter Enyong from the University of Buea for providing samples of *S. damnosum.* This study was partially supported by the DFG-COBE grant (234586079).

References:

Adler, P.H. (2022): World Blackflies (Diptera:Simuliidae): A comprehensive revision of the taxonomy and geographical inventory. – Zootaxa **4455** (1): 145pp.

Adler, P.H., Srisuka, W. Low, v.L., Takaoka, H. & Saeung, A. (2019): High-Elevation Chromosomal Diversity of Black Flies (Diptera: Simuliidae) in Thailand. – Insect Systematics and Diversity **3**(3): 1-10.

Akiba, K. (1970): Leucocytozoonosis of chickens. – National Institute of Animal Health Quarterly **10**: 131-47.

Fain, A., & Elsen, P. (1973): Notes on Simulies of Eastern Cameroon. – Rev. Zoo1. Bot. afr. **87**: 34.

Hajibabaei, M., Janzen, D.H., Burns, J.M., Hallwachs, W. & Hebert, P.D. (2006): DNA barcodes distinguish species of tropical Lepidoptera. – Proceedings of the National Academy of Sciences of the United States of America **103**: 968-671.

Kim, S.R., Kim, K.Y., Jeong, J.S., Kim, M.J., Kim, K.H., Choi, K.H. & Kim, I. (2017): Population genetic characterization of the Japanese oak silkmoth, *Antheraea yamamai* (Lepidoptera: Saturniidae), using novel microsatellite markers and mitochondrial DNA gene sequences. – Genetics and Molecular Research **16**: 19.

Kononov, A., Ustyantsev, K., Wang, B., Mastro, V.C., Fet, V., Blinov, A. & Y. Baranchikov, Y. (2016a): Genetic diversity among eight Dendrolimus species in Eurasia (Lepidoptera: Lasiocampidae) inferred from mitochondrial COI and COII, and nuclear ITS2 markers. – BMC genetics **17**: 157.

Krueger, A. & Hennings, I.C. (2006): Molecular phylogenetics of blackflies of the Simulium damnosum complex and cytophylogenetic implications. – Mol. Phylogenet. Evol. **39**: 83-90.

Li, G.Q., Lin, H.H., Ong, Y.B., Qiu, Z.F., Zhu, J.R. & Zhong, C.H.Y. (1998): Epidemiological surveys of Leucocytozoon caulleryi infection in chicks in Guangdong, Shandong and Fujian, China. – Chinese Journal of Veterinary Science and Technology **28**(8): 17-19.

Mafuyai, H.B., Post, R.J., Vajime, C.G. & Molyneux, D.H. (1996): Cytotaxonomic identifications of the Simulium damnosum complex (Diptera: Simuliidae) from Nigeria. – Tropical Medicine & International Health **1**(6): 779-785.

McCrae, A.W.R. (2008): Ecology and speciation in African Blackflies (Diptera:Simuliidae). – Biological Journal of the Linnean Society **1**(1/2): 43-49.

Prakash, A., Walton, C., Bhattacharyya, D.R., Loughlin, S.O., Mohapatra, P.K. & Mahanta, J. (2006): Molecular characterization and species identification of the Anopheles dirus and An. minimus complexes in north-east India using r-DNA ITS-2. Acta Trop.**100**(1-2):156-161.

Renz, A., Barthlmess, C. & Eisenbeiss, W. (1987): Vectorial capacity of Simulium damnosum s.l. populations in Cameroon. – Tropical Medicine and Parasitology **38**: 344-345.

Ruiz-Arrondo, I., Hernández-Triana, L.M., Ignjatović-Ćupina, A., Nikolova, N., Garza-Hernández, J.A., Rodríguez-Pérez, M.A., Oteo, J.A., Fooks, A.R. & Lucientes Curdi, J. (2018): DNA barcoding of blackflies (Diptera: Simuliidae) as a tool for species identification and detection of hidden diversity in the eastern regions of Spain. – Parasit Vectors **11**(1): 463.

insects

MDPI

Article

Morphological and Molecular Investigation of Non-*Simulium damnosum* Black Flies in Cameroon Using Nuclear ITS 2 and Mitochondrial Cox 1 Genes

Pierre Kamtsap [1,2,*], Archile Paguem [2,3], Flore Nguemaïm Ngoufo [4] and Alfons Renz [1,2]

[1] Institute for Evolution and Ecology, Department of Comparative Zoology, University of Tübingen, Auf der Morgenstelle 28, 72076 Tübingen, Germany; alfons.renz@uni-tuebingen.de
[2] Onchocerciasis Program Field Station of the University of Tübingen, Ngaoundéré, Cameroon; achillepaguem@yahoo.fr
[3] Faculty of Agriculture and Veterinary Medicine, Department Veterinary Medicine, University of Buea, Buea P.O. Box 63, South West, Cameroon
[4] Faculty of Health Sciences, University of Bamenda, Bambili Po. Box 39, Cameroon; ngflorema@yahoo.fr
* Correspondence: kamtsapbuea200@gmail.com

Simple Summary: Using morphological and molecular techniques, we investigated the biodiversity of black flies in Cameroon, with a special emphasis on non-*Simulium-damnosum* species. After gathering 1184 pupae from 13 different locations, we used gill morphology and DNA sequencing (Cox1 and ITS2 genes) to identify 19 species. The first identification of 2 undescribed (based on identification keys we used) *Simulium* species in Cameroon and the validation of the known onchocerciasis vectors, *Simulium vorax* (first time to be described in Cameroon) and *Simulium dentulosum*, are important discoveries. For reference, DNA sequences were uploaded to GenBank. This study emphasizes the advantages of molecular approaches in revealing the diversity of cryptic species and the drawbacks of conventional morphological techniques. The most widely dispersed species was found to be *Simulium cervicornutum*, while species such as *S. alcocki* and *S. kenyae* showed restricted distributions. This study highlights the possibility that, in the right circumstances, non-*damnosum* species could spread illness, urging increased molecular analysis and vector surveillance in Cameroon. To improve vector control techniques and obtain a deeper understanding of species-specific roles in pathogen transmission, future research should integrate whole-genome sequencing and more comprehensive ecological and taxonomy studies.

Abstract: Background: This study enhances knowledge of black fly biodiversity in Cameroon by integrating morphological and molecular analyses. A total of 19 *Simulium* species were identified from 1184 pupae collected across 13 sites, using morphological examination of gills and DNA sequencing of Cox1 and ITS2 markers. Key findings include the first report of 2 not yet described (based on identification keys used) species in Cameroon and confirmation of *S. vorax* and *S. dentulosum* as known vectors of onchocerciasis. DNA sequences have been deposited in GenBank for reference. Methods: Combining morphological and molecular approaches revealed more species diversity than previously described, showing the potential of molecular techniques in black fly study. Notably, the presence of species not typically associated with human-biting behavior (e.g., *S. cervicornutum*) raises the possibility that such flies could act as vectors under favorable conditions. Conclusion: This study underscores the importance of identifying *Simulium* species for understanding their role in pathogen transmission. The results provide a foundation for further research on undescribed *Simulium* species and their potential vectorial capacities. Future studies should explore the ecological and behavioral factors influencing vector status, especially in the context of environmental changes. By bridging morphology and DNA analysis, this

check for updates

Academic Editor: Ding Yang

Received: 31 December 2024
Revised: 17 February 2025
Accepted: 28 February 2025
Published: 28 May 2025

Citation: Kamtsap, P.; Paguem, A.; Nguemaïm Ngoufo, F.; Renz, A. Morphological and Molecular Investigation of Non-*Simulium damnosum* Black Flies in Cameroon Using Nuclear ITS 2 and Mitochondrial Cox 1 Genes. *Insects* **2025**, *16*, 572. https://doi.org/10.3390/insects16060572

research advances the study of black flies and sets the stage for improved vector monitoring and disease control in Cameroon and beyond.

Keywords: *Simuliidae*; Cameroon; molecular; identification; black fly; *vorax*; Menchum; *dentulosum*; Soramboum; Mawong

1. Introduction

Despite the importance of black flies in the transmission of various parasites, few studies have been carried out concerning the general diversity and identification (on the molecular level) of black flies in Cameroon. Larvae and pupae are widespread in fast-flowing water of rivers and tributaries [1,2]. Some adult females are bloodsucking, their life cycle includes feeding on vertebrates, e.g., wild and domestic animals and humans, as blood-hosts [3,4]. Thereby, black flies transmit important pathogens such as *Onchocerca volvulus* and *O. ochengi*, causative agents of human onchocerciasis (river blindness) and bovine onchocercosis [5,6], respectively, *Leucocytozoon* to birds in Asia and North America [7,8], and numerous other filarial parasites of wild and domestic animals (*Onchocerca dukei*, *O. ramachandrini*, *O. lienalis*, *O. lupi*, *O. flexuosa*, *Lappnema* sp.) [3,4,9–12].

Black flies (Diptera: Simuliidae) are present worldwide, there being 31 genera containing 2348 species (2331 living and 17 fossil species) [13]. In Africa, 124 black fly species have been described, mostly in the Ethiopian region in the 1950s [14]. In Cameroon, 55 species have been morphologically described (Table 1) [13] and only a few are as yet well classified [15]. Similarly, species diversity and identity on the molecular level in Cameroon remain insufficiently understood. Not only knowledge of the geographical characteristics and the geographical diversity of the members of the *S. damnosum* complex but also the determination of the differentiation scale and estimation of the distance between populations are necessary for any planned vector control.

Table 1. Comprehensive list of Simuliidae species described from Cameroon [13,15].

Subgenus	Species-Group	Species	Authors	Years
BYSSODON Enderlein	Griseicolle	*griseicolle*	Becker	1903
ANASOLEN Enderlein		*dentulosum*	Roubaud	1915
EDWARDSELLUM Enderlein	Damnosum	*damnosum complex*	Theobald	1903
		damnosum s. str	Dunbar and Vajime	1981
		mengense	Vajime and Dunbar	1979
		sirbanum	Vajime and Dunbar	1975
		squamosum (*complex*)	Enderlein	1921
		yahense	Vajime and Dunbar	1975
		soubrense	Ayissi et al.	2022
LEWISELLUM Crosskey		*atyophilum*	Lewis and Disney	1969
		ovazzae	Grenier and Mouchet	1959
MEILLONIELLUM Rubtsov		*adersi*	Pomeroy	1922
		hirsutum	Pomeroy	1922
METOMPHALUS Enderlein	Bovis	*bovis*	De Meillon	1930
		eouzani	Germain and Grenier	1970
		wellmanni	Roubaud	1906

Table 1. *Cont.*

Subgenus	Species-Group	Species	Authors	Years
METOMPHALUS Enderlein	Medusaeforme	*akouense*	Fain and Elsen	1973
		colasbelcouri	Grenier and Ovazza	1951
		crosskeyi	Lewis and Disney	1970
		futaense	Garms and Post	1966
		hargreavesi	Gibbins	1934
		medusaeforme s. str.	Pomeroy	1920
		ngouense	Fain and Elsen	1973
		tondewandouense	Fain and Elsen	1973
NEVERMANNIA Enderlein	Loutetense	*loutetense*	Grenier and Ovazza	1951
	Ruficorne	*antibrachium*	Fain and Dujardin	1983
		aureosimile	Pomeroy	1920
		ekomei	Lewis and Disney	1972
		katangae	Fain	1951
		nigritarse	Coquillett	1901
		ruficorne	Macquart	1838
PHORETOMYIA Crosskey		*afronuri*	Lewis and Disney	1970
		dukei	Lewis, Disney, and Crosskey	1969
		berneri	Freeman	1954
		kumboense	Grenier, Germain, and Mouchet	1966
		baetiphilum	Lewis and Disney	1972
		lumbwanum	De Meillon	1944
		rickenbachi	Germain, Grenier, and Mouchet	1966
POMEROYELLUM Rubtsov	Alcocki	*alcocki*	Pomeroy	1922
		coalitum	Pomeroy	1922
		djallonense	Roubaud and Grenier	1943
		duodecimum	Gibbins	1936
		vargasi	Grenier and Rageau	1949
		garmsi	Crosskey	1969
		hissetteum	Gibbins	1936
		impukane	De Meillon	1936
		johannae	Wanson	1947
		oguamai	Lewis and Disney	1972
	Cervicornutum	*cervicornutum*	Pomeroy	1920
		leberrei	Grenier, Germain, and Mouchet	1966
		palmeri	Pilaka and Elouard	1999
		unicornutum	Pomeroy	1920
	Kenyae	*kenyae*	De Meillon	1940
	Schoutedeni	*audreyae*	Garms and Disney	1974
		schoutedeni	Wanson	1947

In a previous study [16], we focused on the molecular diversity of members of the *Simulium damnosum* complex in Cameroon, which are the main local vectors of *Onchocerca volvulus*, *O. ochengi*, and *O. ramachandrini*. We now extend this study to the *non-S. damnosum* black flies, which constitute the majority of all species in this country.

A wide range of cytological and molecular markers have been used for population studies in Simuliidae [17,18]. These include chromosomal inversions [19], allozymes [20], and random amplified DNA polymorphisms (RAPD) [21]. Furthermore, the sequencing of mitochondrial Cytochrome oxidase 1 (Cox1) genes [22,23], nuclear genes (ITS), and microsatellite loci analyses [23] have been carried out.

The ITS2 region of nuclear ribosomal DNA is regarded as one of the candidate DNA barcodes because it possesses a number of valuable characteristics, such as the availability of conserved regions for designing universal primers, the ease of its amplification, and sufficient variability to distinguish even closely related species [24].

All the above-mentioned techniques have led to the conclusion that morphological classifications do not distinguish between many populations that should be recognized as true species ('cytospecies', etc.). An ideal barcode should be sufficiently variable to identify closely related species, while carefully identifying distantly related species. Indeed, a prediction has been made that, worldwide, more than 3000 black fly species are potentially undiscovered morpho species and sibling species [2].

Because of their impact on public and animal health, the correct identification of this insect group is of a fundamental importance in order to provide correct information on species distribution and biology so that targeted control measures can be correctly applied. However, standard methods for black fly species identification are mainly based on morphology, which typically requires expert knowledge, and sometimes the resolution can be poor because of the presence of hidden diversity [25–27].

In the present study, we used the morphological aspect of pupal gills and developed a molecular platform based on the ITS2 and the Cox1 in order to support the species identification of the poorly studied black fly fauna of Cameroon.

2. Materials and Methods

2.1. Source of Material and Morphological Identification

Substrates to which pupae were attached were collected by hand in Cameroon (Figure 1, Table 2) and included trailing vegetation, debris, stones, and refuse such as plastic and glass. Pupae which were attached to their substrate were immediately placed in boxes and covered with wet tissue. Pupae were removed from substrates, cleaned using a fine brush and forceps, and preserved in 70 to 96% ethanol.

Table 2. Sampling points with coordinates, sample collectors, sample type, and species collected in each point.

SN	River	Site	Latitude	Longitude	Date	Collector	Morphological Identification.
1	Vina du Nord	Touboro Vina bridge	7.7502	15.3636	16-March-11	AR	*S. bovis*, *S. cervicornutum*, *S. vorax*
2	Benoue	Near Karna Manga	7.7808	13.5874	05-September-14	AE, DE	*S. kenyae*;

Table 2. *Cont.*

SN	River	Site	Latitude	Longitude	Date	Collector	Morphological Identification.
3	Tributary to river Vina du Nord	Aladji Marafat	7.4016	13.5522	10-January-20	PK	*S. nigritarsis*, *S. medusaeforme* f. Pomeroy, *S. adersi*, *S. unicornutum*, *S.* undescribed 2 *S. cervicornutum*
4	Vina du Sud	Vina fall at Galim Pont	7.2100	13.5862	18-June-20	DE	*S. vorax*, *S. nigritarsis*, *S. medusaeforme* f. *hargreavesis*, *S. adersi*
5	Mayo Djouroum	Near Galim	7.2011	13.5930	26-April-19	PK, DE	*S. vorax*, *S. medusaeforme* f. Pomeroy, *S. medusaeforme* f. *hargreavesis*, *S. cervicornutum*
6	Mawong river	Near Befang	6.3242	10.0037	17-November-17	PK	*S. schoutedeni*, *S. unicornutum*, *S. katangae*, *S. hirsutum*, *S. cervicornutum*, *S. medusaeforme* f. *hargreavesis*, *S. alcocki*, *S. dentulosum* *S. ruficorne*, *S. adersi*, *S.* undescribed 1, *S. kenyae*
7	Tunga (Menchum) river	Menchum Falls	6.3069	10.0169	02-August-18	PK	*S. cervicornutum*, *S. unicornutum*, *S. katangae*, *S. dentulosum* *S. hirsutum*, *S. alcocki*, *S.* undescribed 1
8	River near IRAD	Bambui	6.0149	10.2677	29-October-18	PK	*S. cervicornutum*, *S. unicornutum*, *S. katangae*, *S. dentulosum* *S. alcocki*, *S.* undescribed 1
9	Tributary of river Nkam	Mbanga near the slaughter-house	4.5044	9.5719	28-December-21	PK	*No Simulium* found
10	Lele	Nkongsamba	4.9724	9.9289	29-December-21	PK	*S. cervicornutum*, *S. katangae*, *S. dentulosum* *S. medusaeforme* f. Pomeroy, *S. ruficorne*
11	Boriko	Nkongsamba	4.9544	9.9259	29-December-21	PK	*S. cervicornutum*, *S. katangae*, *S. dentulosum* *S. medusaeforme* f. Pomeroy, *S. ruficorne*

Table 2. *Cont.*

SN	River	Site	Latitude	Longitude	Date	Collector	Morphological Identification.
12	Tributary near Total	Nkongsamba	4.9577	9.9300	29-December-21	PK	*S. cervicornutum*, *S. katangae*, *S. dentulosum* *S. medusaeforme* f. Pomeroy, *S. ruficorne*
13	Esoa	Nkongsamba	4.9749	9.9399	29-December-21	PK	*S. cervicornutum*, *S. katangae*, *S. dentulosum* *S. medusaeforme* f. Pomeroy, *S. ruficorne*
14	Vina du Nord	Soramboum	7.7872	15.0061	31-October-16	DE	*S. alcocki*

PK: Pierre Kamtsap; DE: David Ekale; AE: Albert Eisenbarth; AR: Alfons Renz.

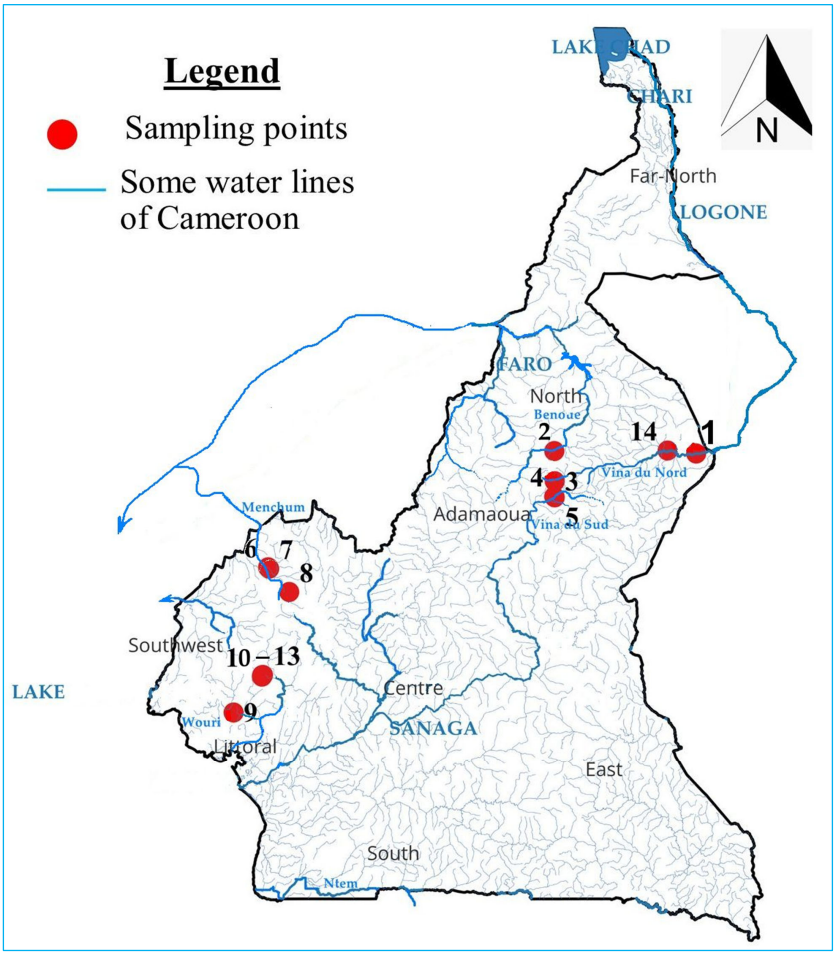


Figure 1. Map of Cameroon showing the localities where samples were collected in this study. Sample points are indicated by red dots with numbers from 1 to 13 (see Table 2 for geographical coordinates).

Pupae were identified under a Wild M5 dissection and a Zeiss Axioplan compound microscope by using standard keys as described by Freeman and de Meillon and others (molecular aspect) [14,28,29]. Identified species were cleared in 10% potassium hydroxide (KOH) solution for about 24 h at room temperature. Gills were cut out carefully using fine needles and forceps, transferred on a clean slide containing a drop (approximately 50 µL) of polyvinyl lactophenol, and covered with a coverslip. All mounted slides were kept on a

heat bloc (Omni lab Jürgens, Germany) set at 60 °C for approximately 24 h. Images were taken with an incorporated Canon EOS-650D camera.

2.2. DNA Extraction, PCR, and Sequencing

The Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) [30] was used, as instructed by the manufacturer, to extract total genomic deoxyribonucleic acid (DNA) from the individual pupae that had been identified morphologically [28]. Gene amplification of the mitochondrial protein-coding gene CoxI, which is about 650 bp long, was performed by the Polymerase Chain Reaction (PCR) with the Lep primers: forward (5′-ATTCAACCAATCATAAAGATATTGG-3′) and reverse (5′-TAAACTTCTGGATGTCCAAAAAATCA-3′) [21], whereas ITS2, which is about 400 bp long, was identified by using forward (5′-TGTGAACTGCAGGACACAT-3′) and reverse (5′-ATGCTTAAATTTAGGGGGT-3′) primers [31,32]. All the PCRs were performed in a final volume of 25 µL comprising 2 µL genomic DNA, 5 µL Promega 5× DNAgo Buffer, 2 mM $MgCl_2$, 0.25 mM each dNTPs, 50 pmol forward and reverse primers and 1 U Promega Taq Polymerase (Promega) [30]. Amplifications were performed in a Master Cycler (Eppendorf Master Cycler). For the Lep primers, PCR consisted of an initial denaturation (95 °C, 2 min), followed by 35 cycles of denaturation at 95 °C for 30 s, an annealing step at 51 °C for 30 s, an extension at 72 °C for 60 s, and then a final extension at 72 °C for 5 min [30]. For the ITS2, PCR consisted of an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 40 s, an annealing step at 51 °C for 60 s, extension at 72 °C for 60 s, and then a final extension at 72 °C, for 5 min [32]. The amplified amplicons were checked by electrophoresis on a 1.5% agarose gel. Finally, PCR products were sent to a commercial sequencing facility (Macrogen, Amsterdam, The Netherlands).

2.3. Sequence Analysis

All bi-directional sequences were combined to produce a single consensus sequence in Geneious Prime v. 2023.2.1. The alignment was performed with ClustalW with default parameters, and the neighbor-joining (NJ) analysis was undertaken using the K2P distance to represent species distribution patterns in the NJ tree. The robustness of the NJ tree was calculated using the bootstrap methodology employing 1000 as pseudoreplicates. All obtained sequences for Simuliidae from this study (GenBank accessions for ITS2: see Supplementary File S1) were chosen to encompass the range of Simuliidae species occurring in Cameroon based on morphology. The optimal tree with the sum of branch length is shown. The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The ME tree was searched using the Close Neighbor Interchange (CNI) algorithm at a search level of 1. The number of nucleotide sequences implicated in the analysis is indicated. Codon positions included were 1st + 2nd + 3rd + Non-coding. All ambiguous positions were removed for each sequence pair. The number of the total position in the final dataset is indicated. We analyzed the dataset in MEGA v.7 [33].

3. Results

3.1. Morphological Identification

Thirteen sample collection points (see Table 2 with collection sites, sample collector, and collection date; Supplementary File S2 with the number of each species per location and percentage of appearance of each species from a specific location) were included in this study. A total of 19 non-*Simulium damnosum* species were identified by morphology based on pupae respiratory gills including *Simulium dentulosum* type A (14 filaments per respiratory gill), *dentulosum* type B (16 filaments per respiratory gill), *dentulosum* type C (12 filaments per respiratory gill); *Simulium adersi*; *Simulium alcocki*; *Simulium bovis*; *Simulium*

cervicornutum; *Simulium medusaeforme* f. Pomeroy; *S. medusaeforme* f. *hargreavesi*; *Simulium hirsutum*; *Simulium katangae*; *Simulium kenyae*; *Simulium nigritarsis*; *Simulium ruficorne*; *Simulium schoutedeni*; *Simulium unicornutum*; *Simulium vorax*; *and* 2 not yet described *Simulium* species based on the identification keys that we used (*Simulium* undescribed 1 and 2) see Figure 2a–s.

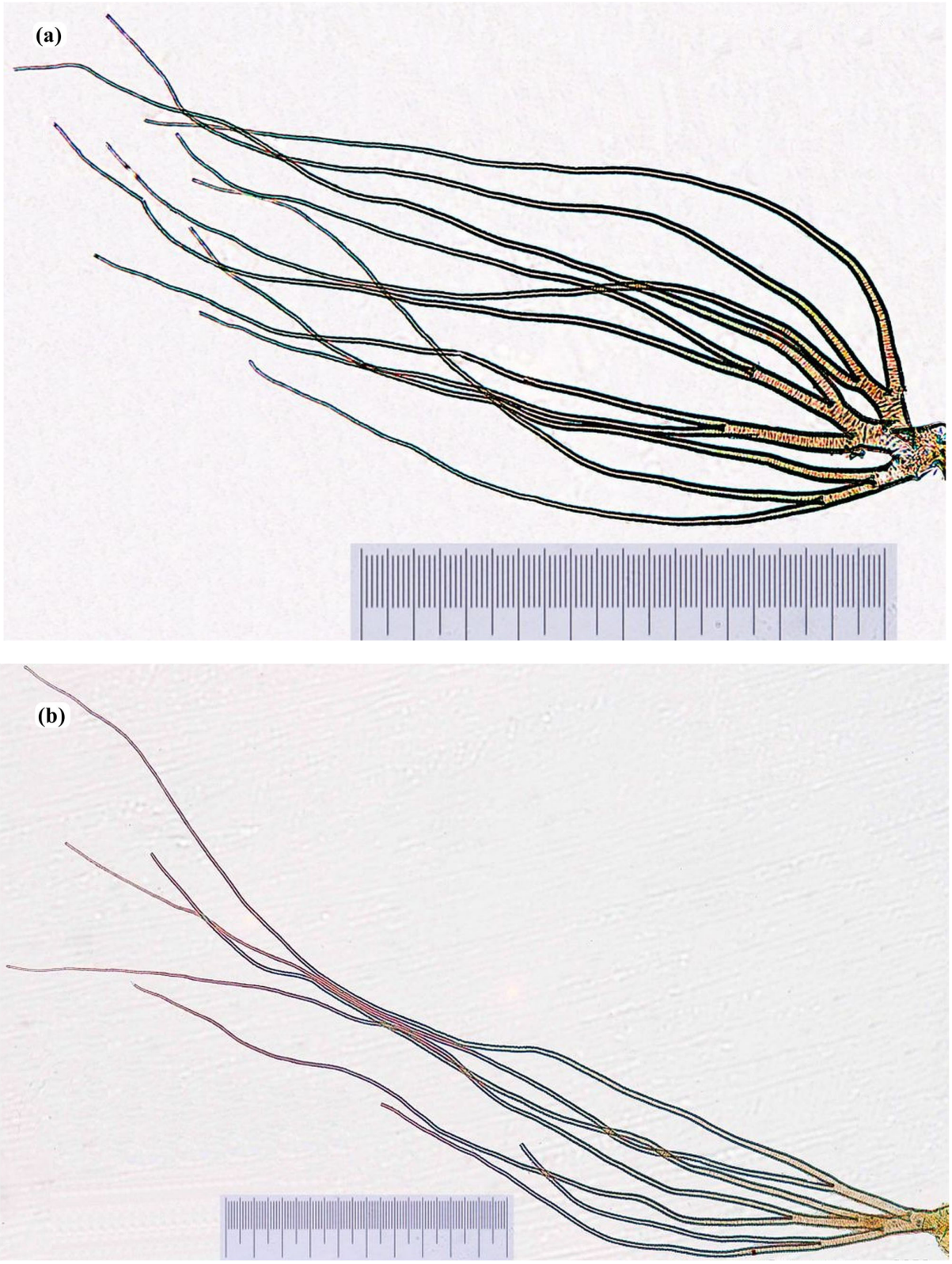


Figure 2. *Cont.*

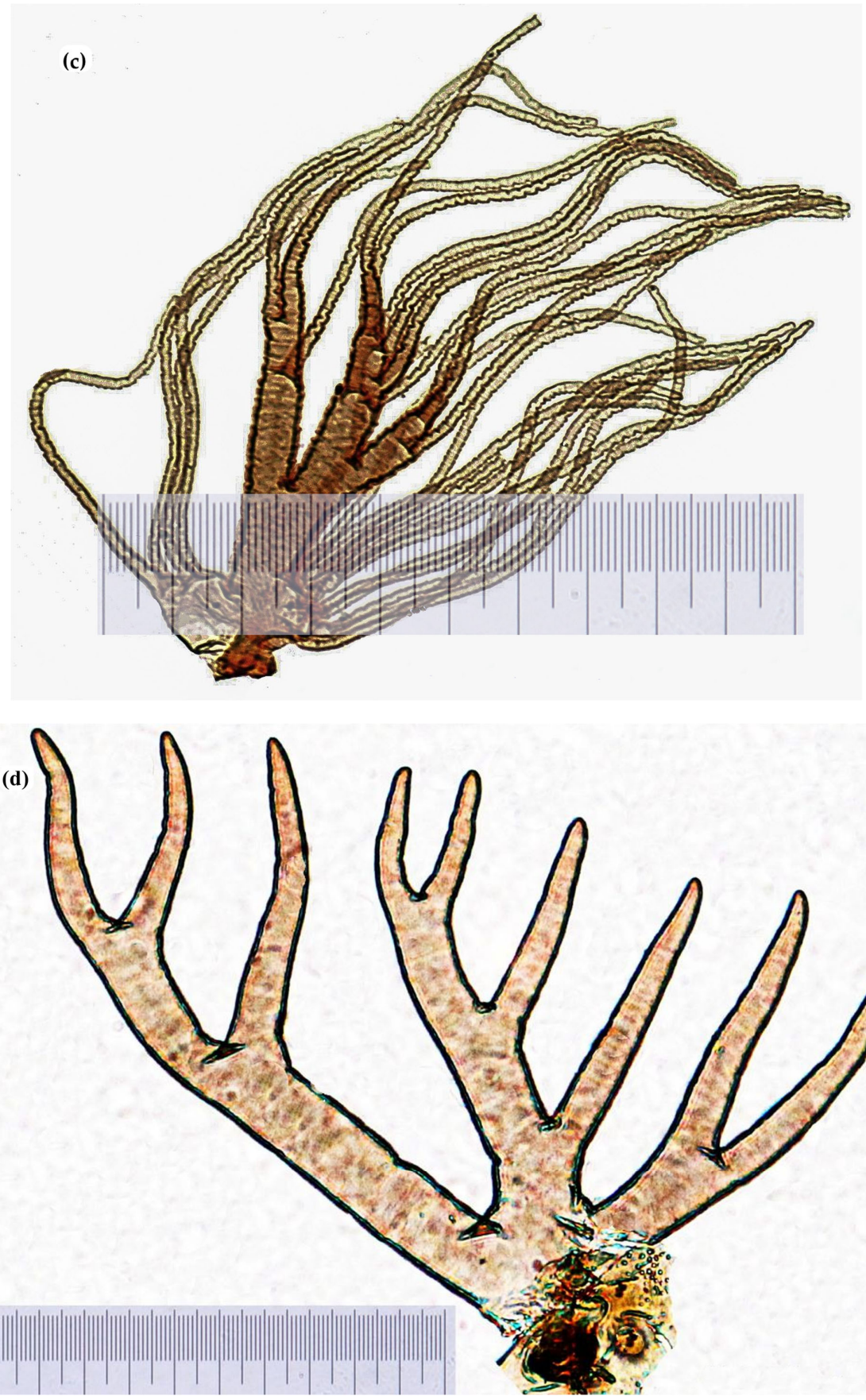


Figure 2. *Cont*.

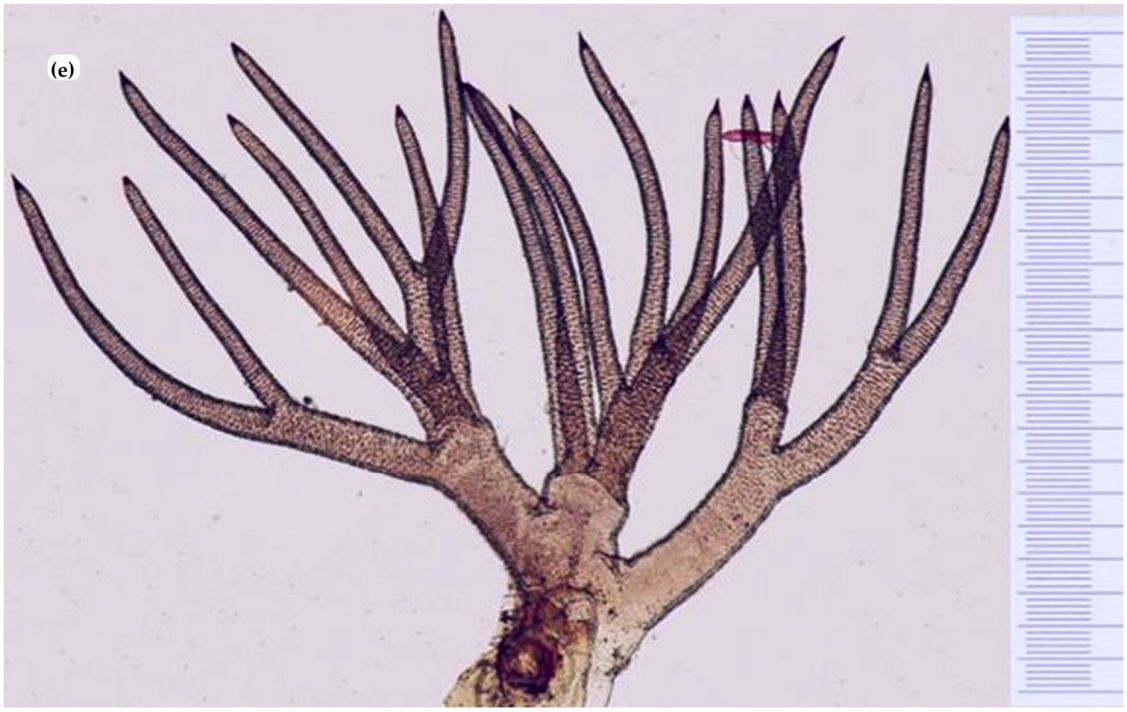


Figure 2. *Cont*.

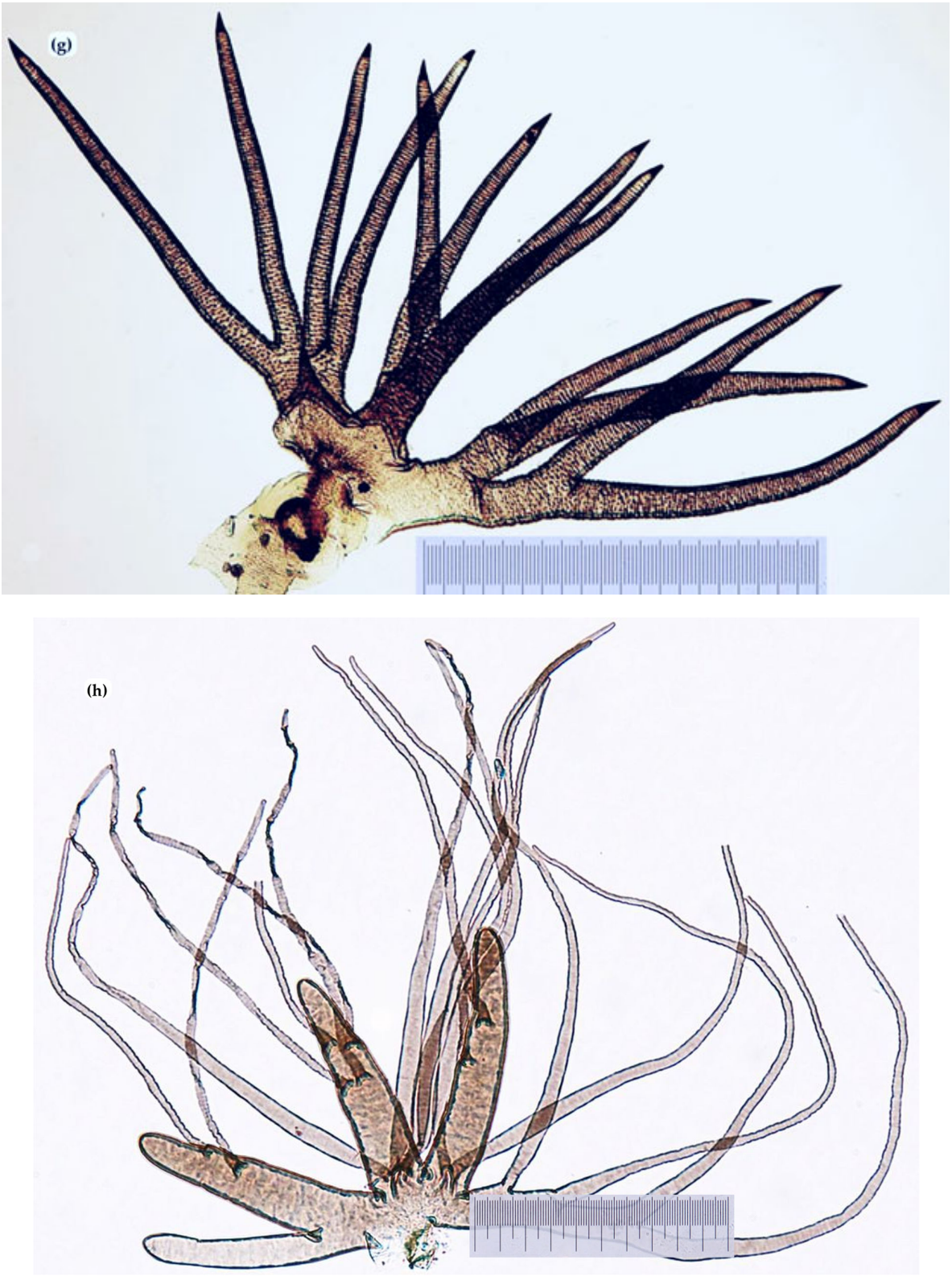
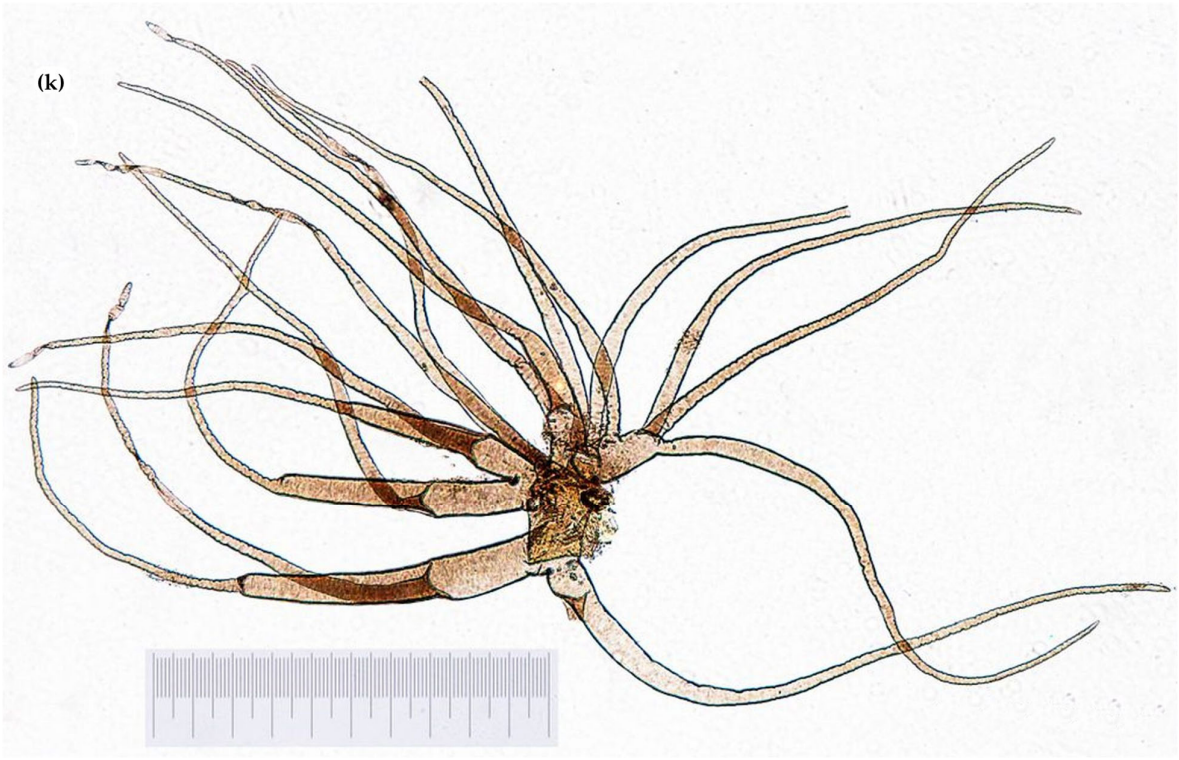


Figure 2. *Cont*.

Figure 2. *Cont*.



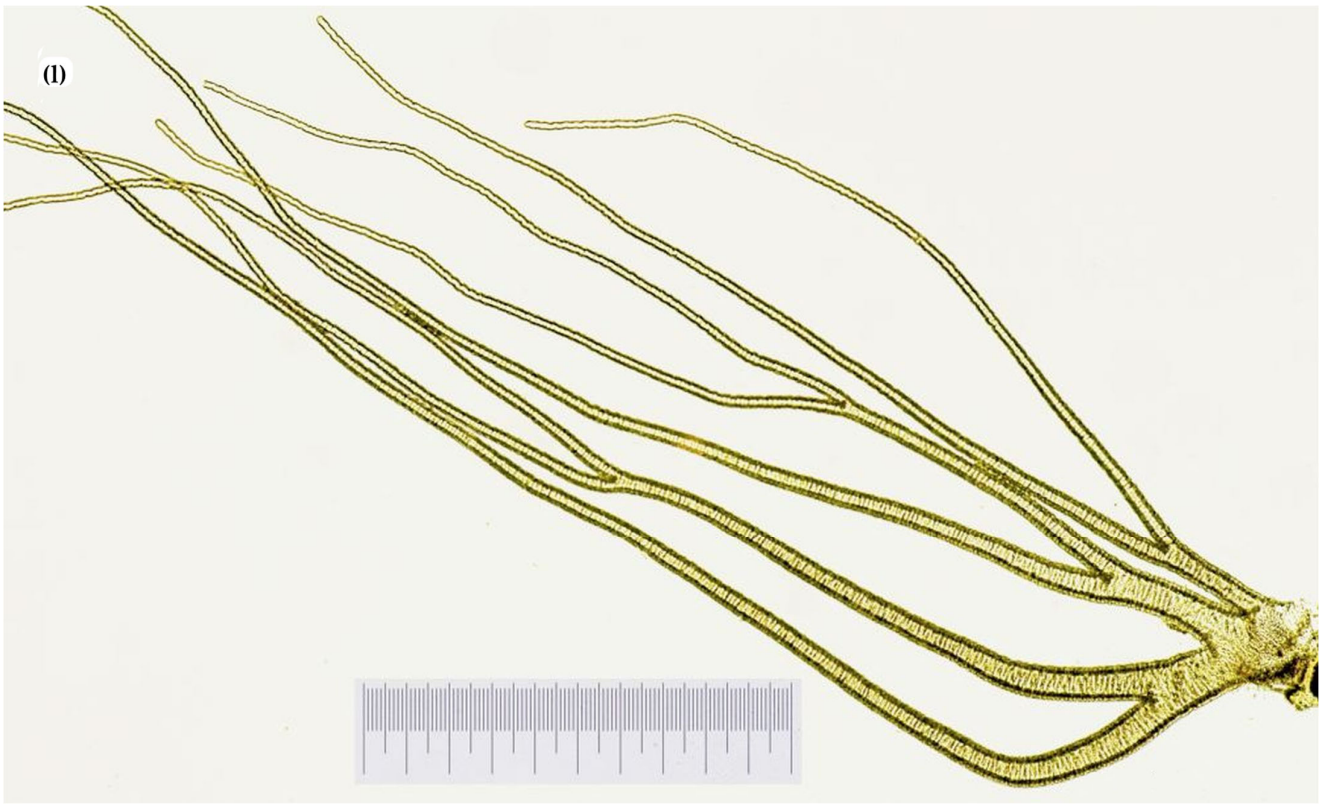


Figure 2. *Cont*.

Figure 2. *Cont*.

Figure 2. *Cont*.

Figure 2. Respiratory gills of pupal stages: (**a**) *Simulium adersi*; (**b**) *Simulium alcocki*; (**c**) *Simulium bovis*; (**d**) *Simulium cervicornutum*; (**e**) *Simulium dentulosum* A; (**f**) *Simulium dentulosum* B; (**g**) *Simulium dentulosum* C; (**h**) *Simulium medusaeforme* f. Pomeroy; (**i**) *S. medusaeforme* forme *hargreavesi* (**j**) *Simulium* undescribed 1; (**k**) *Simulium* undescribed 2; (**l**) *Simulium hirsutum*; (**m**) *Simulium katangae*; (**n**) *Simulium kenyae*; (**o**) *Simulium nigritarsis*; (**p**) *Simulium ruficorne*; (**q**) *Simulium schoutedeni*; (**r**) *Simulium unicornutum*; (**s**) *Simulium vorax*. Scale- 1 mm.

The heatmap (Figure 3) and the Supplementary File S2 reveal strong spatial patterns in species dominance and diversity, with *S. cervicornutum* emerging as the most widely distributed and numerically dominant species across multiple locations, including Mawong

River, Menchum Falls, and around IRAD. Conversely, *S. medusaeforme* f. *hargreavesi* demonstrates extreme localization, dominating Soramboum (96.97%). Species like *S. katangae* and the undescribed species exhibit regional importance, particularly around IRAD and Menchum Falls. High-diversity areas such as Mawong River, despite being dominated by *S. cervicornutum*, host over 12 species. In contrast, sites like Karna Manga and Aladji Marafat show near-monospecific populations.

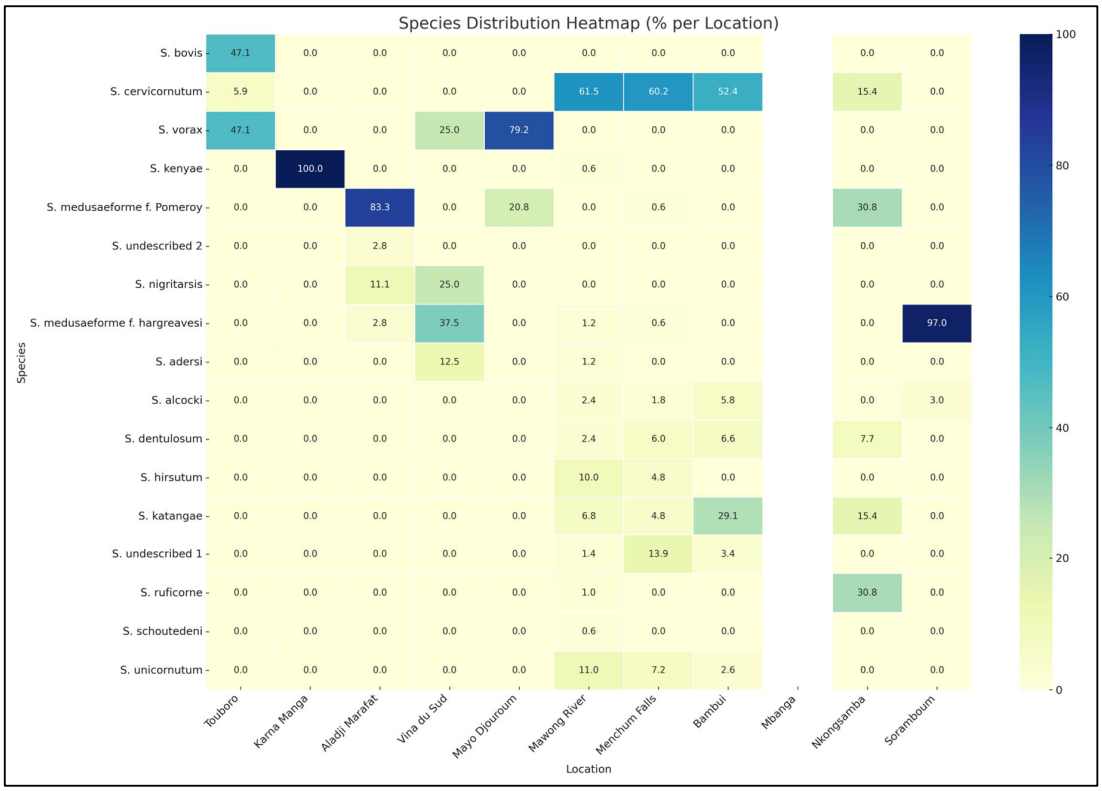


Figure 3. Heatmap showing the percentage distribution of non *Simulium damnosum* species across surveyed locations in Cameroon. Color intensity reflects relative abundance, with dominant species like *S. cervicornutum* and *S. medusaeforme f. hargreavesi* clearly concentrated in specific areas (e.g., Mawong River, Soramboum), while other species show localized or low-frequency patterns.

3.2. Molecular Identification

A total of 1184 non-*Simulium* black fly specimens were collected from 14 distinct geographic locations in the Ethiopian region, encompassing varied ecological zones. Genomic DNA was successfully extracted from and subjected to PCR amplification targeting the nuclear ITS2 region and the mitochondrial Cox1 gene.

Sequences were critically assessed for quality upon receipt from the sequencing facility. Only high-quality sequences with clear chromatograms were included in subsequent analyses. Despite genetic variation, individuals belonging to the same species consistently clustered together regardless of their ecological zone, as illustrated in Figure 4.

PCR amplification of the ITS2 region was successful, producing clear, single bands of approximately 400 bp, as visualized by agarose gel electrophoresis. The absence of non-specific products or primer-dimers confirmed the high quality of both the extracted DNA and the primers. Sanger sequencing of these amplicons yielded high-quality bidirectional chromatograms, with average scores exceeding 30 across both strands, ensuring reliable base calling. Sequence alignment revealed conserved regions with minimal variation, supporting the intra-species consistency of ITS2. BLASTn (Figure 4) analysis against GenBank showed >98% similarity to known non-*Simulium* black fly species, thereby confirming species identity. Notably, pairwise sequence divergence ranged from 1.2% to 4.8%, indicat-

ing the presence of distinct haplotypes and potential cryptic diversity. Geographic analysis suggested clustering of similar ITS2 sequences within specific regions, hinting at possible population structuring.

To assess broader genetic diversity and phylogeographic relationships, samples were sequenced for the Cox1 gene (~650 bp). A Neighbor-Joining phylogenetic tree based on Cox1 sequences (Figure 4b) resolved the samples into six well-supported clades with bootstrap values >70%. Reference sequences from GenBank aided in confirming the taxonomic identity of each clade. Sequences such as *S. vorax* (MT323206) and *S. ruficorne* (KY421710, unpublished) showed notable alignment, supporting the validity of the identified groupings.

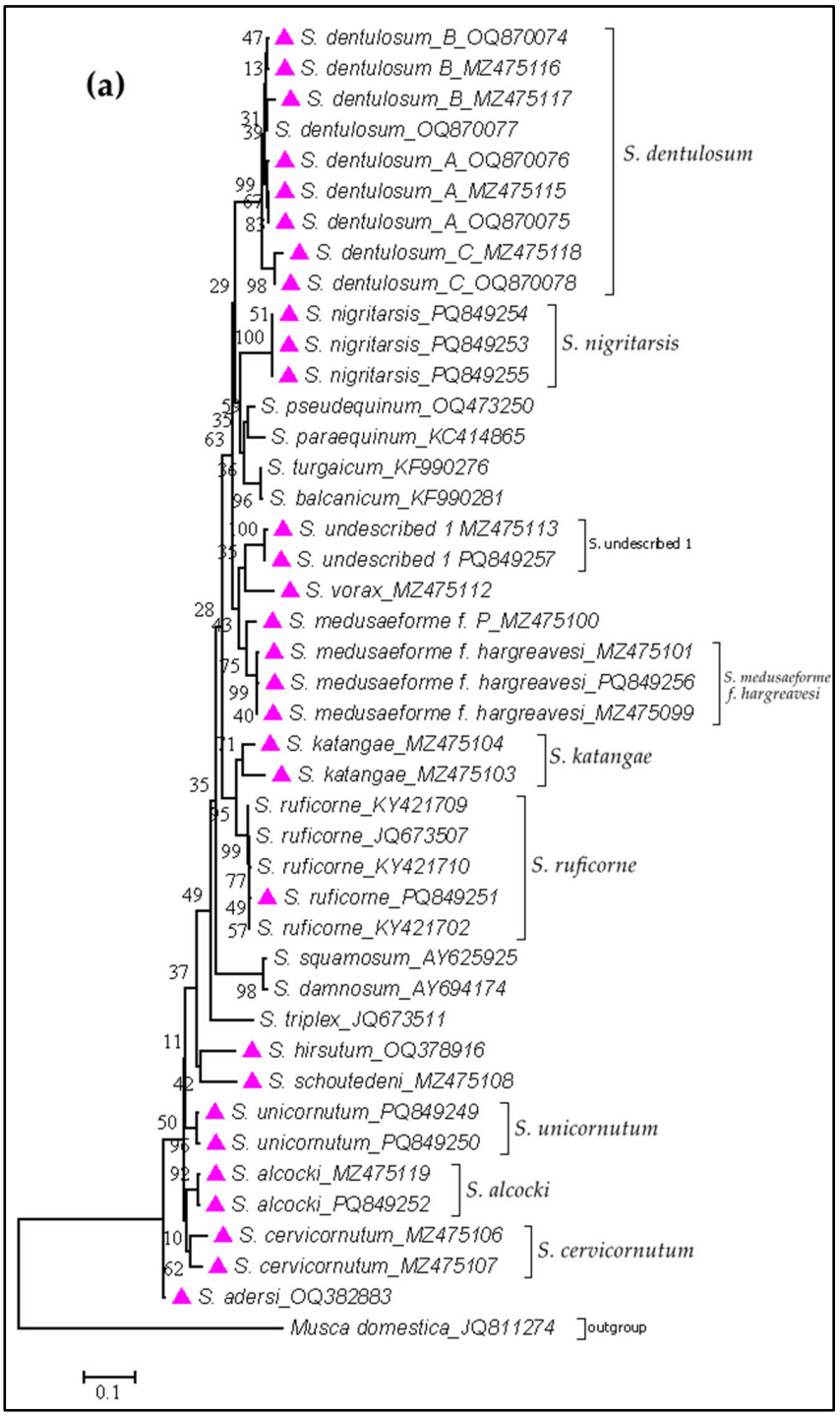


Figure 4. *Cont.*

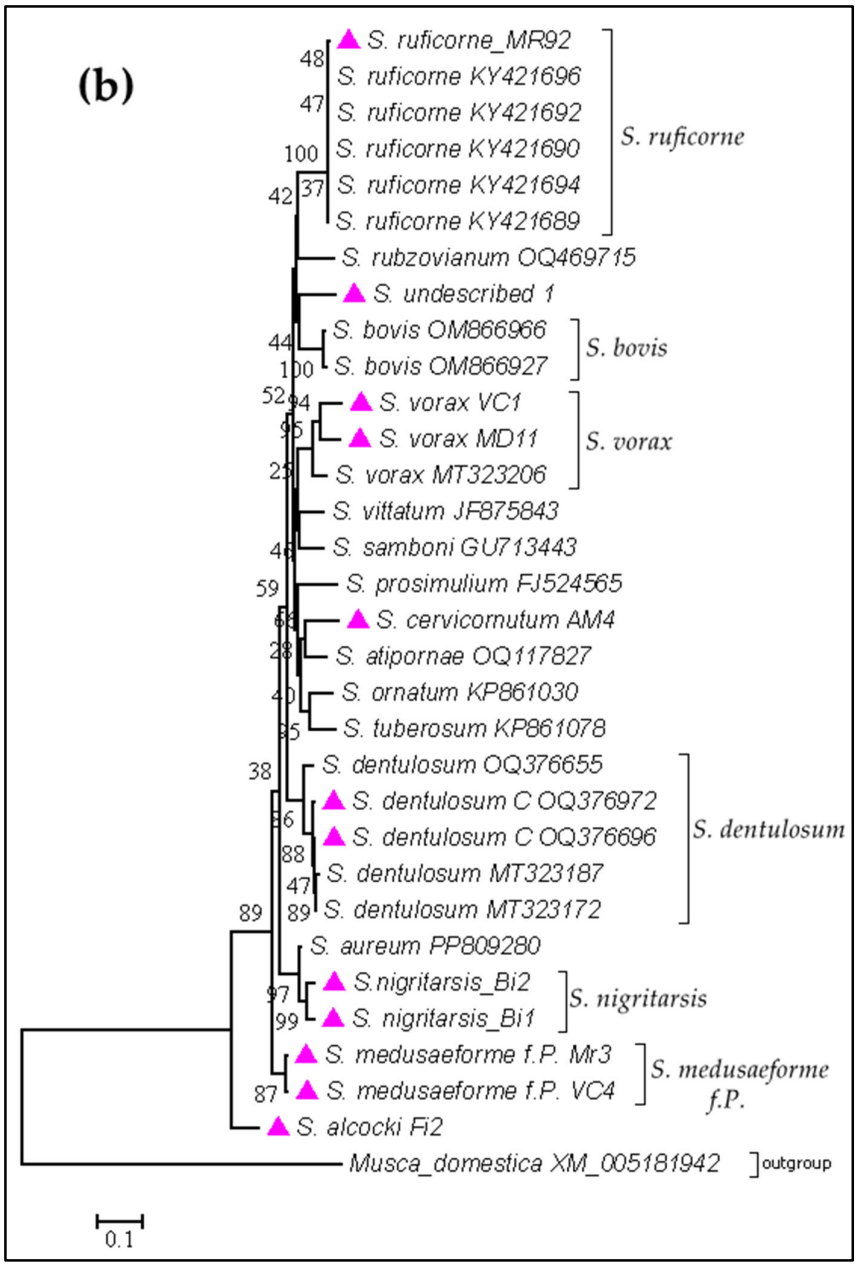


Figure 4. Molecular phylogenetic analysis by neighbor-joining method using ITS2 (**a**) and Cox1 (**b**). Species marked in purple/violet triangles are from this study and others are from the GenBank submitted by other authors (see Supplementary File S3 for references) and used in this analysis to align our findings. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Sequences generated in this study have been deposited in GenBank, with accession numbers provided in Supplementary S1.

Table 3 presents the estimation of Average Evolutionary Divergence over Sequence Pairs within Groups by ITS2 region. The ITS2 region analysis revealed varying levels of intra-species divergence among sampled *Simulium* species. Very low divergence values ($\leq$0.005) were observed in *S. ruficorne* (0.002), *S. undescribed* 1 (0.003), *S. hargreavesi* (0.004), and *S. alcocki* (0.005), indicating high genetic similarity likely due to recent common ancestry or limited geographic separation. Moderate divergence was found in *S. unicornutum* (0.008) and *S. dentulosum* (0.024), suggesting modest genetic variation, with *S. dentulosum* possibly reflecting population sub-structuring or ecological adaptation. High divergence

values (≥0.05) in *S. cervicornutum* (0.053) and *S. katangae* (0.063) point to significant genetic variability, potentially due to cryptic speciation, long-term isolation, or misclassification, warranting further study. *S. nigritarsis* exhibited zero divergence (0.000), indicating identical sequences across individuals, possibly from recent divergence or limited sampling. Divergence for the outgroup could not be calculated, which is expected and does not impact intra-species comparisons.

Table 3. Estimates of Average Evolutionary Divergence over Sequence Pairs within Groups by ITS2 region.

Species	Average Divergence	Standard Error
S. alcocki	0.005	0.004
S. dentulosum	0.024	0.004
S. nigritarsis	0	0
S. hargreavesi	0.004	0.002
S. katangae	0.063	0.013
S. unicornutum	0.008	0.005
S. ruficorne	0.002	0.001
S. cervicornutum	0.053	0.011
Outgroup	n/c	n/c

The presence of n/c in the results denotes cases in which it was not possible to estimate evolutionary distances.

Table 4 represents the substitution matrix where each entry is the probability of substitution (*r*) from one base (row) to another base (column). Transitions (purine↔purine: A↔G; pyrimidine↔pyrimidine: C↔T) exhibit significantly higher substitution rates (15.5722) compared to transversions (purine↔pyrimidine: A↔T, A↔C, G↔T, G↔C), which have lower rates (4.7139). This pronounced transition bias, a common feature in molecular evolution, is due to biochemical constraints, as transitions involve simpler molecular changes between structurally similar bases and thus occur more frequently. The substitution matrix is symmetric, indicating that the rates of substitution are equal in both directions (e.g., A→G equals G→A), reflecting reversible mutation processes typical of models like Kimura 2-parameter (K2P) and General Time Reversible (GTR). The rates are relative and scaled so that the average substitution rate across all pairs equals one, with the highest rate (15.5722) being approximately 3.3 times greater than the lowest (4.7139), signifying that some substitutions are markedly more probable. This pattern, especially the elevated A↔G and C↔T rates, is characteristic of the ITS2 region, which, while moderately conserved, allows sufficient variability to reveal meaningful substitution trends. Such biases can influence phylogenetic tree topology and affect divergence time estimates, underscoring the importance of accurate substitution rate modeling. Maximum Likelihood Estimation ensures that the matrix best fits the sequence data, enhancing the reliability of phylogenetic inference, molecular clock calibration, and insights into evolutionary pressures acting on specific gene regions.

Table 4. Maximum Likelihood Estimate of Substitution Matrix.

From/To	A	T	C	G
A	–	4.7139	4.7139	15.5722
T	4.7139	–	15.5722	4.7139
C	4.7139	15.5722	–	4.7139
G	15.5722	4.7139	4.7139	–

4. Discussion

The molecular analysis presented in Figure 4a confirms the species identity of non-*Simulium damnosum* specimens through ITS2-based Sanger sequencing. Clear and specific PCR amplification products, alongside high-quality sequence data, demonstrate successful differentiation of multiple non-*Simulium damnosum* taxa. These results support the utility of ITS2 as a reliable marker for species-level resolution within *Diptera*, particularly in *Simuliidae* and related groups.

Our findings align with previous studies that underscore the robustness of the ITS2 region in resolving species boundaries among hematophagous insects [34,35]. The distinct ITS2 sequence profiles observed suggest notable genetic diversity, potentially indicating cryptic species or substantial intraspecific variation—both critical considerations for accurate vector surveillance and ecological research.

Importantly, the analyzed samples originated from diverse geographic regions, and the sequence divergence observed corresponds with known patterns of geographical structuring in black fly populations [36,37]. This geographic differentiation likely reflects local adaptation or historical biogeographic separation and underscores the importance of integrating molecular tools into vector control initiatives.

The identification of non-*Simulium damnosum* species contributes significantly to understanding species composition in black fly communities, with direct implications for vectorial capacity and potential disease transmission. This is especially pertinent in areas where onchocerciasis transmission dynamics may be influenced by non-primary vectors, as recent evidence suggests [38,39].

Our application of both ITS2 and Cox1 molecular markers facilitated precise species identification, revealed intra- and inter-specific genetic diversity, and elucidated geographic variation among non-*Simulium damnosum* black flies. ITS2-based Sanger sequencing proved reliable for routine species confirmation, especially in field settings where morphological identification is complicated by cryptic species or degraded specimens. ITS2 sequences displayed minimal intraspecific variation, aligning with its conserved nature, yet provided high-confidence species confirmation via BLAST matching [34,35].

Conversely, the mitochondrial Cox1 gene—owing to its higher mutation rate—revealed significant haplotype diversity and geographic clustering (Figure 4b), offering insights into population structure and phylogeography. These patterns support previous work using Cox1 barcoding to differentiate black fly populations across regions [36,37]. The formation of geographically distinct clades suggests restricted gene flow due to environmental barriers, breeding site isolation, or host specificity. Understanding this population structuring is crucial, as it may influence vector competence, transmission dynamics, and response to control efforts. Notably, even non-primary vectors could contribute to disease transmission, particularly where zoonotic *Onchocerca* spp. have been detected in non-*Simulium* species [38].

Our findings complement and refine the classical morphological taxonomy established by Freeman and de Meillon for *Simuliidae* in Africa [39]. The molecular approaches utilized here provide enhanced resolution and accuracy in species identification, vital for epidemiological monitoring and biodiversity assessments. Additionally, Sanger sequencing remains a cost-effective and accessible method for field laboratories, enabling rapid and reliable surveillance, particularly in resource-limited settings.

In this study, we have, for the first time, undertaken a combined morphological and molecular analysis of non-*Simulium damnosum* black fly populations in Cameroon. Notably, 2 undescribed *Simulium* species were detected for the first time in Cameroon. The presence of such species in distinct locales suggests that ecological or environmental factors

significantly influence their distribution. High species concentration in certain areas may guide conservation and surveillance efforts.

Ecological patterns also emerged: *S. cervicornutum* was found across multiple locations, indicating ecological adaptability. In contrast, species like *S. medusaeforme f. Pomeroy* and *S. vorax* were limited to specific environments (Aladji Marafat), suggesting niche specialization. Similarly, *S. kenyae* and *S. alcocki* were restricted to certain locations, implying highly specialized environmental preferences.

Furthermore, our molecular analysis revealed the presence of *S. vorax* in Mayo Djouroum near Galim, Adamaoua Region. This species clustered with a previously identified *S. vorax* specimen from the Kakoi–Koda focus in the Democratic Republic of Congo, where it has been implicated in onchocerciasis transmission [40]. This suggests that *S. vorax* in northern Cameroon may also play a role in transmission, possibly including novel or zoonotic *Onchocerca* spp.

Additionally, we present molecular data for three subspecies of *S. dentulosum*, which cluster closely together. This species was previously identified as a primary vector in Kakoi–Koda [40]. For the first time, such molecular data for the Cameroonian subspecies of S. dentulosum have been presented.

Historically, studies in Cameroon have focused on the *S. damnosum* complex as the sole vectors of onchocerciasis [16,41]. However, our results suggest that other blackfly species may also serve as potential vectors under favorable environmental conditions, emphasizing the need to broaden the scope of vector surveillance.

Lastly, our data also identified *S. ruficorne*, previously described in a phylogenetic study of *Simulium* on Réunion Island [42], underscoring the broader biogeographic connections and potential for species migration or introduction.

5. Conclusions

According to these data, some species (like *S. cervicornutum* and *S. katangae*) exhibit a large, extensive range, whereas others (like *S. alcocki*) are more location-specific. The diverse distributions of species and their concentration in particular regions imply that ecological elements, including competition, habitat type, and environmental circumstances, are important determinants of species success. To further understand the underlying reasons for these trends and to improve conservation initiatives, more ecological research would be helpful, including habitat surveys and environmental monitoring. Our study should help to provide useful methods or techniques for vector sibling identification and the spatial distribution of sub-Saharan and tropical elements. Nevertheless, other members of the *Simulium*, and not only the *damnosum* complex, need to be included, such as populations from other regions of Cameroon. In addition, analyses of the whole genome might help to improve the resolution of the relationships. Indeed, the application of identification to other African *Simulium* species with a similar geographical distribution might be useful for underlining Simuliid biogeography and evolution in Africa. The implementation of the suggested ideas and methods will aid the planning of undescribed *Simulium* species that could be implicated in the transmission of onchocerciasis and some unknown pathogens that could be transmitted by black flies. Most species in this study showed low to moderate intra-group divergence, supporting the utility of the ITS2 region for species identification and taxonomic resolution among non-*Simulium damnosum* black flies. The elevated divergence in *S. katangae* and *S. cervicornutum* suggests potential taxonomic complexity and the presence of cryptic species. These results provide valuable insights for phylogenetic analysis, species delimitation, and vector control strategies, where accurate identification is crucial.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/insects16060572/s1, Supplementary file S1: Sample details and GenBank accession numbers of individual species used for DNA sequence analysis; Supplementary file S2: species' count and percentage distribution across locations; Supplementary file S3: Species used to align samples in this study using the ITS2 gene.

Author Contributions: Conceptualization, P.K.; methodology, P.K.; software, P.K. and A.P.; validation, P.K., A.P., F.N.N. and A.R.; formal analysis, P.K.; investigation, P.K. and A.P.; resources, P.K., F.N.N. and A.R.; data curation, P.K.; writing—original draft preparation, P.K.; writing—review and editing, P.K., A.P., F.N.N. and A.R.; project administration, A.R. and F.N.N.; funding acquisition, F.N.N. and A.R. All authors have read and agreed to the published version of the manuscript.

Funding: This study is supported by the Medical Research Council of the UK through the Global Challenges Research Fund and granted by the United Kingdom's Official Development Assistance (ODA) through the Centre for Research in Infectious Diseases (CRID), Grant/Award Number MR/P027873/1. This study was partially supported by the DFG-COBE grant (DFG RE-1536/ff) and the Programme Onchocercoses laboratory in Ngaoundéré-Cameroon.

Data Availability Statement: Sequences were deposited in GenBank, and prepared slides are in the Institute for Evolution and Ecology, Department of Comparative Zoology, University of Tübingen, Germany.

Acknowledgments: We acknowledge David Ekale and Jeremi Yembo from the ProgOncho Laboratory in Ngaoundere Cameroon for their involvement in sample collections.

Conflicts of Interest: The authors have no conflicts of interest to declare that are relevant to the content of this article.

References

1. Sankarappan, A.; Mani, K.; Sundaram, D.; Muthukalingan, K. Species diversity of black flies (Diptera: Simuliidae) in Oriental region and molecular phylogeny of the subgenus Gomphostilbia members. *J. Vector Borne Dis.* **2017**, *54*, 7.
2. Currie, D.C.; Adler, P.H. Global diversity of black flies (Diptera: Simuliidae) in freshwater. *Hydrobiologia* **2008**, *595*, 469–475. [CrossRef]
3. Wahl, G. Identification of a Common Filarial Larva in *Simulium damnosum* s.l. (Type D, Duke, 1967) as *Onchocerca ramachandrini* from the Wart Hog. *J. Parasitol.* **1996**, *82*, 520–524. [CrossRef]
4. Bain, O.; Wahl, G.; Renz, A. *Onchocerca ramachandrini n. sp.* from the warthog in Cameroon. *Ann. Parasitol. Hum. Comparée* **1993**, *68*, 139–143. [CrossRef]
5. Crosskey, R.W. *The Natural History of Blackflies*; Wiley: Chichester, UK, 1990; 711p.
6. Wahl, G.; Achu-Kwi, M.D.; Mbah, O.; Renz, A. Bovine onchocercosis in North Cameroon. *Vet. Parasitol.* **1994**, *52*, 297–311. [CrossRef]
7. Akiba, K. Leucocytozoonosis of chickens. *Natl. Inst. Anim. Health Q.* **1970**, *10*, 131–147.
8. Li, G.; Lin, H.; Ong, Y.; Qiu, Z.; Jun, Z.; Zhong, C. Epidemiological surveys of *Leucocytozoon caulleryi* infection in chicks in Guangdong, Shandong and Fujian, China. *Chin. J. Vet. Sci. Technol.* **1998**, *28*, 17–19.
9. Hassan, H.K.; Bolcen, S.; Kubofcik, J.; Nutman, T.B.; Eberhard, M.L.; Middleton, K.; Wekesa, J.W.; Ruedas, G.; Nelson, K.J.; Dubielzig, R.; et al. Isolation of *Onchocerca lupi* in Dogs and Black Flies, California, USA. *Emerg. Infect. Dis.* **2015**, *21*, 789–796. [CrossRef]
10. Wahl, G.; Ekale, D.; Renz, A. The development of *Onchocerca dukei* and *O. ochengi* microfilariae to infective-stage larvae in *Simulium damnosum s.l.* and in members of the *S. medusaeforme* group, following intra-thoracic injection. *Ann. Trop. Med. Parasitol.* **1991**, *85*, 329–337. [CrossRef]
11. Bain, O.; Renz, A. Infective larvae of a new species of Robertdollfusidae (Adenophorea, Nematoda) in the gut of *Simulium damnosum* in Cameroon. *Ann. Parasitol. Hum. Comparée* **1993**, *68*, 182–184. [CrossRef]
12. Schulz-Key, H. Investigations on the filariae of cervids in southern Germany, 1, Nodule formation, sex determination and microfilariae release in *Onchocerca flexuosa* (Wedl, 1856) in red deer (*Cervus elaphus*). *Trop. Med. Parasitol.* **1975**, *26*, 60–69.
13. Adler, P.H.; Crosskey, R.W. *World Blackflies (Diptera: Simuliidae): A Comprehensive Revision of the Taxonomy and Geographical Inventory*; Natural History Museum: London, UK, 2016.
14. Freeman, P.; de Meillon, B. *Simuliidae of the Ethiopian Region*. Paul Freeman and Botha de Meillon. British Museum (Natural History), London, 1953. vii + 224 pp. Illus. £2 10s. *Science* **1954**, *120*, 178–179. [CrossRef]

15. Fain, A.; Elsen, P. Notes on Simulies of Eastern Cameroon. *J. Afr. Zool. Bot.* **1973**, *87*, 34.
16. Kamtsap, P.; Paguem, A.; Renz, A. Molecular Diversity in the *Simulium damnosum* complex (Diptera: Simuliidae) in Cameroon. *Mitt. Dtsch. Ges. Allg. Angew. Ent.* **2024**, *23*, 147–152.
17. Đuknić, J.; Jovanović, V.M.; Popović, N.; Živić, I.; Raković, M.; Čerba, D.; Paunović, M. Phylogeography of *Simulium* Subgenus *Wilhelmia* (Diptera: Simuliidae). Insights From Balkan Populations. *J. Med. Entomol.* **2019**, *56*, 967–978. [CrossRef]
18. McCreadie, J.W.; Adler, P.H. Scale, Time, Space, and Predictability: Species Distributions of Preimaginal Black Flies (Diptera: Simuliidae). *Oecologia* **1998**, *114*, 79–92. [CrossRef]
19. Vajime, C.G.; Dunbar, R.W. Chromosomal identification of eight species of the subgenus *Edwardsellum* near and including *Simulium* (*Edwardsellum*) *damnosum* Theobald (Diptera: Simuliidae). *Trop. Med. Parasitol.* **1975**, *26*, 111–138.
20. Thomson, M.C.; Renz, A.; Davies, J.B. A new PGM electromorph diagnostic for *S. squamosum* from Sierra Leone and Togo but not found in *S. squamosum* from Cameroun. *Acta Leiden.* **1990**, *59*, 303–305.
21. Duncan, G.A.; Adler, P.H.; Pruess, K.P.; Powers, T.O. Molecular differentiation of two sibling species of the black fly *Simulium vittatum* (Diptera: Simuliidae) based on random amplified polymorphic DNA. *Genome* **2004**, *47*, 373–379. [CrossRef]
22. Hajibabaei, M.; Janzen, D.H.; Burns, J.M.; Hallwachs, W.; Hebert, P.D. DNA barcodes distinguish species of tropical Lepidoptera. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 968–971. [CrossRef]
23. Simard, F.; Lehmann, T.; Girod, R.; Brutus, L.; Gopaul, R.; Dournon, C.; Collins, F.H. High amounts of genetic differentiation between populations of the malaria vector *Anopheles arabiensis* from West Africa and eastern outer islands. *Am. J. Trop. Med. Hyg.* **1999**, *60*, 1000–1009. [CrossRef] [PubMed]
24. Krueger, A.; Hennings, I.C. Molecular phylogenetics of blackflies of the *Simulium damnosum* complex and cytophylogenetic implications. *Mol. Phylogenetics Evol.* **2006**, *39*, 83–90. [CrossRef] [PubMed]
25. Yao, H.; Song, J.; Liu, C.; Luo, K.; Han, J.; Li, Y.; Pang, X.; Xu, H.; Zhu, Y.; Xiao, P.; et al. Use of ITS2 Region as the Universal DNA Barcode for Plants and Animals. *PLoS ONE* **2010**, *5*, e13102. [CrossRef]
26. Hernández-Triana, L.; Montes De Oca, F.; Prosser, S.W.J.; Hebert, P.D.N.; Gregory, T.R.; McMurtrie, S. DNA barcoding as an aid for species identification in Austral black flies (Insecta: Diptera: Simuliidae). *Genome* **2016**, *60*, 348–357. [CrossRef]
27. Cywinska, A.; Hunter, F.F.; Hebert, P.D. Identifying Canadian mosquito species through DNA barcodes. *Med. Vet. Entomol.* **2006**, *20*, 413–424. [CrossRef]
28. Hernández-Triana, L.M.; Chaverri, L.G.; Rodriguez-Perez, M.A.; Prosser, S.; Hebert, P.; Gregory, T.; Johnson, N. DNA barcoding of Neotropical black flies (Diptera: Simuliidae): Species identification and discovery of cryptic diversity in Mesoamerica. *Zootaxa* **2015**, *3936*, 93–114. [CrossRef]
29. Ilmonen, J.; Adler, P.H.; Malmqvist, B.; Cywinska, A. The *Simulium vernum* group (Diptera: Simuliidae) in Europe: Multiple character sets for assessing species status. *Zool. J. Linn. Soc.* **2009**, *156*, 847–863. [CrossRef]
30. Zwick, H.; Crosskey, R.W. The taxonomy and nomenclature of the blackflies (Diptera: Simuliidae) described by J.W. Meigen. *Aquat. Insects* **1980**, *2*, 225–247. [CrossRef]
31. Kim, S.R.; Kim, K.Y.; Jeong, J.S.; Kim, M.J.; Kim, K.H.; Choi, K.H.; Kim, I. Population genetic characterization of the Japanese oak silkmoth, *Antheraea yamamai* (Lepidoptera: Saturniidae), using novel microsatellite markers and mitochondrial DNA gene sequences. *Genet. Mol. Res.* **2017**, *16*, 19. [CrossRef]
32. Kononov, A.; Ustyantsev, K.; Wang, B.; Mastro, V.; Fet, V.; Blinov, A.; Baranchikov, Y. Genetic diversity among eight *Dendrolimus* species in Eurasia (Lepidoptera: Lasiocampidae) inferred from mitochondrial COI and COII, and nuclear ITS2 markers. *BMC Genet.* **2016**, *17*, 157. [CrossRef]
33. Prakash, A.; Walton, C.; Bhattacharyya, D.R.; O'Loughlin, S.; Mohapatra, P.K.; Mahanta, J. Molecular characterization and species identification of the *Anopheles dirus* and *An. minimus* complexes in north-east India using r-DNA ITS-2. *Acta Trop.* **2006**, *100*, 156–161. [CrossRef] [PubMed]
34. Thanwisai, A.; Kuvangkadilok, C.; Baimai, V. Molecular phylogeny of black flies (Diptera: Simuliidae) from Thailand, using ITS2 rDNA. *Genetica* **2006**, *128*, 177–204. [CrossRef]
35. Krüger, A.; Strübing, F.; Hennings, I.; Garms, R. Molecular identification and phylogenetic analysis of black flies (Diptera: Simuliidae) from Uganda and Cameroon. *Trop. Med. Int. Health* **2006**, *11*, 1227–1234. [CrossRef]
36. Post, R.J.; Crainey, J.L.; Ramirez, C.R.; Maia-Herzog, M.; Shelley, A.J. A preliminary investigation of mitochondrial DNA variation in the *Simulium damnosum* complex (Diptera: Simuliidae) in Cameroon. *Med. Vet. Entomol.* **2007**, *21*, 345–350. [CrossRef]
37. Adler, P.H.; Cheke, R.A.; Post, R.J. Evolution, epidemiology, and population genetics of black flies (Diptera: Simuliidae). *Infect. Genet. Evol.* **2010**, *10*, 846–865. [CrossRef]
38. Basáñez, M.G.; Walker, M.; Turner, H.C.; Coffeng, L.E.; de Vlas, S.J.; Stolk, W.A. River blindness: Mathematical models for control and elimination. *Adv. Parasitol.* **2021**, *113*, 33–126. [CrossRef]
39. Inci, A.; Yildirim, A.; Duzlu, O.; Onder, Z.; Ciloglu, A.; Seitz, G.; Adler, P.H. Genetic Diversity and Identification of Palearctic Black Flies in the Subgenus Wilhelmia (Diptera: Simuliidae). *J. Med. Entomol.* **2017**, *54*, 888–894. [CrossRef]

40. Post, R.J.; Laudisoit, A.; Dunbar, R.W.; Mandro, M.; Lakwo, T.; Laemmer, C.; Pfarr, K.; Hoerauf, A.; Tortosa, P.; Gomard, Y.; et al. Identification of the onchocerciasis vector in the Kakoi–Koda focus of the Democratic Republic of Congo: Implications for control. *Parasit. Vectors* **2022**, *15*, 26. [CrossRef]
41. Mank, R.; Wilson, M.D.; Rubio, J.M.; Post, R.J. A molecular marker for the identification of *Simulium squamosum* (Diptera: Simuliidae). *Ann. Trop. Med. Parasitol.* **2004**, *98*, 197–208. [CrossRef]
42. Phayuhasena, S.; Baimai, V.; Adler, P.H.; Pramual, P. Phylogenetic Relationships among the Black Fly Species (Diptera: Simuliidae) of Thailand Based on Multiple Gene Sequences. *Genetica* **2010**, *138*, 633–648. [CrossRef]

Received: 9 May 2022 | Accepted: 21 November 2022

DOI: 10.1111/mve.12630

ORIGINAL ARTICLE

Medical and Veterinary Entomology

Testing the susceptibility of larval stages of *Simulium* to temephos and *Bacillus thuringiensis* var *israelensis* in Germany and Northern Cameroon

Pierre Kamtsap[1,2,3] | Paguem Archile[3,4] | Nguemaïm Flore[5] | Flobert Njiokou[6] | Alfons Renz[1,3]

[1]Department of Comparative Zoology, Institute of Evolution and Ecology, University of Tübingen, Tübingen, Germany

[2]Faculty of Science, Department of Cellular and Molecular Parasitology, University of Buea, Buea, Cameroon

[3]Programme Onchocercoses field station, University of Tübingen, Ngaoundéré, Cameroon

[4]Faculty of Agriculture and Veterinary Medicine, Department Veterinary Medicine, University of Buea, Buea, Cameroon

[5]Faculty of Health Sciences, University of Bamenda, Bambili, Cameroon

[6]Laboratoire de Biologie Générale, Université de Yaoundé I, Yaoundé, Cameroon

Correspondence
Pierre Kamtsap, Department of Comparative Zoology, Institute of Evolution and Ecology, University of Tübingen, Tübingen, Germany.
Email: kamtsapbuea200@gmail.com

Funding information
Baden Württemberg Foundation in Germany; Medical Research Council of the UK through the Global Challenges Research Fund and granted by the United Kingdom's Official Development Assistance (ODA) through the Centre for Research in Infectious Diseases (CRID), Grant/Award Number: MR/P027873/1; Programme Onhocercoses laboratory in Ngaoundéré-Cameroon

Abstract

Assays to evaluate the susceptibility of *Simulium* larvae to temephos and *Bacillus thuringiensis* var *israelensis* (*Bti*) were carried out by setting-up an in vitro laboratory test ('bio assay') and a semi-natural test ('système de goutières') to assess the LC50/LC90 values. Larvae of *Simulium* species in Cameroon (*S. damnosum* s.l., *S. hargreavesi*, *S. vorax* and *S. cervicornutum*) and (*S.* (*Odagmia*) *ornatum* and *S. latipes*) in Germany were identified and tested. In the bio-assay, 50 larvae were exposed for 10 min to concentrations from 0.01 to 10 ppm. For the *Simulium* from Germany, the LC50 (LC90) values after 3 and 6 h were 3.1 (27.9) and 0.14 (1.26) ppm for temephos and for *Bti* 7.8 (70.2) and 1.7 (15.3) ppm, respectively. For Cameroonian species, the values of LC50 (LC90) were lower, that is, 0.42 (8.04), 0.14 (2.70) and 0.073 (1.38) ppm, respectively, after 3, 6 and 12 h for temephos. In a semi natural condition, the LC50 of 10 min of application of temephos was 0.84 ppm after 3 h and a working solution (2.6 L) of *Bti* killed 50% after 6 h. To detect an upcoming of any resistance as it happened in Ivory Coast, a study of the occurrence resistance genes should be implemented.

KEYWORDS
Bti, Ngaoundere, *Simulium damnosum*, susceptibility, temephos

INTRODUCTION

Before effective drugs against onchocerciasis were available, the means of control were based on vector control by using insecticides against *Simulium* larval stages. In 1955, Taufflieb carried out the first treatments in the Mayo-Kebbi in North Cameroon (Taufflieb, 1955). At that time, dichlorodiphenyltrichloroethane (DDT) was considered as "the perfect weapon for the target" (Brown, 1962) until when the

persistent and longterm effects of this insecticide became very noticeable. This has given impetus to the testing of less persistent larvicides (Jamnback, 1962; Nagahana et al., 1964; Travis & Wilton, 1965) and in 1970, attempts were made with DDT and to develop a reliable testing method for *S. damnosum* susceptibility as suggested by the WHO (WHO, 1970).

As a result of these trials, temephos, an organophosphate larvicide affecting the nervous system of blackfly larvae through inhibition of cholinesterase (Lima et al., 2003) was considered to be the first choice (Back et al., 1979) because it was environmentally tolerable, biodegradable and responsible of negligible damage to the non-target aquatic fauna (Leveque et al., 1979). It was therefore much used since 1974, in particular by the Onchocerciasis Control Program in West-Africa (WHO, 1995).However, resistance to temephos was quickly detected on the lower Bandama in the Cote d'Ivoire, where it affected two closely related species of the *Simulium damnosum* complex, *Simulium sanctipauli* and *Simulium soubrense* (Guillet et al., 1980). Temephos resistance was later also demonstrated on the downstream section of the river Marahoue (in August 1980), on the Kan (November 1980), the middle Sassandra (January 1981) and the Leraba (February 1981) in the same country (WHO, 1981b). The former *Simulium sanctipauli* species is found almost exclusively in forest rivers while the latter, *Simulium soubrense*, more widespread, prefers large rivers in humid regions but can also develop in savanna areas where local bioclimatic and/or seasonal conditions are favourable (Remme et al., 1986). In this context is it important to remember that resistance only occurred in these two members of the *S. damnosum* complex, but did not spread or develop in the other, more important vectors of *O. volvulus* (Leveque et al., 1988). Larvicide treatment was stopped by OCP in 2002 (Coffeng et al., 2013) and ivermectin became the backbone of the African Program for Onchocerciasis Control (APOC). More than 99% of infected people live in 31 African countries. The disease also exists in some foci in two countries in Latin America (the Yanomani area in Brazil and Venezuela) and Yemen. The Global Burden of Disease Study estimated in 2017 that at least 220 million people required preventive chemotherapy against Onchocerciasis, 14.6 million of the infected people already had skin disease and 1.15 million had vision loss (WHO January, 2022).

In highly endemic foci, ivermectin treatment alone might not be enough to eradicate the parasite (Lakwo et al., 2020). Also, in foci of co-endemicity with *Loa loa*, ivermectin cannot be used for community-based mass-treatments, due to severe side-effects (Nana Djeunga et al., 2020; Boussinesq et al., 2018). Considerable research has been conducted on bacterial insecticides over the last decades, and major successes have been obtained (Guillet et al., 1990). The significant advantages of *Bti* over chemical insecticides have been responsible for their fast introduction into large-scale routine operation for mosquito control in Europe as well as for blackfly (*Simuliidae*) control in Africa (Becker, 1998; Nartey et al., 2013). The advantage of *Bti* in comparison to chemical control is its effectiveness at relatively low doses, safety to humans and non-target wildlife, low cost of product in some cases and lower risk of resistance development.

Still alternative and complementary strategies to drug treatment are needed. Such measures could be the destruction of *Simulium* breeding sites by clearing the vegetation support for larvae "slash & clear" strategy (Lakwo et al., 2020) or the strategic use of safe and uncostly larvicides (Nana Djeunga et al., 2020).

Here, we report the results of the susceptibility of larval stages of *Simulium* to temephos and *Bacillus thuringiensis* var *israelensis* in Northern Cameroon and Germany. Specifically, the aim of this study was to set-up an experimental screen (in-vitro laboratory testing system, 'bio assay') and a semi-field test ('système de goutières') to assess the LC (lethal concentration 50% and 90% which is the concentration of the larvicide at which half or 90% of the members of a population are killed after a specified duration of exposure) of temephos and *Bti* against the larval stages of blackflies.

MATERIALS AND METHODS

Study area

The trials in Tübingen were done from January to March 2020 on last stage of *Simulium latipes* larvae from a stream in the botanic garden (48°32′21.50″N/9°02′19.14″E) of the University of Tubingen and from the Weilerbach River near Hagelloch (48°32′04.56″N/9°01′16.17″E).

Trials in Cameroon were carried out on *Simulium* larvae collected from breeding sites around Ngaoundéré in the rivers and effluents of the "Vina du Nord" and "Vina du Sud", flowing towards the Lac Chad and the Sanaga River, respectively. Both originate on the Adamawa plateau (Figure 1a). The main *S. damnosum s.l.* (*S. squamosum*, mainly) breeding sites are in the "Vina du Sud" falls (7°12′33.92"N/13°35′07.04″E) near Galim (Wakwa) and in the "Vina du Nord", located South-West of Dang at the 'Chutes de la Bini' (Figure 1, 7°24′26.60″N/13°34′27.59″E). Additional *non-damnosum* (*S. hargreavesi*) breeding sites, which are close to the Programme Onchocercoses laboratory in Ngaoundéré are found in the town itself at the Mayo Mardock (7°19′59.73"N/13°33′49.05″E) near the 'pepinières' at ca. 3 km and at the outlet of Lac Bini (behind the university of Ngaoundéré: 7°25′33.16"N/13°30′42.67″E) where the Vina du Nord originates (locally this river is called 'Bini'). The sampling sites were selected from river stretches known to have dense *Simulium* larval population during the dry and rainy seasons.

Source of the insecticides

Temephos (Abate 500 EC: organophosphate insecticide) as delivered by the manufacturer at a concentration of 500 gr of active compound ($C_{16}H_{20}O_{16}P_2S_3$) per litre and the Teknar HP-D (*Bacillus thuringiensis* var *israelensis*, Lot No: 20140023) with 1.6% active dipteran toxin(s) and 98.4% of inert ingredients were bought from the Valent Bioscience corporation, Agrochem Cameroon, Douala.

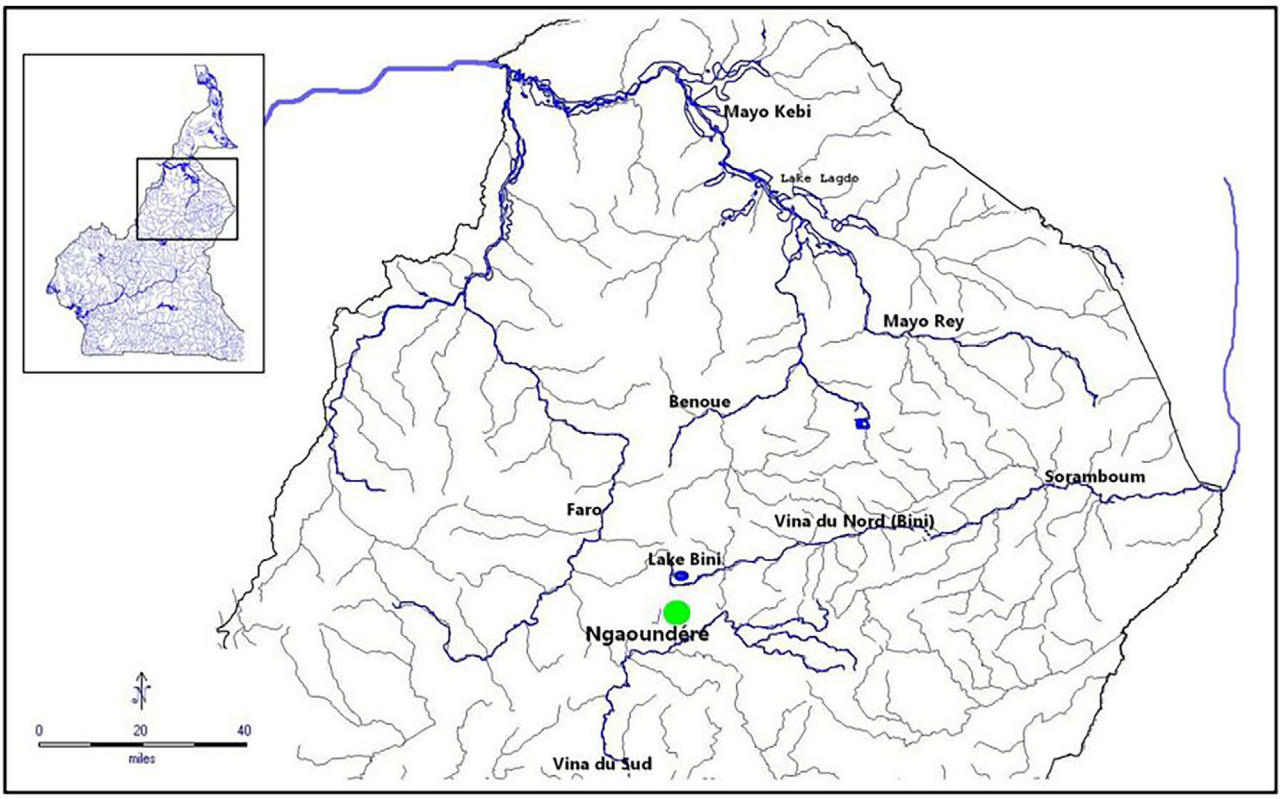


FIGURE 1 Location of *Simulium* breeding rivers and study site in Northern Cameroon

Preparation of insecticide

To prepare the insecticide for the trials, we diluted it in to two steps:

A stock solution of temephos at 1 gram per litre (1 part of active compound per thousand, ppt) is first prepared by diluting 2 ml of insecticide to 1 L of water according to protocol of testing the susceptibility of larvae of *S. damnosum* complex (WHO, 1981a, 1983). This solution is kept protected from the sun in a cool and dry place. The actual amount of larvicide used in the trial is taken from this stock solution. The solution of *Bti* is prepared by adding 2 ml of the main solution to 998 ml of water from the breeding site. The solutions were mixed thoroughly by inverting the container several times.

The study is structured in two stages

Laboratory trials to test the susceptibility (Lethal Concentration (LC50 and LC90)) of *Simulium* larvae to temephos and *Bacillus thuringiensis* var *israelensis* in a laboratory-based in-vitro test ('bio-assay'), and *Field trials* in the Vina du Nord river, including the construction of a 'système de gouttières'.

Design of the laboratory trials of larvicide activity (assessment of LC50 and LC90 in the bio-assay)

The bioassay was based on established methods developed elsewhere (Rutschke & Deschle, 1988). While still attached to their substrates, *Simulium* larvae were collected as fresh as possible in a cool box covered by wet tissue. A plastic container was filled with water from the breeding site and used for the trial. Larvae were incubated in aerated glass-beakers (usually 50 larvae in a 1000 ml beaker) following a modified biotest-system, (Rutschke & Deschle, 1988) (Figure 2). In our study, larvae are incubated at room temperature for 1 h in beakers containing 1 L of water from the breeding site to allow larvae to accommodate in their new environment instead of 1 day as described originally (Figure 3).

Increasing concentrations (0.01; 0.02; 0.04; 0.08; 0.16; 0.32; 0.64; 1.28; 2.56; 5.20 and 10.40 ppm, Table A1) of insecticide (temephos and *Bti*) were applied for 10 min, and an untreated control (larvae without insecticide) was observed under the same conditions. The treated water was removed using a siphon and replaced by clean water from the breeding site. The survival of the *Simulium* larvae was recorded at 3, 6 and 12 h post treatment (p.t.) and the larvicide concentration, achieving $LC50_s$ and $LC90_s$ calculated for these time points (Figure 4). Two replicas were set up for each concentration.

The design of the laboratory test system ('bio-assay') was developed at the Institute for Evolution and Ecology of the University of Tübingen in Germany before being later used (the 'bio-assay') in the Programme Onchocercoses laboratory in Ngaoundéré.

Preparation of larvae in the laboratory and larvicide application for the trial

Simulium larvae from the box were detached from their support with the help of a small and fine brush. Fifty larvae were transferred per

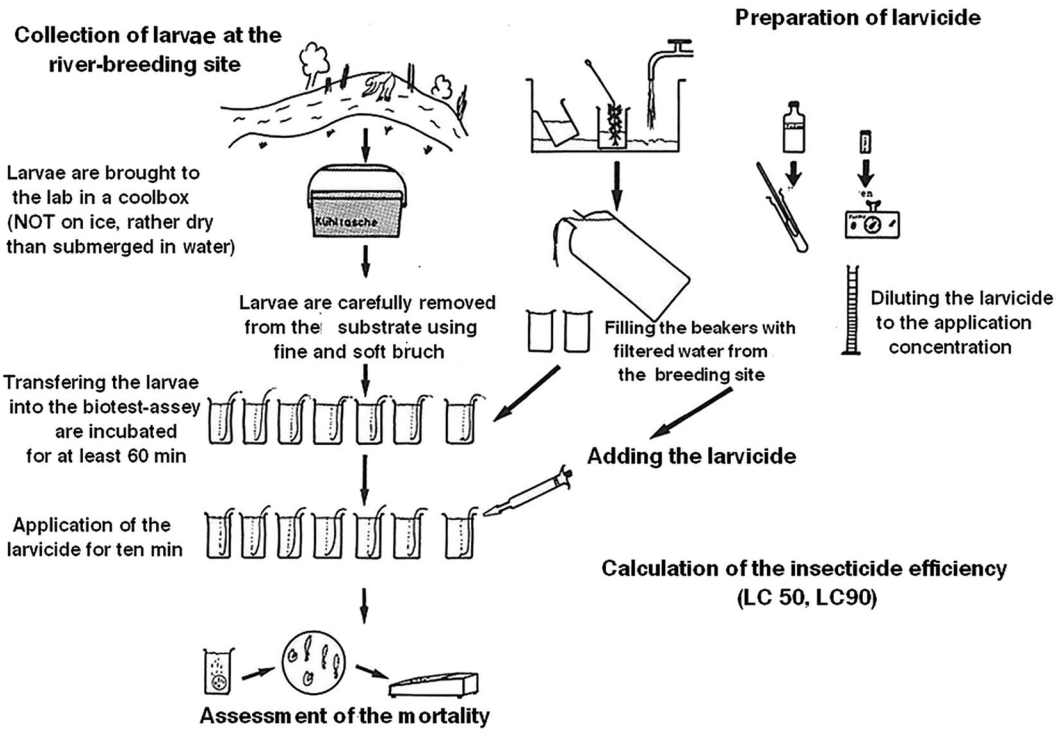


FIGURE 2 Set-up of bio-assay larvicide-trials in the laboratory (modified from Rutschke & Deschle, 1988).

beaker containing water from the breeding site and left to accommodate for 1 h. All beakers were aerated using an air-pump (Eheim air 400) and valves to keep water in motion to provide enough current for larvae survival. Syringe needles (size G20 × 1 1/2 in. 0.90 × 40 mm) were connected on silicone tubes and to the valves in such a way as to deliver at least 10 air bubbles/s in each beaker (Figure 3). Then the calculated amount of insecticide was added to the beakers and the larvae were incubated for 10 min and thereafter, the water was. The survival of the treated larvae and control larvae was recorded and scored at 3, 6 and 12 h post treatment (p.t.).

To determine larval viability, each larva was touched with the tip of a needle in Tübingen and with a grass stalk in Ngaoundere which seems more appropriate than a needle and its reaction observed visually. If the larva reacted immediately by bending and continued this sort of motion for at least several seconds, it was classified "alive". If the larvae did not respond at all it was classified "dead". If the larva flexed its body incompletely and only once, before returning to a straightened position with the mouthparts quivering, it was considered moribund.

Experimental field trials

Construction and running of the "Système de goutières"

The 'système de goutières' (Figure 5) was built in the Programme Onchocercoses laboratory in Ngaoundéré and then set up at a fish trap near the outlet of the Lake Bini when the water level was high enough to operate the trap. A polyvinyl chloride (PVC) pipe (diameter 15 cm) collected the water from upstream and the test-system is installed below the fish-trap.

The substrate on which *Simulium* larvae (approximately 50) are attached were clamped on each of the five channels of the "goutières" and used for the larvicide tests in the droughts. The aluminium plate (originally used for roofing a house) was 200 cm long and 90 cm large and had a slope of 20%. The five channels were 2 cm high and 2.5 cm large.

Solutions of larvicides were spread through the channels of the set-up, using infusion bags equipped with valves (Figure 6). Each larvicide solution is poured one after another through a tap of the devise which was directed on one of the rectangular channels of the sheet metal on which the larvae are fixed on their support. A piece of filter gauze was placed at the end of the sheet metal with a filter incorporated inside to collect the larvae that will detach under the effect of the insecticide. At the end of flowing time (3 and 6 h), larvae collected from the filter were separated into three groups, live, moribund and dead.

In a first series of experiments, based on results of the experimental bioassay, we adopted the technique of "increasing volume" from 1, 2, 3, 4, 5 to 20 L of insecticide which means that a concentration of 0.42 ppt for a series of six tests each for a time "t" was used and the volume of water flowing through the counter is measured. The insecticide flowing time is recorded, the water meter reading is taken and the count of alive, moribund and dead larvae is recorded after 3 and 6 h.

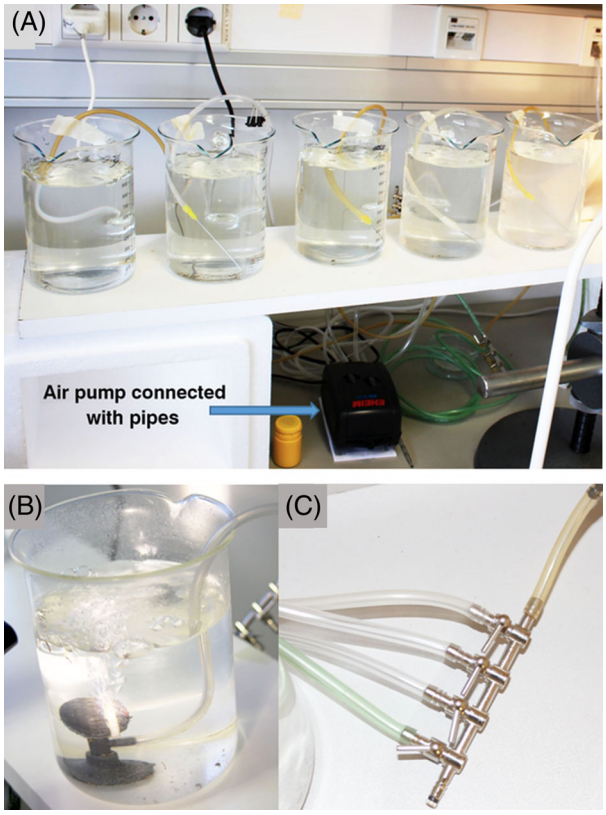


FIGURE 3 Aeration system using air-pumps and valves: 1. beakers filled with diluted larvicide. 2. production of air bubbles. 3. valves connected with pipes

Later-on, the volume of temephos solution in the infusion bags was maintain at 1000 ml but with increasing concentrations (0, 15, 30, 60, 120 and 240 ml of stock solution), respectively, for each channel of the metal sheet. The time it takes for each volume to flow is recorded as well as the number of dead larvae after 3 and 6 h.

In a final series of experiments, the time of larvicide contact was regulated to 10 min. approximately, and the water discharge in each channel was measured by taking the time it needed to fill a beaker of 1 L. Then the discharge per 10 min of contact time was calculated and the infusion bags filled with the appropriate amount of larvicide solution to drain during 10 min.

Field trial using *Bti* on larvae from the Vina du Sud falls

In the field, Teknar HP-D was used to test the LC50 of *Simulium* larvae from the Vina Sud. For this, we prepared separately a working solutions by diluting 2 ml of *Bti* in 1 L of water from the breeding site. A series of five different amount (1; 2; 3; 4 and 5 L) of this working solution is taken. Larvae where treated with larvicide for 10 min, mortalities are recorded (Table 5) after 3 and 6 h and the graphs were constructed (Figure 7).

Morphological and molecular identification of *Simulium* species

Larvae and pupae collected at the Vina du Nord and Vina du Sud falls were preserved in 95% alcohol and examined at the University of Tubingen in Germany.

Pupae were morphologically identified by their respiratory gills and examined under a Wild M5 dissection and a Zeiss Axioplan compound microscope using standard keys as described by Freeman and de Meillon for African and Lindner and Rubtsov (Rubtsov, 1969) for European species. Identification of mature larvae of the *Simulium damnosum* species complex was done using a hand lens to check for cuticular setae extending forward to the proleg, and pairs of prominent dorsal, tubercles or bumps on the segments of the abdomen.

Molecular genetic identification

The Winzard Genomic DNA Purification Kit (Promega, Germany) was used to extract total genomic DNA from individual pupae that were identified morphologically (Kim et al., 2017) following the manufacturer instructions. A fragment of the mitochondrial Cytochrome c oxidase subunit 1 (CoxI) which is about 658 bp long was amplified by Polymerase Chain Reaction (PCR) using the Lep primers forward (5′-ATTCAACCAATCATAAAGATATTGG-3′) and reverse (5′-TAAACTTCTGGATGTCCAAAAAATCA-3′) (Hajibabaei et al., 2006). As a nuclear gene, a fragment of the internal transcribed spacer 2 which is about 400 bp long was amplified using the ITS2 primers forward (5′-TGTGAACTGCAGGACACAT-3′) and reverse (5′-ATGCTTAAATTTAGGGGGT-3′) (Kononov et al., 2016).

The PCR reaction with a final volume of 25 µl was performed using the Go Taq Hot start PCR Kit (Promega) with the following volumes: 2 µl of DNA extract and 23 µl of Master mix (13.8 µl of ddH_2O; 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 primer and 1 U of Promega Taq Polymerase) (Kim et al., 2017). Amplifications were performed in a Master Cycler (Eppendorf Master Cycler). For the Lep primer, an initial denaturation (95°C, 2 min); 35 cycles denaturation (95°C, 30 s); annealing step (51°C, 30 s); extension (72°C, 60 s) and incubation (72°C, 5 min) were used. For the ITS2, initial denaturation (94°C, 2 min); 35 cycle denaturation (94°C, 40 s); annealing step (51°C, 60 s); extension (72°C, 60 s) and incubation (72°C, 5 min) were used (Prakash et al., 2006). The amplified DNA was checked by electrophoresis using 1.5% agarose gel with a generator set at 100 V for 25 min. Finally, PCR products were sequenced on the EZ – Seq V2.0 apparatus (Macrogen, Netherlands).

Sequence alignment

Mitochondrial coding gene

Contigs of genes were assembled and edited in Geneious Prime 2019.0.4. Protein coding sequence of *Simulium* flies was imported into

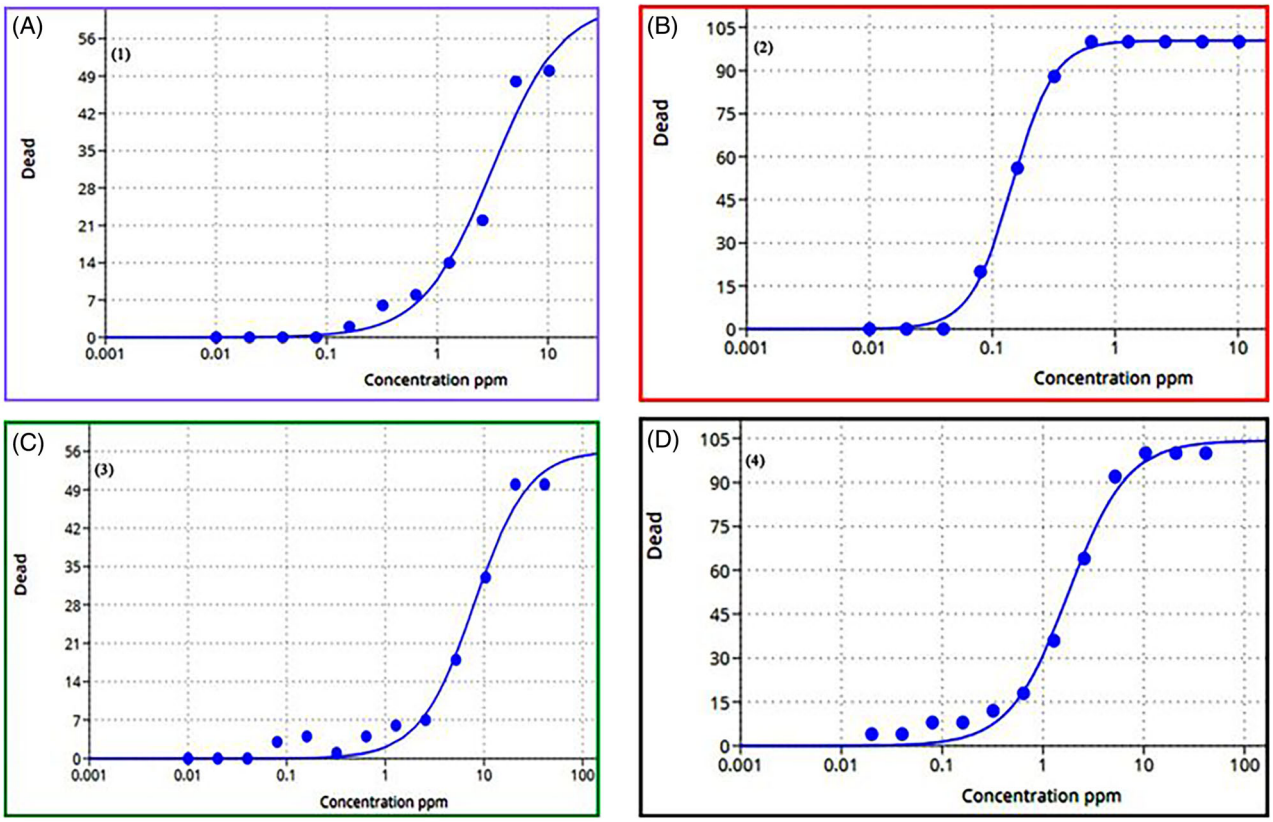


FIGURE 4 Results of susceptibility tests (LC50) with temephos and *Bti* in the bioassay in Tübingen: 1. temephos = 3.1 ppm after 3 h. 2. temephos = 0.14 ppm after 6 h. 3. *Bti* = 7.8 ppm after 6 h. 4. *Bti* = 1.7 ppm after 12 h.

MEGA 7.0.26 (Krueger & Hennings, 2006) for alignment according to amino acid. After determining the open reading frame, sequences were translated to amino acid and then aligned with the MUSCLE program with published sequences from the GenBank. The aligned amino acid was then back-translated to nucleotides, retaining the codon positions. The evolutionary history was inferred using the UPGMA method (Alzahrani et al., 2021). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Figure 8). The evolutionary distances were computed using the Tamura 3-parameter method (Tamura & Nei, 1993) and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair.

Internal transcribed spacer 2

The sequences of ITS2 were aligned over a consensus length of approximately 245 bp with published sequences from GenBank. Phylogenetic analysis was performed using neighbour joining methods based on the Tamura-Nei model (Kumar et al., 2018). Confidence limits were assessed using the bootstrap procedure (1000 replicates) (Kumar et al., 2018).

The Minimum Evolution (ME) tree was searched using the CNI algorithm (Saitou & Nei, 1987) at a search level of 1. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA 7.0.26 (Kumar et al., 2016).

Data analysis

Percentage mortality of larvae was calculated from moribund and dead larvae for each set of tests. The online software (https://www.aatbio.com/tools/lc50-calculator) was used to calculate the LC50, the GraphPad-QuickCalcs was used to determine the LC90, and the ratio of LC50 to LC90 (http://www.graphpad.com/quickcalcs/ConfInterval1.cfm).

RESULTS

Bio assay tests done in Tübingen

A series of 11 concentrations of temephos and *Bti* including a control were tested against *Simulium* (*Ogdamia*) *ornat*um and *Simulium latipes* (Tables A1 and A2) and the plots of percentage mortalities are shown in Figure 4.

For these two European species of *Simulium*, the LC50 (LC90) values after 3 and 6 h, respectively, were 3.1 (27.9) and 0.14 (1.26) ppm for temephos; and 7.8 (70.2) ppm and 1.7 (15.3) ppm for *Bti* after 6 and 12 h, respectively. The proportion of LC50/LC90 without specific indication of specie was 0.11 (Table 1).

FIGURE 5 "System de goutières" build at the Programme Onchocercoses laboratory in Ngaoundéré.

FIGURE 6 Set up of the "System de gouttières" at the local fish trap at the out let of the lake Bini near the University of Ngaoundéré with plastics bags containing diluted insecticide.

Bio assay tests in Ngaoundéré

The survival of the *Simulium* larvae from the breeding site located at the outlet of Lake Bini near the University of Ngaoundere is recorded (Table A3) at 3, 6 and 12 h post treatment (p.t.). The susceptibility test is done, data are recorded (Table 2), calculations (LC50, LC90 and LC50/LC90 – Table 2) are done and graphs are constructed (Figure 8). After 3 hours, the LC50 of temephos was 0.42 ppm and was respectively LC50 = 0.14 ppm and LC50 = 0.073 ppm after 6 hours 12 hours (Table A3).

All species (*S. damnosum s.l.*, *S. hargreavesi*, *S. cervicornutum* and *S. vorax*) found from the breeding sites were tested and no difference regarding to their fan's filtering was observed.

"Système de goutières" in a semi natural treatment assay was done with larvae from the Vina du Sud falls in Ngaoundéré.

"Increasing volume" with 0.42 ppt of temephos experiment

Respectively 1, 2, 3, 4, 5 and 20 L of 0.42 ppt of temephos (or *Bti*) were added to 66, 85, 67, 80, 52 and 80 L of water that ran off in 4, 20, 4, 6, 10 and 11 min, active concentrations (0.79, 0.61, 0.76, 0.63, 0.93 and 0.49 ppm) were recorded (Table 3). For 10 min incubation, 59% of dead larvae were recorded after 3 h with an active concentration of 0.93 ppm. For 20 min incubation with insecticide, 43% of dead larvae were recorded and no dead larvae was recorded in the control test.

"Increasing concentration" of larvicide (temephos) added to 1 L of water from breeding site.

After 10 min incubation of larvicide together with larvae attached on channels, 53% of dead larvae were recorded for an active concentration of 1 ppm for 3 h corresponding to 60 L of flowing water in the corresponding channel. No mortality in the control was registered. (Table 4). The plots of percentage mortalities are showed in Figure 9 and the LC50 is 0.95 ppm.

Field trial using *Bti* on larvae from the Vina Sud.

After 10 min incubation of larvae with *Bti*, mortalities were checked after 1, 2, 3, 4 and 5 ltr of working solution have been flown out in corresponding channel for 3 and 6 hours. It is shown that 4.6 and 2.6 L could kill 50% of larvae respectively after 3 and 6 hours.

Species diversity from the study sites around Ngaoundéré

Ninety *Simulium* pupae were identified from the morphology of their pupal gills and four *Simulium* species were found (Table 6). *Simulium hargreavesi* was mostly (100%) prevalent at the Vina du Nord (outlet of Lake Bini) and *S. cervicornutum*, *S. vorax* at Vina du Sud. Pupae of *S. damnosum s.l.* were only seen at the Vina du Sud near Galim and identified by sequencing the Cox1 and the ITS2, to be *S. squamosum* Savannah type. Five from each identified species were also sequenced with Cox1 and ITS2 (Figure 10).

We evaluated the phylogenetic relationship of *Simulium* species of this study with other deposited sequences in the GenBank. We used some accession numbers (DQ869241) from Kinshasa,

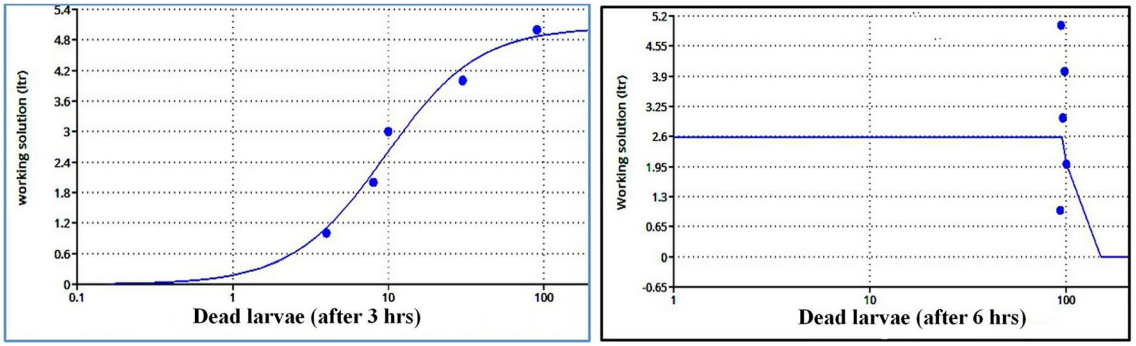


FIGURE 7 Results of susceptibility tests (LC50) with *Bti* after 3 and 6 h with larvae from the Vina du Sud falls. *Bti* LC50 = 4.6 L of working solution after 3 h, *Bti* LC50 = 2.6 L of working solution after 3 h.

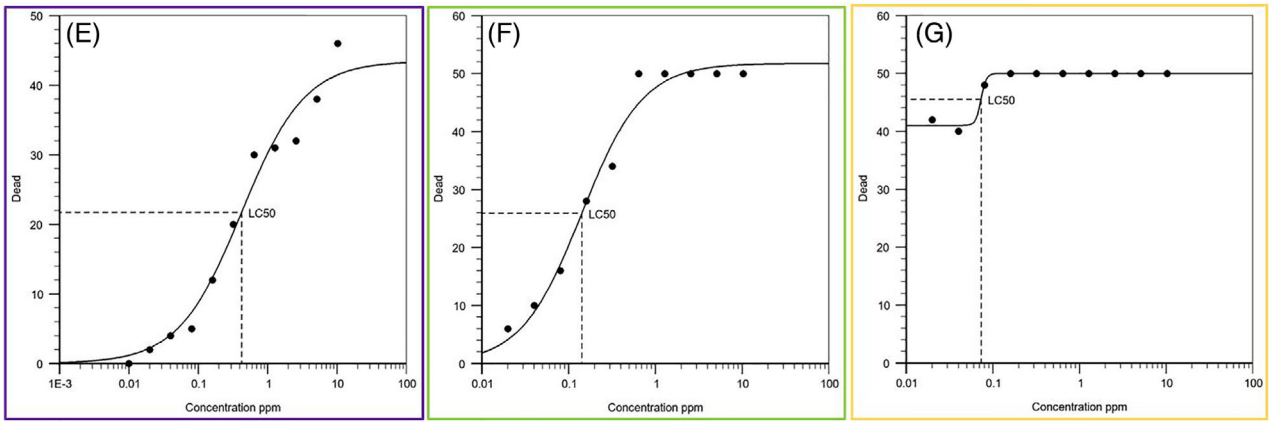


FIGURE 8 Results of susceptibility tests (LC50) with temephos in the bioassay from Ngaoundere: E- temephos LC50 = 0.42 ppm after 3 h, f-temephos LC50 = 0.14 ppm after 6 h, g- Temephos LC50 = 0.07 ppm after 24 h.

TABLE 1 Results of LC_{50} and LC_{90} after 3 and 6 h of temephos and *Bti* on *Simulium* larvae from Tübingen

	Temephos lethal concentration ppm		*Bti* lethal concentration ppm	
	After 3 h	After 6 h	After 3 h	After 6 h
LC50	3.1	0.14	7.8	1.7
LC90	27.9	1.26	70.2	15.3
LC50/LC90	0.11	0.11	0.11	0.11

Abbreviations: *Bti*: *Bacillus thuringiensis* var *israelensis* LC50/LC90; LC, lethal concentration; ppm, part per million.

TABLE 2 Results of LC50 and LC90 of temephos after 3, 6 and 12 h on *Simulium* larvae from Ngaoundéré

	After 3 h ppm	After 6 h ppm	After 12 h ppm
LC 50	0.4234	0.142	0.073
LC 90	8.0446	2.698	1.387
LC50/LC90	0.052	0.052	0.052

Abbreviations: *Bti*, *Bacillus thuringiensis* var *israelensis* LC50/LC90; LC, lethal concentration; ppm, part per million.

Democratic Republic of Congo, (AY625925) Kalagala Falls, Uganda as reference.

DISCUSSION

We describe the development and set-up of an experimental in vitro laboratory testing system, (bio-assay) and a semi-natural in-vivo test (système de goutières) to assess the LC50 and LC90s of temephos and *Bti* against the larval stages *Simulium*.

From the results of our experiments, it is concluded that *Simulium* larval populations in the local rivers were susceptible to temephos and that this compound could be used in large scale to properly control blackfly populations in endemic area especially with co-endemicity with *Loa loa*. *Loa loa* is not endemic in the Adamaoua region but its occurrence is very high in the rain-forest

TABLE 3 Record data of "increasing volume" with 0.42 ppt temephos

Volume of larvicide in ppm (ltr)	Volume of stock-solution (ml)	Incubation time (min) with insecticide flowing	Volume of water flowing in each channel (ltr)	Active concentration ppm	Mortality of larvae attached on the channel of the "système de goutières"		
					Alive	Mortality Nber (%)	
CONTROL			57		58	0	0
1 L	50.4	6	66	0.79	196	4	2
2 L	100.8	20	85	0.61	47	36	43
3 L	151.2	4	67	0.76	28	05	15
4 L	201.6	6	80	0.63	30	10	25
5 L	252	10	52	0.93	12	17	59
20 L	1008	11	80	0.49	23	51	69

TABLE 4 Record of data of insecticide of identical concentration at 1 ppt

Final volume of water in infusion bags	Volume of stock-solution (ml)	Volume of water flowing in each channel	Active concentration ppm	Mortality of larvae attached in the channel of the "système de goutières"		
				Alive Nber	Mortality Nber (%)	
1000 ml	0	75	0		0	0
985 ml	15	75	0.2	59	10	14
970 ml	30	60	0.5	50	8	14
940 ml	60	60	1	24	27	53
880 ml	120	67	2	20	87	81
760 ml	240	75	4.2	36	79	69

Note: After 60 L of water have flown out with 3 L of working solution of insecticide at 1 ppt for 10 min, more than 50% of dead larvae were recorded.

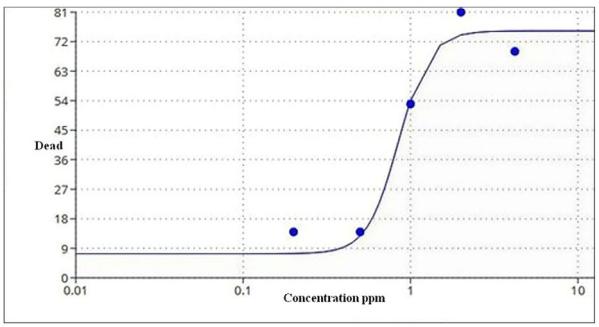


FIGURE 9 Increasing concentration of larvicide added to 1 L of water from breeding Temephos LC50 = 0.95 ppm after 3 h.

regions of Cameroon (BOUSSINESQ, 1997). From the experience of the Onchocerciasis Control Programme (OCP) areas, larvae of *Simulium* were regarded to be resistant to temephos when they survive concentrations higher than 1.0 ppm for 10 min. (Davies, 1994).

In our experiments, for the *Simulium* from Germany, the LC50 (LC90) values after 3 and 6 h were 3.1 (27.9) and 0.14 (1.26) ppm for temephos and for *Bti* 7.8 (70.2) and 1.7 (15.3) ppm respectively. For Cameroonian species, the values of LC50 (LC90) were lower, that is, 0.42 (8.04), 0.14 (2.70) and 0.073 (1.38) ppm, respectively, after 3, 6 and 12 h for temephos. In a semi natural condition, the LC50 of temephos was 0.84 ppm after 3 h and a working solution (2.6 L) of *Bti* killed 50% after 6 h.

The Cameroonian subspecies did not show any particular difference in susceptibility to each other, nor did those from Germany. The molecular study shows a great genetic diversity between the *Simulium* species involved in this study.

During the bio-assay, one could well observe how larvae were filtering the insecticide using the fans of the mouthparts. However, this observation was not feasible with the "système de goutières". In both

TABLE 5 Susceptibility of Simulium larvae to *Bti*, Ngaoundere – Cameroon in the systeme de Goutières

PIIVeC data collection: Susceptibility of *Simulium* larvae to *Bti* Ngaoundere - Cameroon								
Date: January 2022 larvae from: Vina falls sheet; No: 02; Observers: PK, DE, JY								
		Incubation time (min) with insecticide flowing		Volume of water (L) in 10 min	Mortality of larvae attached on the channel of the "système de goutières"			
					3 h		6 h	
Larvicide main solution (2 ml in 1 L of water)	Volume litre (L)	Start	End		Alive Nber	Dead Nber	Alive Nber	Dead Nber
CONTROL	0	//	//	50	59	0	59	0
Channel I	1	9 h35	9 h45	43	96	4	7	93
Channel 2	2	9 h50	10 h00	75	45	4	0	100
Channel 3	3	9 h55	10 h05	86	45	5	2	96
Channel 4	4	10 h06	10 h16	86	69	21	2	98
Channel 5	5	10 h12	10 h22	67	14	122	6	94

TABLE 6 Percentage on the basis of different breeding site of sample analysed for molecular aspect

Species	*S. hargreavesi* Numbers (%)	*S. Damnosum s.l.*	*S. vorax*	*S. Cervicornutum*
Vina du Nord	40 (100)	0 (0)	0 (0)	0 (0)
Vina du Sud	0 (0)	25 (50)	10 (20)	15 (30)

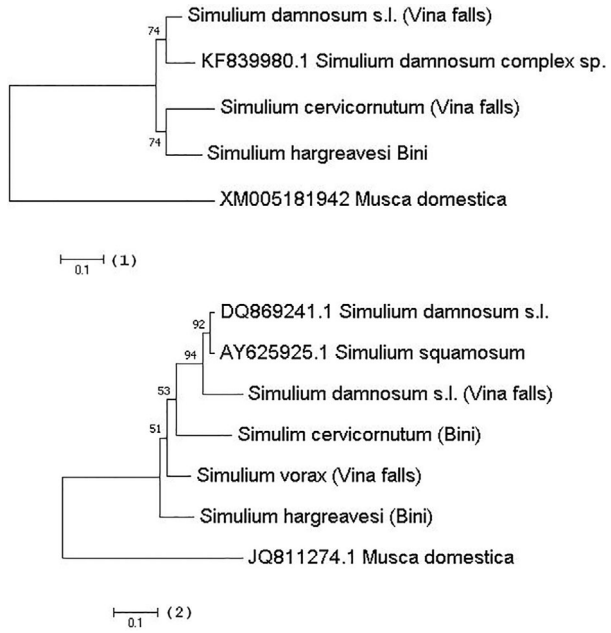


FIGURE 10 Evolutionary relationships of *Simulium* pupae collected from the study site based on Cox1 (1) and ITS2 (2).

trials, some dead larvae detached from the support on which they were attached, but not all.

From the experience of the Onchocerciasis Control Program (OCP) in West African, the first evidence of *S. damnosum* s.l. resistance developing to temephos was reported approximately 5 years after continuous intensive application in the upstream parts (WHO, 1995), while the resistant population was found in downstream stretches. That implies that a diluted concentration of the product was reaching downstream populations for a relatively long time before resistance was detectable. The identification of "resistance genes" and hybridization could be a subject of deep studies.

The savanna area along the "Vina du Nord" has a history of intensive use of organophosphate pesticides in the cotton fields although concrete documentation was not so easy to have. So it is likely that some of these pesticides trickled into the drainage system such as streams and eventually into the river systems which form breeding habitats for *Simulium* species, some of which are vectors of human onchocerciasis. The presence of this in any ecological habitat could presumably cause insecticide tolerance to both target and non-target aquatic fauna.

In 1986, a field-study done by J. Rutsche, W. Deschle, C. Barthelmess and A. Renz showed that a dose of 1 ppm of *Bti* for 10 min achieved a mortality of 93% after 9.7 km in the river Vina du Nord at Soramboum after 12 h. Even after 18 km, a mortality of 28% could still be established and at this distance from the application site, 20 h after application, moribund larvae could still be found (Rutschke & Deschle, 1988).

Considering for example the Ntem River which takes its source in Gabon at about 1100 m with an annual average flow rate of about 300 m^3/s at Ngoazik (Olivry, 1986) and drains a watershed of 31,000 km^2, one will need for 1 ppm approximately 300 ml of insecticide per second for 10 min. This calculation shall be considered for any national *Simulium* larviciding program.

CONCLUSION

At the end of this study, we have seen that for the European species of *Simulium*, the LC50 (LC 90) values after 3 and 6 h were 3.1 (27.9) and 0.14 (1.26) ppm for temephos and for *Bti* 7.8 (70.2) ppm and 1.7 (15.3) ppm, respectively. For Cameroonian species, the values of LC50 (LC90) were lower, that is, 0.42 (8.04); 0.14 (2.70) and 0.073 (1.38) ppm respectively after 3, 6 and 12 h for temephos. *Simulium* species used in this work are genetically distinct. To detect an upcoming of any resistance as it happened in Ivory Coast, a study of gene resistance shall be implemented.

AUTHOR CONTRIBUTIONS

Pierre Kamtsap, Nguemaïm Flore, Alfons Renz and Flobert Njiokou conceived the study. Pierre Kamtsap and Alfons Renz collected the samples. Pierre Kamtsap and Paguem Archile build the système de gouttières, conducted the molecular studies and analysed the data. Pierre Kamtsap drafted the primary manuscript. Alfons Renz and Joachim Rutschke did the experimental larvicide trial in 1985. All authors read and approved the manuscript.

ACKNOWLEDGMENTS

Our thanks go to David Ekale and Jeremi Yembo, Lab. Technician assistants from the Programme Onchocercoses laboratory in Ngaoundéré and to the Centre for Research in Infectious Diseases (CRID)-Cameroon for their commitment in acquiring administrative documents which made this work possible and to J. Rutschke., W. Deschle and C. Barthelmess for making their data and unpublished results available. Open Access funding enabled and organized by Projekt DEAL.

FUNDING INFORMATION

This work was partially funded by the Medical Research Council of the UK (grant number MR/P027873/1) through the Global Challenges Research Fund granted by the United Kingdom's Official Development Assistance (ODA) through the Centre for Research in Infectious Diseases (CRID), by the Baden Württemberg Foundation in Germany and by the Programme Onhocercoses laboratory in Ngaoundéré-Cameroon. The larvicide trails done in 1986 were financed by a grant of the European Commission.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare that are relevant to the content of this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Pierre Kamtsap https://orcid.org/0000-0002-8124-4466

REFERENCES

Alzahrani, D.A., Albokhari, E.J. & Khoj, A. (2021) Taxonomic studies on some members of the Genus Abutilon Mill. (Malvaceae). *American Journal of Plant Sciences*, 12, 199–220.

Back, C., Lanouette, J.-G. & Aubin, A. (1979) Preliminary tests on the use of temephos for the control of black flies (Diptera: Simuliidae) in Northern Quebec. *Mosquito News*, 39, 6.

Becker, N. (1998) The use of bacillus thuringiensis subsp. Israelensis (*Bti*) against mosquitoes, with special emphasis on the ecological impact. *Israel Journal of Entomology*, 32, 63–69.

BOUSSINESQ, M. (1997) Prevalences of Loa loa microfilaraemia throughout the area endemic for the infection. *Annals of Tropical Medicine and Parasitology*, 91, 17.

Boussinesq, M., Fobi, G. & Kuesel, A.C. (2018) Alternative treatment strategies to accelerate the elimination of onchocerciasis. *International Health*, 10, i40–i48.

Brown, A.W.A. (1962) A survey of Simulium control in Africa. *Bulletin of the World Health Organization*, 27, 511–527.

Coffeng, L.E., Stolk, W.A., Honorat, G.M., Zouré, J.L., Veerman, K.B., Agblewonu, M.E. et al. (2013) African Programme for onchocerciasis control 1995–2015: model-estimated health impact and cost. *PLoS Neglected Tropical Diseases*, 7, e2032.

Davies, J.B. (1994) Sixty years of onchocerciasis vector control: a chronological summary with comments on eradication, reinvasion, and insecticide resistance. *Annual Review of Entomology*, 39, 23–45.

Djeunga, N., Clotaire, H., Domche, A., Niamsi, Y., Moungui, H., Walker, M. et al. (2020) Situation analysis of onchocerciasis in Cameroon: a protocol for systematic review of epidemiological studies and impact of disease control interventions. *Systematic Reviews*, 9, 1.

Guillet, P., Escaffre, H., Ouedraogo, M. & Quillévéré, D. (1980) Mise en évidence d'une résistance au téméphos dans le complexe Simulium damnosum (S. sanctipauli et S. soubrense) en Côte d'Ivoire (zone du programme de lutte contre l'onchocercose dans la région du bassin de la Volta). *Cahiers ORSTOM, Série Entomologie Médicale et Parasitologie*, 18, 291–299.

Guillet, P., Kurtak, D.C., Philippon, B. & Meyer, R. (1990) Use of bacillus thuringiensis israelensis for onchocerciasis control in West Africa. In: de Barjac, H. & Sutherland, D.J. (Eds.) *Bacterial control of Mosquitoes & Black Flies: biochemistry, Genetics & Applications of bacillus thuringiensis israelensis and Bacillus sphaericus*. Dordrecht: Springer Netherlands.

Hajibabaei, M., Janzen, D.H., Burns, J.M., Hallwachs, W. & Hebert, P.D. (2006) DNA barcodes distinguish species of tropical Lepidoptera. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 968–971.

Jamnback, H. (1962) An eclectic method of testing the effectiveness of chemicals in killing blackfly larvae (Simuliidae: Diptera). *Mosquito News*, 22, 6.

Kim, S.R., Kim, K.Y., Jeong, J.S., Kim, M.J., Kim, K.H., Choi, K.H. et al. (2017) Population genetic characterization of the Japanese oak silkmoth, Antheraea yamamai (Lepidoptera: Saturniidae), using novel microsatellite markers and mitochondrial DNA gene sequences. *Genetics and Molecular Research*, 16, 1–19.

Kononov, A., Ustyantsev, K., Wang, B., Mastro, V.C., Fet, V., Blinov, A. et al. (2016) Genetic diversity among eight Dendrolimus species in Eurasia (Lepidoptera: Lasiocampidae) inferred from mitochondrial COI and COII, and nuclear ITS2 markers. *BMC Genetics*, 17, 157.

Krueger, A. & Hennings, I. (2006) Molecular phylogenetics of blackflies of the Simulium damnosum complex and cytophylogenetic implications. *Molecular Phylogenetics and Evolution*, 39, 83–90.

Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, 35, 1547–1549.

Kumar, S., Stecher, G. & Tamura, K. (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33 7, 1870–1874.

Lakwo, T., Oguttu, D., Ukety, T., Post, R. & Bakajika, D. (2020) Onchocerciasis elimination: Progress and challenges. *Research and Reports in Tropical Medicine*, 11, 81–95.

Leveque, C., Fairhurst, C.P., Abban, K., Paugy, D., Curtis, M.S. & Traore, K. (1988) Onchocerciasis control programme in West Africa. *Chemosphere*, 17, 421–440.

Leveque, C., Odei, M. & Pugh, T.M. (1979) Ecological effects of pesticides. In: Perring, F.H. & Plellanby, K. (Eds.) *Linnean Society Symposium Series*, London: Academic Press, Vol. 5, p. 11.

Lima, J.B., Da-Cunha, M.P., Da Silva, R.C., Galardo, A.K., Soares Sda, S., Braga, I.A. et al. (2003) Resistance of Aedes aegypti to organophosphates in several municipalities in the state of Rio de Janeiro and Espírito Santo, Brazil. *The American Journal of Tropical Medicine and Hygiene*, 68, 329–333.

Nartey, R., Owusu-Dabo, E., Kruppa, T., Baffour-Awuah, S., Annan, A., Oppong, S. et al. (2013) Use of bacillus thuringiensis var israelensis as a viable option in an integrated malaria vector control Programme in the Kumasi Metropolis. *Parasites & Vectors*, 6, 116.

Olivry, J.C. (1986) Fleuves et Rivières du Cameroun. *Monographies Hydrologiques ORSTOM*, 9, 781.

Prakash, A., Walton, C., Bhattacharyya, D.R., Loughlin, S.O., Mohapatra, P. K. & Mahanta, J. (2006) Molecular characterization and species identification of the Anopheles dirus and an. Minimus complexes in north-East India using r-DNA ITS-2. *Acta Tropica*, 100, 156–161.

Remme, J.H.F., Ba Ouleye, K.Y., Dadzie, Y., & Karam, M. (1986) A force-of-infection model for onchocerciasis and its applications in the epidemiological evaluation of the onchocerciasis control Programme in the Volta River basin area. *Bulletin of the World Health Organization*, 64, 667–681.

Rubtsov, I.A. (1969) Short keys to the bloodsucking Simuliidae of the USSR. *Zoological Institute of the Academy of Sciences of the USSR*, 77, 228.

Rutschke, A.R. & Deschle, W. (1988) Untersuchungen zur Bekampfung der Simuliiden-Larven (Diptera) mit Bacillus thuringiensis H-14, Dissertation. 207.

Saitou, N. & Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4(4), 406–425.

Tamura, K. & Nei, M. (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, 10, 512–526.

Taufflieb, R. (1955) Campaign against Simulium damnosum in Mayo Kebbi. *Bull Soc Pathol Exot Filiales*, 48, 564–576.

Travis, B.V. & Wilton, D.P. (1965) A progress report obsimulated stream tests of blackfly larvicides. *Mosquito News*, 25, 7.

WHO. (1970) Insecticide resistance and vector control. Seventeenth report of the WHO expert committee on Insecticides'. *World Health Organization Technical Report Series*, 443, 1–279.

WHO. (1981a) Instructions for determining the Susceptibility or Resistance of Black fly Larvae to Insecticides, Man, WHO/VBC/81.811.

WHO. (1981b) Onchocerciasis Control Programme in the Volta River basin area, Progress report, OCP/PR/81.4. 33.

WHO. (1983) Protocol for Testing the Susceptibility of Larvae of Simulium damnosum s.l. to Insecticides as used in Vector Control Unit of the Onchocerciasis Control Programme (OCP), Man, WHO/VBC/81. 811.

WHO. (1995) Onchocerciasis and its control. Report of a WHO expert committee on onchocerciasis Control'. *World Health Organization Technical Report Series*, 852, 1–104.

WHO. (2022) Key facts of onchocerciasis.

How to cite this article: Kamtsap, P., Archile, P., Flore, N., Njiokou, F. & Renz, A. (2022) Testing the susceptibility of larval stages of *Simulium* to temephos and *Bacillus thuringiensis* var *israelensis* in Germany and Northern Cameroon. *Medical and Veterinary Entomology*, 1–14. Available from: https://doi.org/10.1111/mve.12630

APPENDIX A

TABLE A1 Bio-assay-data record sheet of temephos on *Simulium* larvae from Tübinge – Germany

Larvicide	Susceptibility of *Simulium* larvae to temephos in Tübingen - Germany													
	Date: 11.02.2020/12.02.2020/13.02.2020 larvae from: Weilerbach river; Sheet No:01; Observers: PK													
	Incubation before treatment		Larvicide (10 min contact)		Alive			Moribund			Dead			
Concentration ppm	Start time	End time	Start time	end time	0	3 h	6 h	0	3 h	6 h	0	3 h	6 h	
CONTROL	15 h00	16 h00	16 h00		50	50	50	00	00	00	00	00	00	
0.01	15 h00	16 h25	16 h25	16 h35	50	50	50	00	00	00	00	00	00	
0.02	15 h25	16 h45	16 h47	16 h57	50	50	50	00	00	00	00	00	00	
0.04	12 h00	13 h18	13 h18	13 h28	50	50	40	00	00	02	00	00	08	
0.08	12 h25	13 h35	13 h38	13 h48	50	50	22	00	00	06	00	00	22	
0.16	11 h35	12 h35	12 h35	12 h45	50	48	06	00	02	04	00	00	40	
0.32	14 h45	15 h45	15 h46	15 h56	50	44	00	00	00	08	00	06	42	
0.64	14 h50	15 h50	15 h50	16 h00	50	46	00	00	04	04	00	00	46	
1.28	14 h50	15 h54	15 h54	16 h06	50	36	00	00	00	00	00	12	50	
2.56	14 h55	16 h10	16 h13	16 h23	50	28	00	00	00	00	00	22	50	
5.20	15 h00	16 h20	16 h20	16 h30	50	02	00	00	00	00	00	48	50	
10.40	15 h20	16 h29	16 h29	16 h39	50	00	00	00	00	00	00	50	50	

Note: Incubation time means the time for acclimatization of the larvae in the beakers: Collected larvae are distributed into the beakers (50 larvae per well aerated beaker) and larvae are let to settle for about 1 hr. The time is noted. After 1 hr of incubation, the larvicide is added for 10 min, then the water and the insecticide are removed and replaced with fresh water from the breeding site.

TABLE A2 Bia-assay-data record sheet of *Bti* on *Simulium* larvae from Tübingen – Germany

Larvicide	PIIVeC data collection: Susceptibility of *Simulium* larvae to *Bti* in Tübingen - Germany														
	Date: 27.03.2020–28.03.2020 larvae from: Weilerbach river sheet; No: 02; Observers: PK														
	Incubation before treatment		Larvicide (10 min contact!)		Alive				Moribund			Dead			
Concentration ppm	Start time	End time	Start time	End time	0	3 h	6 h	12 h	3 h	6 h	12 h	3 h	6 h	12 h	
CONTROL	15 h30	16 h40	16 h41	16 h51	50	50	50		00	00	00	00	00	00	
0.02	15 h30	16 h40	16 h41	16 h51	50	50	50	48	00	00	00	00	00	02	
0.04	15 h30	16 h40	16 h42	16 h52	50	50	50	48	00	00	00	00	00	02	
0.08	15 h30	16 h40	16 h44	16 h54	50	49	47	46	00	00	00	01	03	04	
0.16	15 h30	16 h40	16 h46	16 h56	50	48	46	46	00	00	00	02	04	04	
0.32	15 h50	17 h00	17 h05	17 h15	50	49	49	44	00	00	00	01	01	06	
0.64	15 h50	17 h05	17 h08	17 h18	20	47	46	41	00	00	00	03	04	09	
1.28	15 h50	17 h10	17 h13	17 h26	50	46	44	32	00	00	00	04	06	18	
2.56	15 h55	17 h15	17 h18	17 h28	50	44	43	18	00	00	00	06	07	32	
5.20	16 h00	17 h20	17 h20	17 h30	50	34	33	00	00	00	00	16	18	50	
10.40	16 h05	17 h25	17 h25	17 h35	50	26	17	00	00	00	00	24	33	50	
20.80	16 h10	17 h30	17 h30	17 h40	50	18	00	00	00	00	00	32	50	50	

TABLE A3 Bio-assay-data record sheet of temephos on *Simulium* larvae from Lake Bini

Larvicide	Susceptibility of *Simulium* vector larvae to temephos in Northern Cameroon															
	Date: 09–15/Oct 2020 larvae from: Lac Bini sheet; No: 03; Observers: PK, DE,JY															
	Incubation before treatment		Larvicide (10 min contact!)		Alive				Moribund				Dead			
Concentration ppm	Start time	End time	Start time	end time	0	3 h	6 h	12 h	0	3 h	6 h	12 h	0	3 h	6 h	12 h
CONTROL	9 h55	10 h40	10 h40	10 h50	50	50	50	48	00	00	00	02	50	0	0	0
0.02	10 h10	10 h55	10 h55	11 h05	50	48	44	8	00	00	00	00	50	2	6	42
0.04	10 h15	10 h58	10 h58	11 h08	50	46	40	10	00	00	00	00	50	4	10	40
0.08	10 h40	11 h11	11 h11	11 h21	50	45	44	2	00	00	00	01	50	5	16	47
0.16	11 h00	11 h29	11 h29	11 h39	50	38	22	0	00	00	00	08	50	12	28	42
0.32	11 h10	11 h43	11 h43	11 h53	50	30	16	0	00	00	02	04	50	20	34	46
0.64	10 h00	10 h39	10 h39	10 h49	50	20	0	0	00	00	00	1	50	30	50	44
1.28	10 h05	10 h44	10 h44	10 h54	50	19	0	0	00	00	04	06	50	31	46	44
2.56	10 h10	10 h53	10 h53	11 h03	50	18	0	0	00	00	00	00	50	32	50	50
5.20	10 h20	10 h59	10 h59	11 h09	50	12	0	0	00	00	00	00	50	38	50	50
10.40	10 h30	11 h09	11 h09	11 h19	50	4	0	0	00	00	00	00	50	46	50	50

Note: Incubation time means the time for acclimatization of the larvae in the beakers: Collected larvae are distributed into the beakers (50 larvae per well aerated beaker) and larvae are let to settle for about 30 min. The time is noted. After about 30 min of incubation, the larvicide is added for 10 min, then the water and the insecticide are removed and replaced with fresh water from the breeding site.

microorganisms

MDPI

Article

Knowledge and Practices of Four Onchocerciasis-Endemic Communities in Cameroon

Kamtsap Pierre [1,2,*], Nguemaïm Ngoufo Flore [3], Paguem Archile [2,4,*] and Renz Alfons [1,2]

[1] Institute for Evolution and Ecology, Department of Comparative Zoology, University of Tübingen, Auf der Morgenstelle 28, 72076 Tübingen, Germany; alfons.renz@uni-tuebingen.de
[2] Programme Onchocercoses Field Station, University of Tübingen, Ngaoundéré Box 65, Cameroon
[3] Faculty of Health Sciences, University of Bamenda, Bambili P.O. Box 69, Cameroon; ngflorema@yahoo.fr
[4] Faculty of Agriculture and Veterinary Medicine, Department Veterinary Medicine, University of Buea, Buea P.O. Box 63, Cameroon
* Correspondence: kamtsapbuea200@gmail.com (K.P.); achillepaguem@yahoo.fr (P.A.)

Simple Summary: This study examines the knowledge and practices of four onchocerciasis-endemic communities in Cameroon regarding black flies and the disease. Onchocerciasis, or river blindness, is caused by *Onchocerca volvulus* and transmitted by *Simulium* black flies. Despite over 20 years of ivermectin distribution, no vector control has been implemented, leading to high black fly densities. A survey of 452 individuals from Mawong, Menchum, Soramboum, and Galim revealed significant knowledge gaps. While 90% recognized black flies as a nuisance, only 9.3% knew their bites could cause blindness, and many mistakenly associated them with malaria. About 34.1% correctly identified evening as the main biting period, but misconceptions about breeding sites and transmission were common. Most respondents were unaware of precautionary measures beyond wearing appropriate gear. Misconceptions differed by location, depending on the education level and occupation. The study emphasizes the need for better community education on vector ecology, disease transmission, and prevention. Integrating targeted health education with ivermectin delivery, as well as implementing vector control measures, could help to eliminate onchocerciasis. Future studies should broaden geographical scope and include qualitative methods to better understand community attitudes and improve intervention techniques.

Abstract: In onchocerciasis-endemic communities, black fly vectors of *Onchocerca volvulus* cause annoyance. No vector control was performed in Cameroon to complement ivermectin treatment despite high fly densities in the savannah and forest areas. This study assessed the knowledge and practices of four onchocerciasis-endemic communities in Sudan and the Guinea savannah in Cameroon. We surveyed four communities using a structured questionnaire. We interviewed 452 individuals (Mawong: 136, Befang: 160, Soramboum: 88, and Galim: 68) aged 14–50 years. Most respondents (90%) knew about black flies being a nuisance but had misconceptions about their bite's effect, location, and season. Only 9.3% knew that black fly bites could lead to blindness, whereas 34.1% correctly regarded evenings as the biting period. In Savannah, Sudan, 30.9% of the interviewees stated that black flies bite both outdoors and indoors, and 37.0% of the enrolees believed that black flies bite any exposed body part. In the Guinea savannah, 61.1% of respondents agreed that black flies typically bite during the dry season. Proper clothing can protect against black fly bites, but the transmission mode and vector biology are poorly understood. Implementing community-based vector surveillance can help fight onchocerciasis and reduce black fly nuisance.

Academic Editor: Sonia Almeria

Received: 20 January 2025
Revised: 18 February 2025
Accepted: 22 March 2025
Published: 25 March 2025

Citation: Pierre, K.; Flore, N.N.; Archile, P.; Alfons, R. Knowledge and Practices of Four Onchocerciasis-Endemic Communities in Cameroon. *Microorganisms* **2025**, *13*, 736. https://doi.org/10.3390/microorganisms13040736

Keywords: onchocerciasis; black fly; knowledge; practice; Cameroon; Befang; control; Soramboum; Mawong; Galim

1. Introduction

Onchocerciasis or 'river blindness' is a neglected tropical parasitic disease caused by *Onchocerca volvulus*, which is transmitted in Africa mainly by black fly members of the *Simulium damnosum* complex, which breed in rapid-flowing rivers and streams [1–3]. Adult female *O. volvulus* worms live in subcutaneous nodules for over a decade [1,4,5]. A female worm is capable of releasing 1300–1900 microfilariae per day during the intermittent periods of reproduction, and these microfilariae are mainly found in the skin and eyes [6,7], where they survive for months or even years, waiting for a female black fly to pick them up during its blood meal. The infection is responsible for severe itching and depigmentation of the skin, as it occurs in African rainforest areas, and profound impairment of visual acuity, often leading to irreversible blindness, which tends to predominate in African savannah [8–10].

Black flies of the *S. damnosum* complex are the main vectors of human onchocerciasis in Cameroon. At least four sub-species have been identified in the rainforest and savannah of Cameroon: *Simulium sirbanum*, a small pale species discovered only in the Sudan savannah; *S. damnosum sensu stricto*, savannah mainly, and now observed in 1986 in the forest near Kumba; *Simulium squamosum*, forest and Guinea savannah [11–13]. Several subspecies of *S. squamosum* have been identified, including dark species in forests and pale species in savannah. *Simulium mengense* occurs mostly in the forest; however, it has occasionally been observed in the savannah [11,14,15].

Onchocerciasis is caused by *Onchocerca volvulus*, a parasitic worm that lives for up to 14 years in the human body. It is spread through the bite of a blackfly of the *Simulium damnosum* species complex, which breeds in fast-flowing rivers and streams. When the fly bites, it deposits the larvae of the parasitic worm, which matures to adulthood and produces millions of tiny worms, called microfilaria. Adult's flies emerge after 8–12 days and live for up to four weeks, during which they can cover hundreds of kilometers in flight. Each adult female worm, thin but more than 0.5 m in length, produces millions of microfilariae (microscopic larvae) that migrate throughout the body. After mating, the female blackfly seeks a blood meal and may ingest microfilariae if the meal is taken from a person infected with onchocerciasis. A few of these microfilariae may transform into infective larvae within the blackfly, which are then injected into the person from whom the next meal is taken and subsequently develop into adult parasites, thus completing the life cycle of the parasite [16,17].

One of the most effective public health campaigns for managing onchocerciasis is mass distribution (MDA) of ivermectin. By lowering the microfilarial load in the skin, ivermectin reduces both the degree of clinical symptoms—such as severe itching, skin depigmentation, and eye lesions and the possibility of transmission by black flies. Although MDA programs show remarkable effectiveness, community involvement and compliance are absolutely crucial for their success. Several studies have noted that even after years of mass ivermectin distribution, local populations may still have significant gaps in their understanding of the disease and its vectors [18,19]. For example, many communities in endemic areas continue to misattribute black fly bites to other conditions like malaria, or they may lack knowledge about the specific behavior and breeding sites of these vectors [20]. This disconnect between biomedical intervention and local perceptions can hinder the overall effectiveness of MDA efforts. An inadequate understanding of the vector's role means that community members

might not fully appreciate the importance of consistent drug uptake, nor might they adopt complementary practices that reduce exposure to black flies. Therefore, integrating targeted health education with MDA programs is crucial. Educational campaigns that explain the life cycle of *Onchocerca volvulus*, the role of black flies in its transmission, and the benefits of ivermectin can help bridge the gap between biomedical strategies and local knowledge. In turn, better-informed communities are more likely to participate fully in MDA and support additional vector control measures, ultimately contributing to a more sustainable reduction in onchocerciasis transmission.

Related filarial parasites may be present in cattle and other livestock in endemic areas in addition to humans. Cattle parasite *Onchocerca ochengi* is one well-researched example [21,22]. The same black fly vectors that transport *O. volvulus* also carry *O. ochengi*, despite the fact that the latter does not infect humans. Our knowledge of parasite development, vector competence, and host immune responses has improved as a result of comparative investigations between these parasites. Black flies' eating habits can also be impacted by the presence of livestock in endemic areas. In certain situations, cattle may act as substitute hosts (a process called zooprophylaxis), which could deter flies from attacking people. This relationship is complicated, though, because in some ecological contexts, cattle may actually boost local black fly abundance by providing more blood supplies, which could raise the likelihood of transmission overall.

In 2016, we performed a study in the northwest region of Cameroon where 1491 *Simulium damnosum s.l.* flies were captured during 6 days of catching from two catching sites: one in Mawong village near river Mawong, a tributary of the Menchum River, and another at the village of Befang near the Menchum River at the sand-digging site close to the Menchum Falls. Of the 558 parous flies dissected, 9.50% were infected with *Onchocerca* larvae (Table 1).

Table 1. Number of flies dissected by site and infection rate.

Capture Site	Number of Parous Flies Dissected (%)	Number of Flies Carrying *Onchocerca* Larvae (% of Parous Flies Infected)			
		L1	L2	L3	
				O. v.	***O. o.***
Befang	520 (61.3)	31 (5.96)	9 (1.73)	6 (1.15)	1 (0.19)
Mawong	38 (16.5)	2 (5.26)	0 (0.0)	4 (10.53)	0 (0.0)
Total	558 (51.8)	33 (5.91)	9 (1.61)	10 (1.79)	1 (0.18)

L1: number of parous flies carrying *Onchocerca* larvae stage 1 found; L2: carrying *Onchocerca* lavae stage 2; L3: carrying *Onchocerca* stage 3 (infective stage larvae); *O. v.*: *Onchocerca volvulus*; *O. o.*: *Onchocerca ochengi*.

In 2016, the annual biting rates at the savannah sites were approximately 16,000 flies/man/year at Soramboum and 13,500 flies/man/year at Galim [21]. In 21,897 flies dissected from these two catching sites, only a few infective stage larvae of *O. volvulus* were observed in Soramboum, whereas no cases were reported in Galim [21].

The surveillance of the infectivity status of *Simulium* vectors biting people along river systems is vital for monitoring the transmission of onchocerciasis [7,22]. Understanding community perceptions and concerns related to onchocerciasis vectors is essential for the global elimination of the disease [18,23]. Community involvement may be a key component of onchocerciasis control activities, especially in the pre-elimination stages of vector control. To achieve community participation and design socially and locally acceptable control strategies, health program planners and implementers need to be familiar with the knowledge of local people and their attitudes toward onchocerciasis [24].

Much attention has been paid to the mass administration of ivermectin; however, insufficient information is available concerning the knowledge and perception of local populations regarding onchocerciasis vectors in Cameroon. Therefore, we investigated the knowledge and practice of black fly vectors of onchocerciasis in people living along the Menchum Valley, Galim in the Guinea savannah, and Soramboum in the Sudan savannah in Cameroon after more than 20 years of mass drug distribution to optimize the control strategy.

2. Methods

2.1. Description of Study Areas and Populations

A community-based cross-sectional survey was conducted in four localities in Cameroon: Befang near the river Menchum (6°18′25″ N, 10°01′01″ E) and Mawong near its tributary Mawong (6°19′26″ N, 10°00′13″ E) in the Northwest Region, Soramboum near the river Vina du Nord (7°47′14″ N, 15°0′22″ E) in the North region, and Galim close to the Mayo Djouroum, a tributary of the river Vina du Sud (7°12′10″ N, 13°35′46″ E) in the Adamawa region (Figure 1). These localities are crossed by, or located near, fast-flowing rivers, which serve as breeding sites for black fly vectors of onchocerciasis. Humans mainly live in livestock farming, agriculture, and sand collection areas.

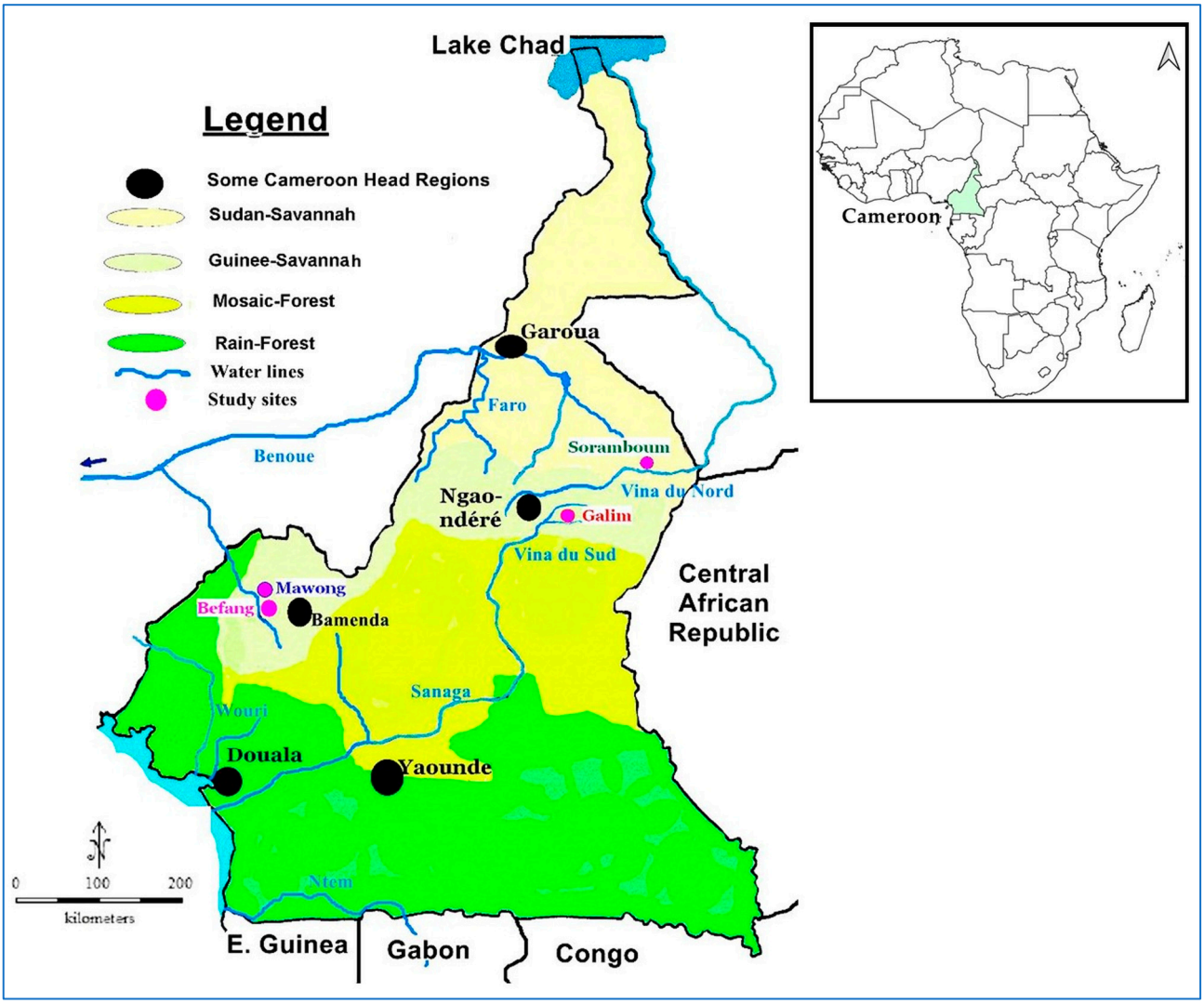


Figure 1. Map of Cameroon showing the study areas.

The Menchum Valley subdivision has a total area of 4469 square km, 48 inhabitants/km^2 with a growth rate of 3% per year, and as of 2005, a total population of 161,998 [25]. The Menchum River drains this area west into Nigeria and joins the Benue River. Generally, tropical grassland vegetation is composed of spear grass with non-uniform soil. Numerous fast-running streams merge with the Menchum River, creating a dendritic network that forms the main tributary of Katsina-Ala in Nigeria. The climate is warm and moist, with heavy rains and high temperatures reaching 37 °C between March and April, as well as hot days and cold nights [25].

Soramboum, a former hyperendemic village for onchocerciasis, is situated close to the Vina du Nord River in the Sudan savannah and has been the site of ivermectin mass treatments conducted since 1987 [26]. Today, the village has approximately 1500 inhabitants (personal observation). The Vina du Nord River flows perennially, with a maximum water discharge of ca. 500 m^3 per second during the height of the rainy season and 5 m^3/s at the end of the dry season (at Touboro, ca. 50 km downstream of Soramboum) [27].

The village of Galim is located on the Adamawa Plateau in a Guinea savannah region 15 km southwest of Ngaoundéré, approximately 3 km from the large river Vina du Sud (mean annual discharge 37 m^3/sec, 1050 m altitude) [27]. The Adamawa Plateau is necessary for livestock production in Cameroon, and agriculture is the main economic activity practiced by the inhabitants.

Simulium damnosum sensu stricto and *S. sirbanum* are the predominant vector species in the Vina du Nord River, whereas *S. squamosum* is the predominant vector species in the Vina du Sud River [28,29].

2.2. Sampling

2.2.1. Calculation of Sample Size and Selection of Recruitment Sites

Insufficient information is available concerning the level of community knowledge and practices regarding onchocerciasis along the Menchum Valley and in the other localities visited. We hypothesized that at least 50% of the population in the target area would not have a good level of knowledge regarding the transmission of onchocerciasis. Accordingly, the sample size was considered to have a 95% confidence level, a 5% margin of error, and 50% accuracy. By assuming maximum variability (50%), we ensured that our sample size would be large enough to provide reliable and precise estimates of the population's knowledge and practices regarding onchocerciasis. We then came out with n = 452.

These communities were selected because of the observed symptoms of onchocerciasis (Figure 2) and the geographical characteristics of these areas, which are savannah-type drained by rivers favorable for the development of black flies. The main activities of the residents are agriculture, sand digging, and cattle and sheep rearing, which are also favorable for black fly activities.

2.2.2. Community Mobilization and Data Collection

Structured questionnaires (Supplementary Table S1) were prepared in English and French and thoroughly explained in the relevant local language. Participants in Menchum Valley were interviewed in 'Pidjin', an English dialect mainly used in the villages of Befang and Mawong. In Galim and Soramboum, Fulfulde was used in the northern part of Cameroon with the aid of local guides.

Information concerning sociodemographic characteristics (including age, sex, occupation, body shape, and educational level), risk factors for the transmission of onchocerciasis, and variables related to participants' knowledge and practices were included in the questionnaires. Age was grouped into intervals of 10 years. The questions were closed, and the participants included in this study were born or had lived in the study areas for several

years. Residents who refused to answer all the questions or did not understand the questions despite explanations were excluded, as were immigrants from other countries and non-residents within the household.

Figure 2. Personal observation during preliminary entomological survey in 2015: Leopard skin seen at the sand digging pool near the Menchum fall.

2.2.3. Ethical Approval

Ethical clearance and approval were obtained from the Institutional Review Board of the University of Douala (CEIUD/371/01/2016/M). The administrative authorization was obtained from the public health authorities of Cameroon. Informed consent was obtained from all voluntarily involved individuals after they received detailed explanations of the study in their local language. Each participant agreed verbally, and participants aged < 18 years participated only when their parents agreed to participation.

2.2.4. Statistical Analysis

Recorded data were transferred to SigmatPlot version 15.0 and analyzed according to the study objectives. Chi-square tests were used to compare categorical variables (sex, occupation, and knowledge of the different villages). Frequencies and percentages were used to summarize the data. Continuous variables (age) are described as the median and interquartile range (IQR).

3. Results

3.1. Sociodemographic Characteristics of Study Participants

A total of 452 individuals were included: 136 from Mawong Village near Mawong, 160 from Befang near Menchum, 88 from Soramboum near Vina du Nord, and 68 from Galim near Mayo Djouroum. More males (67.7%) (the majority coming from Befang) participated in the study than females (Table 2).

The age of the respondents ranged from 14 to 50 years. In Befang, 65.0% of the participants were under 20 years old, whereas 36.4% of the participants in Soramboum were between 40 and 50 years old, with the lowest quartile being 25 years old, the upper quartile being 45 years old, the IQR being 20 years old, and the majority falling within the age group under 20 years. In contrast, sand dredging was prominent in Befang (58.8%) but absent elsewhere, and Galim displayed greater occupational diversity, including motorcycle cab drivers, shepherds, and builders.

Table 2. Sociodemographic characteristics of the study populations.

		Guinea Savannah		Sudan Savannah		*p*-Value
	Variables	**Mawong N (%)**	**Befang N (%)**	**Galim N (%)**	**Soramboum N (%)**	
Sex	Male	80 (58.8)	136 (85.0)	44 (64.7)	46 (52.3)	
	Female	56 (41.2)	24 (15.0)	24 (35.3)	42 (47.7)	<0.001
Age group	Less than 20	16 (11.8)	104 (65.0)	6 (8.8)	14 (15.9)	
	20 to 30	40 (29.4)	32 (20.0)	28 (41.2)	30 (34.1)	
	30 to 40	48 (35.3)	16 (10.0)	10 (14.7)	12 (13.6)	0.002
	40 to 50	32 (23.5)	8 (5.0)	24 (35.3)	32 (36.4)	
Occupation	School children	30 (22.1)	16 (10)	0 (0.0)	0 (0.0)	
	Sand dredging	2 (1.5)	94 (58.8)	0 (0.0)	0 (0.0)	
	Students	4 (2.9)	0 (0.0)	0 (0.0)	0 (0.0)	
	Farmers	56 (41.2)	32 (20.0)	20 (29.4)	80 (90.9)	
	Housekeeper	44 (32.3)	18 (11.3)	18 (26.5)	2 (2.3)	<0.001
	Carpenter	0 (0.0)	0 (0.0)	2 (2.9)	0 (0.0)	
	Motorcycle cab	0 (0.0)	0 (0.0)	8 (11.8)	0 (0.0)	
	Builder	0 (0.0)	0 (0.0)	2 (2.9)	0 (0.0)	
	Shepherd	0 (0.0)	0 (0.0)	10 (14.7)	3 (3.4)	
	Seamstress	0 (0.0)	0 (0.0)	2 (2.9)	0 (0.0)	
	Shopkeeper	0 (0.0)	0 (0.0)	2 (2.9)	0 (0.0)	
	Retired	0 (0.0)	0 (0.0)	2 (2.9)	3 (3.4)	
	Health worker	0 (0.0)	0 (0.0)	2 (2.9)	0 (0.0)	
Educational Status	Primary school	72 (52.9)	80 (50.0)	28 (41.2)	46 (52.3)	
	Secondary school	48 (35.3)	80 (50.0)	2 (2.9)	14 (15.9)	
	University	16 (11.8)	0 (0.0)	0 (0.0)	0 (0.0)	<0.0001
	Did not attend school	0 (0.0)	0 (0.0)	38 (55.9)	28 (31.8)	

%: percentage. The difference in the proportion of males and females between the Guinea Savannah and Soudan Savannah regions is statistically significant. The null hypothesis (H_0) for the table assumes that there is no significant difference in the distribution of demographic characteristics (sex, age group, occupation, and educational status) across the four locations (Mawong, Befang, Galim, and Soramboum). Since the *p*-values are all < 0.001, the null hypothesis is likely rejected for all these variables, meaning that there are significant differences in sociodemographic characteristics between the locations.

A chi-square test of independence was performed to examine the difference between the localities and the capability of respondents with knowledge of the vectors of onchocerciasis based on educational level. This educational levels varied markedly; Mawong and Befang had higher primary and secondary school attendance, whereas Galim and Soramboum had notable rates of illiteracy (55.9% and 31.8%, respectively). In Mawong, 16 individuals (11.8%) had attended university, while no university attendance was reported in the other sites. (Table 2). The difference between the variables was statistically significant (chi-square = 220.91, *p*-value < 0.001). A significant difference was observed among the age groups, in which participants less than 20 years old were from Menchum and were mostly sand diggers (chi-square = 467.06, *p*-value < 0.001; Table 2).

3.2. Knowledge Practice of Community Respondents Regarding Biting Activities of Black Flies

All respondents had some knowledge of the vector of onchocerciasis, with 34.1% assuming that it bites in the evening. However, 0.4% had yet to learn when it was usually a bite. Of the participants from Mawong, Befang, Galim, and Soramboum, 17.6%, 30.0%, 5.9%, and 11.4%, respectively, were confident that black flies would bite in the morning. Regarding the effect of bites on humans, 70.0% of Menchum and 64.7% of Mawong reported that black fly bites caused malaria, whereas 11.8% of Mawong reported that black fly bites caused blindness (Table 3).

Table 3. Comprehensive knowledge of community respondents regarding the biting activities of black flies.

		Guinea Savannah		Sudan Savannah		
	Variables	**Mawong N (%)**	**Befang N (%)**	**Galim N (%)**	**Soramboum N (%)**	***p*-Value**
Biting period	Morning	24 (17.6)	48 (30.0)	4 (5.9)	10 (11.4)	<0.001
	Afternoon	8 (5.9)	8 (5.0)	18 (26.5)	30 (34.1)	
	Evening	48 (35.3)	64 (40.0)	28 (41.2)	14 (15.9)	
	Anytime	56 (41.2)	40 (25.0)	18 (26.5)	32 (36.4)	
	No idea	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.3)	
Biting season	Dry season	88 (64.7)	144 (90.0)	24 (35.3)	20 (22.7)	<0.001
	Rainy season	32 (23.5)	16 (10.0)	42 (61.8)	22 (25.0)	
	Both	16 (11.8)	0 (0.0)	2 (2.9)	46 (52.3)	
Preferred biting site	River	48 (35.3)	120 (75.0)	8 (11.8)	24 (27.3)	<0.001
	Farms	56 (41.2)	24 (15.0)	18 (26.5)	26 (29.5)	
	House	0 (0.0)	8 (5.0)	6 (8.8)	4 (4.5)	
	Any place	32 (23.5)	8 (5.0)	36 (52.9)	34 (38.6)	
Breeding sites	Stagnant water	64 (47.1)	40 (25.0)	0 (0.0)	2 (2.3)	<0.001
	Tree holes	24 (17.6)	88 (55.0)	6 (8.8)	4 (4.5)	
	fast flowing water	40 (29.4)	0 (0.0)	2 (2.9)	36 (40.9)	
	Grass	0 (0.0)	0 (0.0)	2 (2.9)	0 (0.0)	
	Not known	8 (5.9)	32 (20.0)	30 (44.1)	6 (6.8)	
	Falls	0 (0.0)	0 (0.0)	28 (41.2)	40 (45.5)	
Preferred biting parts	Leg	48 (35.3)	152 (95.0)	0 (0.0)	4 (4.5)	<0.001
	Face	0 (0.0)	0 (0.0)	24 (35.3)	0(0.0)	
	Hands	0 (0.0)	0 (0.0)	4 (5.9)	4 (4.5)	
	Any exposed part	88 (64.7)	8 (5.0)	40 (58.8)	80 (91.0)	
Effect of fly bite	Blindness	16 (11.8)	0 (0.0)	4 (5.9)	22 (25.0)	<0.001
	Malaria	88 (64.7)	112 (70.0)	12 (17.6)	22 (25.0)	
	Pruritus	32 (23.5)	48 (30.0)	20 (29.4)	42 (47.7)	
	Tuberculosis	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.3)	
	No idea	0 (0.0)	0 (0.0)	32 (47.1)	0 (0.0)	

The null hypothesis (H_0) for this table states that there is no significant difference in the reported perceptions and experiences of fly-biting behaviors, breeding sites, and health effects across the four locations (Mawong, Befang, Galim, and Soramboum). Since the table shows *p*-values < 0.001 for all variables, we can conclude that the null hypothesis is rejected, indicating that the reported fly-biting patterns, breeding sites, and health effects differ significantly across the four locations.

More than 44% of the study participants thought that most black fly bites occurred near rivers, and 27.4% believed that farms (not close to rivers) were the principal areas in which black flies predominantly occurred. Some respondents (24.3%) answered that black flies bite throughout the region, and 4.0% claimed that they bite within houses. Most participants (75.0%) from Befang affirmed that flies always bite along rivers, while 41.2% from Mawong believed that farms were the preferred places where black flies were observed. In Soramboum, 2.3% had no idea of the times at which bites mostly occurred; however, 52.3% assumed that black flies bite during both the dry and rainy seasons, and 40.9% affirmed that black flies breed in fast-flowing water. In contrast, 41.2% of participants

from Galim thought that black flies bite in the evening, 29.4% agreed that bites cause pruritus, and 26.5% assumed that black flies usually bite on farms. Generally, black flies were thought to bite at any time by 32.3%, in the morning by 19.0%, in the evening by 34.1%, and in the afternoon by 14.2% (Table 3). A significant difference was observed between our study areas (chi-square = 93.815, *p*-value < 0.001), where 10.6% of Befang reported that black flies acted in the morning and only 0.9% in Galim. However, 4.9% of Soramboum and 0.0% of Menchum reported that black fly bites could lead to blindness.

3.3. Knowledge of Community Respondents About Attractants to Black Flies

Four colors (white, black, red, and mixed) were included in the questionnaire. Of the respondents from Befang, 40.0% said that red was the most attractive color for flies, whereas 58.8% in Mawong considered all colors to attract black flies independently (Table 4).

Table 4. Proportions of respondents regarding biting preferences of black flies.

			Guinea Savannah		Sudan Savannah	
	Variables	**Mawong N (%)**	**Befang N (%)**	**Galim N (%)**	**Soramboum N (%)**	***p*-Value**
Dress colour	White	32 (23.5)	24 (15.0)	12 (17.6)	14 (15.9)	<0.001
	Black	24 (17.6)	24 (15.0)	34 (50.0)	42 (47.7)	
	Red	0 (0.0)	64 (40.0)	4 (5.9)	6 (6.8)	
	All colours	80 (58.8)	48 (30.0)	18 (26.5)	26 (29.5)	
Body size	Fat	16 (11.8)	16 (10.0)	17 (25.0)	24 (27.3)	<0.001
	Slim	8 (5.9)	8 (5.0)	2 (2.9)	12 (13.6)	
	All	88 (64.7)	136 (85.0)	49 (72.1)	48 (54.5)	
	Not known	24 (17.6)	0 (0.0)	0 (0.0)	4 (4.5)	
Height	Short	0 (0.0)	0 (0.0)	108 (14.7)	10 (11.4)	<0.001
	Tall	8 (5.9)	16 (10.0)	6 (8.8)	14 (15.9)	
	All	104 (76.5)	144 (90.0)	38 (55.9)	62 (70.5)	
	Not known	24 (17.6)	0 (0.0)	14 (20.6)	2 (2.3)	

In Soramboum and Galim, 54.5% and 72.1% of the participants thought that black flies bite independently of their body size, respectively. Regarding height, 8.8% of people in Galim believed that black flies mainly bite tall people, 76.5% in Mawong, 90.0% in Befang, and 70.5% in Soramboum, assuming that hosts are affected independently of their size and irrespective of their height. In Galim, 3.1% had no idea whether tall or short people were bitten.

3.4. Consolidation of All Data from the Investigated Regions

Below is a heatmap (Figure 3) consolidating the view of knowledge about onchocerciasis across four investigated regions. The data include responses to key factors related to blackfly behavior, breeding sites, and the effects of fly bites.

Befang has the strong awareness of blackfly activity in dry seasons and near rivers, but misconceptions about breeding sites. Galim recognizes fast-flowing water as a breeding site, which is correct. Soramboum has a more balanced understanding of seasons, preferred sites, and the effects of bites, but lacks specific knowledge on breeding. Mawong has some knowledge of biting behavior but incorrectly associates blackflies with malaria.

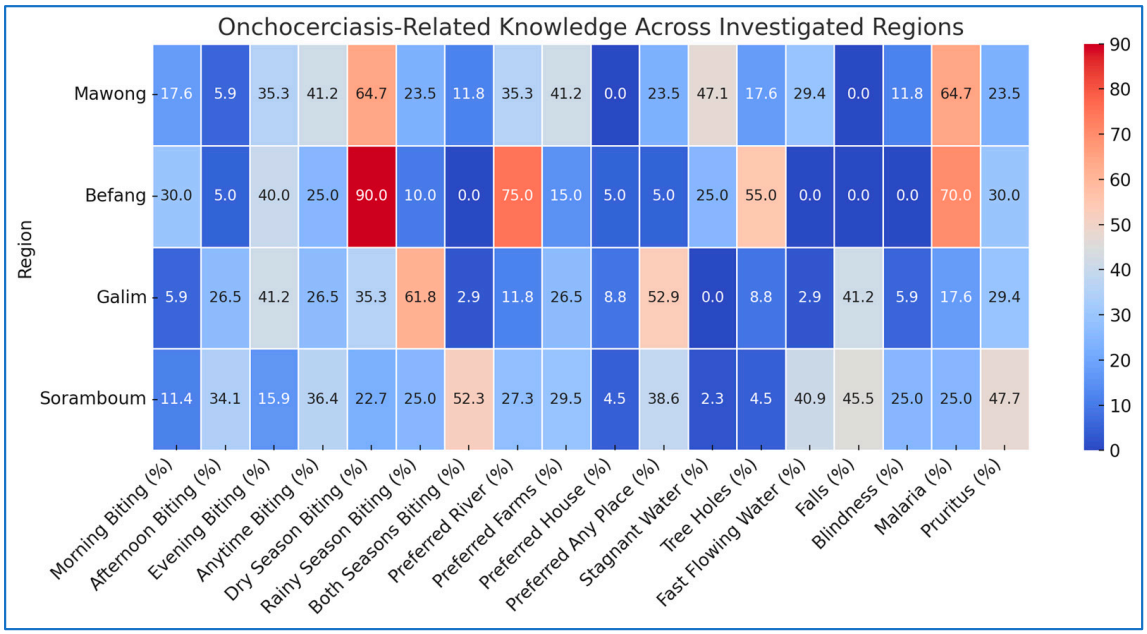


Figure 3. Heatmap of onchocerciasis-related knowledge across investigated regions. Red areas represent higher percentages, meaning a greater proportion of respondents in that region gave a specific answer; blue areas indicate lower percentages, suggesting fewer people identified that particular response; white/light shades represent intermediate values.

4. Discussion

In this study, we assessed the knowledge and practices in three localities in the Guinea savannah and one in the Sudan savannah. Our findings provide a scientifically relevant assessment of the knowledge and practices related to onchocerciasis along the Menchum Valley after 20 years of mass drug distribution and in two other localities (Galim and Soramboum) after 25–35 years of distribution [21]. The obtained data are crucial for evaluating the long-term impact of control strategies and identifying persistent knowledge gaps that may hinder disease elimination efforts.

A large proportion of residents were familiar with black flies, affirming their presence along riversides and corroborating established scientific findings that individuals working near rivers are at a heightened risk of *Simulium* biting and onchocerciasis transmission [30]. Our study also provides new insights into the community's knowledge about black flies, an essential factor in designing culturally appropriate educational interventions. However, despite this familiarity with the vector, our study revealed significant deficiencies in community knowledge regarding disease causation, transmission, and prevention.

Only 9.2% of respondents correctly identified onchocerciasis as a consequence of black fly bites, while more than half mistakenly associated it with malaria. This highlights a major gap in disease awareness, potentially undermining prevention efforts and treatment adherence. Previous studies in African countries have documented variations in knowledge about onchocerciasis manifestations, with communities in high-prevalence areas demonstrating better awareness [24–32]. Our findings suggest that knowledge gaps persist despite decades of intervention, reinforcing the necessity of targeted educational campaigns. Notably, misconceptions about the disease were prevalent, with some respondents erroneously linking black fly bites to tuberculosis, and only 9.3% correctly identifying blindness as a major consequence of infection. This underscores the critical need for improved health education to enhance disease recognition and encourage early intervention.

A significant scientific contribution of this study is the documentation of persistent misconceptions regarding vector ecology. Only 17% of participants correctly identified

fast-flowing rivers and streams as black fly breeding sites. This lack of knowledge presents a potential risk for ongoing transmission, as individuals may unknowingly engage in high-risk activities near these breeding grounds. Consistently with earlier reports from Cameroon and other African countries [20,33,34], some respondents (ranging from 4.5% in Soramboum to 55.0% in Menchum) incorrectly attributed black fly breeding to tree holes. These findings are scientifically relevant as they highlight an urgent need for community education on vector ecology to enhance control measures and reduce exposure.

The study also established a strong correlation between educational attainment and disease awareness, with those having higher levels of education demonstrating better knowledge and practices. In our study, 14.6% of participants were illiterate (primarily from Galim and Soramboum), and up to 50% had only attended primary school. This finding aligns with experience from Southwest Ethiopia, where illiteracy was also linked to poor knowledge of onchocerciasis [35]. The Vina du Nord Valley, where Soramboum is located, is one of the most isolated areas in the country, limiting access to education and health information. This geographic and socioeconomic barrier may contribute to the observed knowledge gaps and reinforces the importance of tailored educational interventions in remote communities.

Another key scientific insight from our study relates to seasonal variations in vector activity and community perceptions. While 61.1% of respondents correctly associated increased biting rates with the dry season, a discrepancy exists between this perception and previous studies in the savannah, which reported high monthly biting rates during the rainy season [36,37]. These discrepancies highlight the need for further entomological studies to refine vector control strategies based on local transmission patterns.

Our study also uncovered a lack of awareness regarding black fly attraction behavior, with more than 18% of respondents believing that white clothing attracts black flies. This finding is scientifically relevant as it informs community-based vector control strategies, such as personal protective measures [38,39].

The broader implication of our findings is that inadequate knowledge of onchocerciasis and its vector may compromise control efforts. Effective vector control campaigns must integrate scientific knowledge dissemination to ensure community engagement and behavioral change. Previous research has demonstrated that educational interventions significantly enhance knowledge and adherence to control measures [40,41]. Our study reinforces this by showing that persistent misinformation and inadequate awareness may contribute to the continued transmission of onchocerciasis despite decades of mass drug administration.

Public health interventions could use these data to target misconceptions (e.g., correcting malaria-blackfly confusion). Community education programs could focus on the role of fast-flowing water in breeding and pruritus/blindness as key symptoms. Prevention strategies (e.g., riverbank vegetation management, protective clothing) can be adapted based on where people believe blackflies are most active.

While this study provides valuable scientific insights, it has limitations. The absence of qualitative methods such as focus group discussions and epidemiological investigations limits the depth of understanding of community perceptions. Additionally, the study was conducted in only four rural communities, making it difficult to generalize the findings to all Cameroonian localities. Nevertheless, the data collection was rigorous, and the questionnaire was designed for clarity and reliability. Despite potential limitations in response options, our findings provide crucial evidence for guiding future intervention strategies.

In conclusion, our study highlights key deficiencies in knowledge and practices related to onchocerciasis, which may affect the effectiveness of mass drug distribution programs. Strengthening community awareness through targeted health education campaigns is essential to improving disease control efforts. Future research should expand to all affected

localities and incorporate the operational aspects of drug distribution to enhance the effectiveness of onchocerciasis elimination programs. The scientific significance of this study lies in its contribution to understanding long-term community knowledge trends, informing evidence-based policy decisions, and optimizing intervention strategies for sustainable disease control.

5. Conclusions

Ultimately, this study offers significant fresh viewpoints on the knowledge and practices about *Simulium* black fly vector of onchocerciasis in Cameroonian endemic populations. Even with long-term mass ivermectin distribution, community knowledge of the disease's spread still lags greatly, especially with regard to the part black fly vectors play. Although the majority of participants perceive the presence of black flies, many have misconceptions about the implications of their bites—for example, only a small percentage associate black fly bites with blindness, whereas a large number incorrectly correlate them with malaria. Additionally, the data show statistically significant regional variations in knowledge and practices, which are influenced by sociodemographic variables as age, occupation, and educational attainment. Misconceptions about vector ecology and disease transmission were especially noticeable in areas with lower literacy rates and less access to health education. These differences highlight the need for specialized, community-based educational initiatives that enhance vector surveillance, correct local misconceptions, and supplement current ivermectin distribution programs.

In general, effective onchocerciasis control and eventual elimination in these areas depend on raising public knowledge and dispelling myths through focused communication tactics. To improve these intervention techniques even more, future studies should include qualitative approaches and a wider geographic focus.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/microorganisms13040736/s1, Table S1. Structured questionnaire.

Author Contributions: Conceptualization, K.P.; methodology, K.P.; software, K.P. and P.A.; validation, K.P., P.A., N.N.F. and R.A.; formal analysis, K.P.; investigation, K.P. and P.A.; resources, K.P., N.N.F. and R.A.; data curation, K.P.; writing—original draft preparation, K.P.; writing—review and editing, K.P., P.A., N.N.F. and R.A.; project administration, R.A. and N.N.F.; funding acquisition, K.P., N.N.F. and R.A. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by a DFG-COBE grant (RE-1536/ff) and the Programme Onchocercoses Laboratory in Ngaoundéré-Cameroon. We acknowledge support from the Open Access Publication Fund of the University of Tübingen.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of the University of Douala (CEIUD/371/01/2016/M).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The structured questionnaire is shown in Supplementary Table S1. All the data are available upon request.

Acknowledgments: The authors thank the local communities in the study area for their interest in and cooperation with this study. We thank the German Research Foundation DFG through the COBE (Can Onchocerciasis Be Eliminated?) project (DFG RE-1536/ff) and its local laboratory at the University of Bamenda.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Gebrezgabiher, G.; Yirga, D.; Abreha, A. Reaching the Last Mile: Main Challenges Relating to and Recommendations to Accelerate Onchocerciasis Elimination in Africa. *Infect. Dis. Poverty* **2019**, *8*, 60. [CrossRef] [PubMed]
2. Taylor, H.R.; Duke, B.O.; Muñoz, B. The Selection of Communities for Treatment of Onchocerciasis with Ivermectin. *Trop. Med. Parasitol.* **1992**, *43*, 267–270.
3. Ozoh, G.A.; Boussinesq, M.; Boatin, B.A.; Remme, J.H.F.; Sékétéli, A. The African Programme for Onchocerciasis Control: Impact on Onchocercal Skin Disease. *Trop. Med. Int. Health* **2011**, *16*, 875–883. [CrossRef] [PubMed]
4. Schulz-Key, H.; Karam, M.; Awadzi, K.; Albiez, E.J. Efficacy of Ivermectin in the Treatment of Concomitant *Mansonella perstans* Infections in Onchocerciasis Patients. *Trans. R. Soc. Trop. Med. Hyg.* **1993**, *87*, 227–229. [CrossRef] [PubMed]
5. Noma, M.; Zouré, H.G.M.; Tekle, A.H.; Enyong, P.; Nwoke, B.E.B.; Remme, J.H.F. The Geographic Distribution of Onchocerciasis in the 20 Participating Countries of the African Programme for Onchocerciasis Control: (1) Priority Areas for Ivermectin Treatment. *Parasites Vectors* **2014**, *7*, 325. [CrossRef]
6. Duke, B.O. The Population Dynamics of *Onchocerca volvulus* in the Human Host. *Trop. Med. Parasitol.* **1993**, *44*, 61–67.
7. Opara, K.N.; Fagbemi, B.O. Population Dynamics of *Onchocerca volvulus* Microfilariae in Human Host after Six Years of Drug Control. *J. Vector Borne Dis.* **2008**, *45*, 29–37.
8. Duke, B.O.; Anderson, J.; Fuglsang, H. The *Onchocerca volvulus* Transmission Potentials and Associated Patterns of Onchocerciasis at Four Cameroon Sudan-Savanna Villages. *Trop. Med. Parasitol.* **1975**, *26*, 143–154.
9. Anderson, J.; Fuglsang, H.; Duke, B.O. Studies on Onchocerciasis in the United Cameroon Republic. IV. A Four-Year Follow-Up of Six Rain-Forest and Six Savanna Villages. The Incidence of Ocular Lesions. *Trans. R. Soc. Trop. Med. Hyg.* **1978**, *72*, 513–515. [CrossRef]
10. Cheke, R.A.; Garms, R. Indices of Onchocerciasis Transmission by Different Members of the *Simulium damnosum* Complex Conflict with the Paradigm of Forest and Savanna Parasite Strains. *Acta Trop.* **2013**, *125*, 43–52. [CrossRef] [PubMed]
11. Mustapha, M.; Post, R.J.; Krüger, A. The Cytotaxonomy and Morphotaxonomy of *Simulium mengense* (Diptera: Simuliidae). *Ann. Trop. Med. Parasitol.* **2004**, *98*, 509–523. [CrossRef] [PubMed]
12. Hendy, A.; Slatko, B.E.; Steen, R.G.; Brindley, P.J.; Wanji, S.; Tchioffo, M.T.; Makepeace, B.L.; Kengne-Ouafo, J.A. *Onchocerca volvulus* Transmission in the Mbam Valley of Cameroon Following 16 Years of Annual Community-Directed Treatment with Ivermectin, and the Description of a New Cytotype of *Simulium squamosum*. *Parasites Vectors* **2021**, *14*, 563. [CrossRef]
13. Mustapha, M.; Cheke, R.A.; Boakye, D.A.; Post, R.J. A New Cytotype of *Simulium squamosum* from South-West Cameroon. *Med. Vet. Entomol.* **2004**, *18*, 296–300. [CrossRef] [PubMed]
14. Post, R.J.; Cheke, R.A.; Boakye, D.A. A Guide to the *Simulium damnosum* Complex (Diptera: Simuliidae) in Nigeria, with a Cytotaxonomic Key for the Identification of the Sibling Species. *Ann. Trop. Med. Parasitol.* **2011**, *105*, 277–297. [CrossRef]
15. Krueger, A.; Hennings, I.C. Molecular Phylogenetics of Blackflies of the *Simulium damnosum* Complex and Cytophylogenetic Implications. *Mol. Phylogenet. Evol.* **2006**, *39*, 83–90. [CrossRef] [PubMed]
16. Lawrence, P.O. Simulium spp., Vectors of *Onchocerca volvulus*: Life Cycle and Control. In *Encyclopedia of Entomology*; Capinera, J.L., Ed.; Springer: Dordrecht, The Netherlands, 2008; pp. 3380–3382.
17. Taylor, M.J.; Hoerauf, A.; Bockarie, M. Lymphatic filariasis and onchocerciasis. *Lancet* **2010**, *376*, 1175–1185. [CrossRef]
18. Alvarez-Moreno, C.; Pardo-Turriago, R.; Rodríguez-Morales, A.J. Long-Term Consequences of the Misuse of Ivermectin Data. *Lancet Infect. Dis.* **2021**, *21*, 1624–1626. [CrossRef] [PubMed]
19. Ōmura, S.; Crump, A. The Life and Times of Ivermectin—A Success Story. *Nat. Rev. Microbiol.* **2004**, *2*, 984–989.
20. Domche, A.; Pion, S.D.S.; Kamga, G.R.; Nana-Djeunga, H.C.; Ntep, M.M.; Mba, R.M.; Epee, E.; Wanji, S.; Boussinesq, M. Knowledge/Perception and Attitude/Practices of Populations of Two First-Line Communities of the Centre Region of Cameroon Regarding Onchocerciasis and Black Fly Nuisance and Bio-Ecology. *Parasites Vectors* **2021**, *14*, 546. [CrossRef]
21. Eisenbarth, A.; Achukwi, M.D.; Renz, A. Ongoing Transmission of *Onchocerca volvulus* after 25 Years of Annual Ivermectin Mass Treatments in the Vina du Nord River Valley, in North Cameroon. *PLoS Negl. Trop. Dis.* **2016**, *10*, e0004392. [CrossRef]
22. Duke, B.O. Studies on Factors Influencing the Transmission of Onchocerciasis. IV. The Biting-Cycles, Infective Biting Density and Transmission Potential of Forest *Simulium damnosum*. *Ann. Trop. Med. Parasitol.* **1968**, *62*, 95–106. [CrossRef] [PubMed]
23. Tekle, A.H.; Elhassan, E.; Isiyaku, S.; Amazigo, U.V.; Bush, S.; Noma, M.; Cousens, S.; Abiose, A.; Remme, J.H. Impact of Long-Term Treatment of Onchocerciasis with Ivermectin in Kaduna State, Nigeria: First Evidence of the Potential for Elimination in the Operational Area of the African Programme for Onchocerciasis Control. *Parasites Vectors* **2012**, *5*, 28. [CrossRef]
24. Weldegebreal, F.; Medhin, G.; Weldegebreal, B.; Legesse, M. Assessment of Community's Knowledge, Attitude and Practice about Onchocerciasis and Community-Directed Treatment with Ivermectin in Quara District, North-Western Ethiopia. *Parasites Vectors* **2014**, *7*, 98. [CrossRef] [PubMed]
25. Ministry of Economy. *Cameroon, Benakuma Council Development Plan Agenda of Municipality*; Ministry of Economy: Yaoundé, Cameroon, 2012; Volume 1, p. 165.

26. Seidenfaden, R.; Fischer, A.; Bonow, I.; Ekale, D.; Tanya, V.; Renz, A. Combined Benefits of Annual Mass Treatment with Ivermectin and Cattle Zooprophylaxis on the Severity of Human Onchocerciasis in Northern Cameroon. *Trop. Med. Int. Health* **2001**, *6*, 715–725. [CrossRef]
27. Olivry, J.C. *Rivers and Streams of Cameroon*; ORSTOM Hydrological Monographs; Mesres-Orstom: Paris, France, 1986; Volume 9, 781p.
28. Renz, A.; Wenk, P.; Anderson, J.; Fuglsang, H. Studies on the Dynamics of Transmission of Onchocerciasis in a Sudan-Savanna Area of North Cameroon. V. What Is a Tolerable Level of Annual Transmission Potential? *Ann. Trop. Med. Parasitol.* **1987**, *81*, 263–274. [CrossRef] [PubMed]
29. Wahl, G.D.; Ekale, D.; Schmitz, A. *Onchocerca ochengi*: Assessment of the *Simulium* Vectors in North Cameroon. *Parasitology* **1998**, *116*, 327–336. [CrossRef]
30. Brieger, W.R.; Otusanya, S.A.; Oke, G.A.; Oshiname, F.O.; Adeniyi, J.D. Factors associated with coverage in community-directed treatment with ivermectin for onchocerciasis control in Oyo State, Nigeria. *Trop. Med. Int. Health* **2001**, *6*, 802–810.
31. Yahaya, A.; Oyeyi, T. Current Status of Onchocerciasis in Tudun Wada and Doguwa Local Government Areas of Kano State. *Niger. J. Parasitol.* **2007**, *24*, 77–88.
32. Yirga, D.; Negussu, N.; Dagne, H.; Taye, H. Knowledge and Belief about Cause and Prevention of Onchocerciasis in Bebeka, Southwest Ethiopia. *Ethiop. J. Health Sci.* **2008**, *18*, 3.
33. Adewale, B.; Mafe, M.A.; Oyerinde, J.P. Infectivity and Transmission Dynamics of *Simulium damnosum* s. around Owena Dam (Ondo State). *West Afr. J. Med.* **1999**, *18*, 257–260.
34. Dozie, I.N.; Onwuliri, C.O.; Nwoke, B.E. Onchocerciasis in Imo State, Nigeria. Community Knowledge and Beliefs about Transmission, Treatment and Prevention. *Public Health* **2004**, *118*, 128–158. [CrossRef] [PubMed]
35. Tadele, A. Knowledge, Attitude and Practices Towards Onchocerciasis Prevention and Control in Bench Maji Zone, South Western Ethiopia. *Jpn. J. Ophthalmol.* **2019**, *7*, 8. [CrossRef]
36. Renz, A.; Wenk, P. Studies on the Dynamics of Transmission of Onchocerciasis in a Sudan-Savanna Area of North Cameroon. II. Seasonal and Diurnal Changes in the Biting Densities and in the Age-Composition of the Vector Population. *Ann. Trop. Med. Parasitol.* **1987**, *81*, 229–237. [CrossRef] [PubMed]
37. Ayisi, F.; Takougang, I.; Njiokou, F.; Simo, G.; Nana-Djeunga, H.C. Onchocerciasis in the Cameroon-Chad Border Area after More than 20 Years of Annual Mass Ivermectin Distribution. *Parasites Vectors* **2024**, *17*, 219. [CrossRef]
38. Renz, A.; Wenk, P. The Distribution of the Microfilariae of *Onchocerca volvulus* in the Different Body Regions in Relation to the Attacking Behaviour of *Simulium damnosum* s.l. in the Sudan Savanna of Northern Cameroon. *Trans. R. Soc. Trop. Med. Hyg.* **1983**, *77*, 748–752. [CrossRef]
39. Afolabi, O.; Okaka, C.; Oke, I.; Oniya, M. Knowledge, Attitude and Perception of Onchocerciasis and Ivermectin Treatment in Idogun Community, Ondo State, Nigeria. *Br. J. Med. Med. Res.* **2016**, *13*, 1–7. [CrossRef]
40. Nandha, B.; Krishnamoorthy, K. Impact of Education Campaign on Community-Based Vector Control in Hastening the Process of Elimination of Lymphatic Filariasis in Tamil Nadu, South India. *Health Educ. Res.* **2011**, *27*, 585–594. [CrossRef]
41. Okanlawon, F.A.; Osanyintolu, G.D. Pilot Training of Community Mobilizers as Health Educators to Prevent Onchocerciasis in Bugai, Kaduna State, Nigeria. *Int. Q. Community Health Educ.* **2011**, *32*, 171–176. [CrossRef]