

Genetic Diversity and Emerging Zoonotic Onchocerca Species in Human Populations in Taraba State, Nigeria

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Abstract

Background: A better understanding of parasite population genetic processes in specific biogeography is needed to support onchocerciasis elimination goals. The genetic diversity of *Onchocerca* microfilariae was explored by amplifying a fragment of the 16S rRNA gene in the endemic area in Taraba State, Nigeria,

Methods: Eight (8) communities were selected including six onchocerciasis endemic communities with records of ivermectin treatment having been annually distributed for 10 to 16 years, and two non-onchocerciasis endemic areas. The participants were 211 from endemic and 110 from non-endemic areas as control. Skin snips were taken from consenting participants by convenience sampling methods using a sterile sclera punch, from males and females residing within the communities for ten years and above or since birth, microfilaria and residual skin snips were preserved in RNALater® in a 1.5 ml micro-centrifuge tube. DNA was extracted from microfilariae recovered and from those in residual skin snip specimens. Polymerase Chain Reaction (PCR) amplification using specific primers for 16S genes was done to detect the identity of *Onchocerca* species. The amplified products were sequenced and analyzed for species identity.

Results: Multiple sequence alignment and phylogenetic analysis results showed distinct diversity of two sample sequences (G49_O.v. Gashaka and Y02_O.v. Yorro) from other samples from the study area and other regions, indicating emergence of a new polymorphic strain of *O. volvulus*. Report of a preliminary case of emerging zoonosis of *O. ochengi* infection in human (skin snips) sample (*O. ochengi* G44) in this study.

Conclusions: It is clear there is genetic diversity of *Onchocerca* species and emerging zoonosis in the study site. We suggest further investigation on the extent and potentials of emerging zoonotic onchocerciasis by *O. ochengi*, in the light of cattle, *Simulium* vectors, environmental and humans overlap in the study area.

Background

Onchocerciasis is called river blindness because the blood-feeding black flies that transmit the ultimately blinding disease are prevalent throughout many major river basins in Africa in lush, fertile land alongside the rivers in which they breed [1].

It is estimated that 120 million people are worldwide are at risk of onchocerciasis. Numbers of people infected with *Onchocerca volvulus* are 26 million, with dermal microfilariae in 31 sub-Saharan African countries [2].

Nigeria ranks among those countries with the highest burden of the disease in the world [2], accounting for about a third of the global prevalence with estimated 8 million Nigerians infected with *O. volvulus*. Nigeria has about 50 million persons in 40,000 communities at risk with over 6.5 million suffering from severe itching or dermatitis, 500,000 are blind [3].

The annual treatment regimen with ivermectin has achieved a high degree of onchocerciasis control in some areas [4,5]. Several field studies have shown the reduction in the transmission of infection [6]. However, in some parts of Nigeria there are still places where active transmission continues and where the prevalence of *O. volvulus* is still high [7].

About one-third of the *Onchocerca* filariidae are in Africa, where the *Simulium damnosum* sibling species are not the only the main vectors of *O. volvulus* but also those species that transmit the cattle parasite *Onchocerca ochengi* and *Onchocerca ramachandrini*, a parasite of warthogs [8]. A reliable and rapid taxonomic identification of parasitic disease agent is essential for their correct diagnosis; hence molecular detection of species diversity has become increasingly popular [9]. The close similarity of *Onchocerca* species necessitates the exact identification of the developmental stages as a condition for assessing transmission indices in endemic areas [10].

In Northern Cameroon, which borders Taraba State, Nigeria, the prevalence of microfilariae of *O. ochengi* in cattle can be as high as 66 – 71% [11,12]. However, since *O. ochengi* is closely related to *O. volvulus*, the routinely used O-150-target, commonly used as a diagnostic marker for *O. volvulus* clusters with other *Onchocerca* species and therefore discrimination is problematic [8].

However, molecular phylogenetic analysis of three ribosomal DNA gene fragments [12S, 16S rRNA genes, and NADH dehydrogenase subunit 5 (ND5)] from five *Onchocerca* species in Mali has shown potentials for use in species separation and segregation in diversity studies [13]. Also, the development of DNA probes specific for *O. volvulus* and *O. ochengi* have become an additional tool in population genetics studies and for discriminating among *Onchocerca* species specifically [14, 15].

Almost all vectors of human onchocerciasis are also partially zoophilic. Thus they can transmit infective larvae of animal *Onchocerca* species from cattle, warthogs and antelopes [16, 17] and other zoonotic species. *Onchocerca volvulus* populations may misdiagnose during surveillance studies affecting the accuracy of epidemiological data and improper interpretation of human onchocerciasis [18].

In a multi-site study evaluating five Community Directed Treatment with Ivermectin (CDTI) projects in Nigeria and Cameroon, over one-quarter of the age-eligible people in the study communities were low complaints and may act as a reservoir for continued transmission of onchocerciasis [19, 20].

Thus, the pressure on the parasite due to over two decades of Mass Drug Administration (MDA) regime of Ivermectin distribution in this endemic area and the potential co-transmission of human and animal filariae presents confounding issues regarding emerging species diversity are the key findings in this article.

Methods

Study Area

Out of a total of 8 communities selected in 4 Local Government Areas of Taraba State, six were onchocerciasis endemic while two were from non-onchocerciasis endemic communities (Figure 1).. The study area is characteristic of southern Guinea Savanna with annual mean temperatures ranging between 27 to 37°C, relative humidity between 62 - 97%) and an average rainfall of 180mm. The physical relief, which surrounds the study area are mountains about 1000m in height [7]. The onchocerciasis endemic focus extends across the international border to Northern Cameroon. The area is innervated with rivers and tributaries of streams traversing the communities and draining into the river Benue, which provide numerous breeding sites for the blackflies [21]

Ethical clearance and Participants consent

Ethical approval from ABU Zaria Nigeria, Committee for Human Subject Research (ABUCUHSR/Zoology/2020/006). Oral informed consent was obtained from the participants.

Skin snip collection

Superficial skin biopsies (“skin snips”) were taken from around the iliac crests previously described by [19], following Rapid Epidemiological Assessment (REA) guidelines [22]. Each snip from individual participants was immediately placed into the 1.5ml microcentrifuge tubes with approximately 100 µl physiological saline and incubated for 12–24 hours [23]. The emerged microfilariae were examined microscopically at 40–400× magnification. Then, the microfilariae and the corresponding residual skin snips were transferred to a 1.5ml Eppendorf tube containing 200 µl of *RNA*later® preservative [24] and stored at -20°C until used for PCR amplification.

DNA Extraction, PCR Amplification, Purification and Sequencing

Genomic DNA was extracted from skin biopsies samples using the QIAamp Investigator kit (Qiagen, Germany). The molecular identification of *Onchocerca* species was done by amplification of ribosomal DNA (rDNA) targeting a fragment of the 16S gene, with specific sets of primers: FW-primer: 5´-TGGCAGCCTTAGCGTGATG-3´, RV-primer: 5´-CAAGATAAACCGCTCTGTCTCAC-3´ [15]. The PCR mix was in a total reaction volume of 25 µl consisting 0.5 µl MgCl₂, 0.5 µl dNTP, 0.5 µl forward and reverse primer, 2.5 µl Green Dream Taq buffer, 0.5 µl Taq polymerase and 1 µl of DNA template. The thermocycler conditions were calibrated to run an initial denaturation for 4 min at 94°C, denaturation at 94°C for 30 s, annealing at 52.5°C for 30s and elongation at 72 °C for 90 s for 35 cycles and a final elongation of 7 min at 72 °C and held at a temperature of 4°C overnight till further analyses.

The PCR products were resolved in 3% TBE agarose gel electrophoresis process, stained with Stain G (SERVA Electrophoresis, Germany) for 1 hour. Amplicons were visualized in a gel documentation system consisting of UV Benchtop Imaging System, Transilluminator (Hero®, Germany) and the images photographed and saved as JPEG formats. The sequencing was achieved when the amplicons in the agarose gels were excised and purified using the **GeneJET** Gel extraction and purification kit as described by the manufacturer's instructions (Thermo Scientific, Dreieich, Germany). Sanger sequencing was carried out by the Microsynth Sequencing Laboratories in Göttingen, Germany. The sequenced samples were analysed using Geneious and Basic Local Alignment Search Tools (BLAST) algorithms similarity search of 16S rRNA *Onchocerca* species in the GenBank in the BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>).

Partial DNA sequence of 16S genes of the samples was edited in Bioedit (<http://www.mbio.ncsu.edu/BioEdit>) and further aligned with sequences retrieved from published 16S sequences of *O. volvulus*, *O. ochengi*, *O. siisa*, *O. gutturosa*, and *O. gibsoni* as well as published *O. volvulus* from other regions and an out-group sequences in the GenBank for comparison and validation of inter and intra-specific variability [25] in MEGA X (<http://www.megasoftware.net>) algorithms using neighbour-joining consensus trees construction [26].

Results

Species identification and sequence characteristics

Table 1 and 2 show the reference sequences (16S) derived from Mali, Yemen, Cameroon, Ghana, Uganda and the sample sequences from the study area. The pairwise nucleotide distance in the p-distance model showed no p-distance (0.00 %) between a sample in this study (Y07_ *O. volvulus* Nigeria) with those from Cameroon, Mali, Yemen, Uganda and Ghana (Table 1). However, there was pairwise-distance between two samples in this study (Y02_ *O.v.* Yorro and G49_ *O.v.*_Gashaka), 3.00% and 7.00% respectively with *O. volvulus* sequences obtained from some countries (Table 1).

The study revealed pairwise-distance between the in-group *Onchocerca* species (GenBank) with sequences in this study. Sample (Y07_ *O. volvulus*, Nigeria) showed no pairwise-distance (0.00%) with *O. volvulus* sequence from GenBank (AY462902) (Table 2). The nucleotide sequence distance between the two samples in this study (Y02_ *O.v.* Yorro and G49_ *O.v.*_Gashaka) against *O. volvulus* sequence from GenBank (AY462902) was 4.00% each (Table 2).

Onchocerca volvulus sequence multiple alignments

Figure 2 presents alignments of 16S clusters of *O. volvulus* from the study area (Nigeria) with Cameroon (KC167346.1), Ghana and Uganda (DQ523754.1), Mali (AY462903.1). The sequences of samples from the present study and *O. volvulus* from Mali, Yemen, Uganda, Ghana and Cameroon (16S-GenBank: AY462902; 16S-GenBank: DQ523754; 16S-GenBank: DQ523754.1; GenBank: AY462903) revealed numbers of nucleotides differences. G49-Nigeria and Y02- Nigeria showed distinct nucleotide diversity at the point of non-star positions and indicated by white colouration (Fig. 2).

Alignments of 16S clusters of *O. volvulus* from the study area (Nigeria) with Cameroon (KC167346.1), Ghana and Uganda (DQ523754.1), Mali (AY462903.1). Non-Stars indicate the position of an *O. volvulus* specific single nucleotide polymorphism (SNP).

Phylogeny of the genus *Onchocerca*

Sequence data from the study and GenBank of some in-group species *O. volvulus*, *O. ochengi* 'Siisa', *O. ochengi*, *O. gibsoni*, *O. gutturosa* and *O. ramachandrini* and an outgroup species *D. immitis* obtained from GenBank and placed as basal species of the genus with high bootstrap support and their relationship.

Cluster 1 in Figure 3 is the closeness of the sequence data of *O. volvulus* in some countries (Cameroon, Uganda, Mali and Ghana) from GenBank to the sample (*O. volvulus*_Y07_Yorro_Nigeria) in this study.

There was diversity observed in two samples sequences in cluster 2 indicated in black words to *O. volvulus* sequence from some countries obtained from the GenBank (Fig 3). The species in cluster 3 in red words is *O. ochengi* identified in this study (Fig 3).

The tree show the *Onchocerca* evolutionary diversity in this study and other members of in-group species of some countries from the GenBank. Clusters: 1 in green colour, 2 in black colour and 3 in red colour.

Emerging zoonosis of *Onchocerca ochengi* and *Onchocerca volvulus* diversity

The sequencing result revealed an emerging Zoonotic sample (*O. ochengi* G44 Gashaka) which clustered with *O. ochengi* GenBank entries from Cameroon 16S-GenBank: DQ523753.1 indicated in red (Fig.4). Another noteworthy finding of our study is that two samples (G49_O. v. Gashaka and Y02_O.v. Yorro) showed distinct variation (diversity), in a cluster, indicated in blue (Fig.4).

The tree showed the *Onchocerca* evolutionary diversity in this study and some in-group species sequences obtained from the GenBank indication in colours: **blue** = *O. volvulus* species diversity identified; **green** = similarity of sample of study site and *O. volvulus* obtained from GenBank; **red** = similarity of sample of study site and *O. ochengi* obtained from GenBank; **black** = other in-group species obtained from GenBank.

Discussion

This study showed the presence of microfilariae (9 of 211 persons examined by skin-snips; 2.47%) as observed by previous parasitological examination carried out by other studies in the same study area [7, 21, 27, 28]. The molecular identification of *Onchocerca* species in this study is much simpler methods needed for diagnosis of *Onchocerca* species [29].

We observed some genetic diversity within the populations of *O. volvulus*. Two samples (Y02_O.v. Yorro and G49_O.v._Gashaka) showed a diversity of 3.0% and 7.0% respectively (Table 1) in the p-distance model, suggesting the review of generalized diagnostic test assay for transmission assessment based on the peculiarity of the endemic areas. The pairwise nucleotide diversity (Table 2) obtained from GenBank as presented in this study confirms the submission by [30] and [25] that *O. ochengi* and *O. Siisa* are much closer to each other – indeed, they freely interbreed and thus belong to one species [25] than to *O. volvulus* [29].

Another noteworthy finding of our study are the two samples (G49_O. v. Gashaka and Y02_O.v. Yorro) that showed distinct variation (diversity) if compared to those from other regions, as indicated in Single Nucleotide Polymorphism (Figure. 2). This might point towards the emergence of a new strain of *O. volvulus* in our study area. No previous similar work has been carried out in this region to confirm this claim. So we suggest further investigation is needed to verify this finding.

This work suggests the likelihood of a diverse sister species emerging nearer to *O. volvulus* than to *O.ochengi* or *O. siisa* [25]. These may be due to pressure of the drug and systematic non-compliance to ivermectin in this endemic area known for continual transmission despite nearly two decades of Mass Drug Administration of Ivermectin [7, 28] which might be leading to changes or genetic drift among the parasite populations in the study area.

The study recorded a preliminary novel incidence of *O. ochengi* (Figure 3) in the human skin biopsies. The patient is a healthy 48 years female farmer that was born and residing in Kwaitap village in Gashaka LGA, Taraba State, Nigeria. A punch of skin snip was obtained from the upper hip (buttock) of the patient, who have no nodule neither any clinical manifestation associated with filariasis, the study site is characterized with abundance of cattle populations that cohabit and graze among the human population. The status of animal onchocerciasis is still unknown in Nigeria even though there is a high cattle population in human onchocerciasis endemic areas who serve as reservoirs for bovine onchocerciasis (*O. ochengi*); almost all

vectors of human onchocerciasis are zoophilic, thus transmitting both animal and human *Onchocerca* species might likely occur [31]

Although intensive studies have been carried out in Cameroon [18], there is no report of *O. ochengi* being isolated from human populations yet. Nonetheless, in Japan [32], zoonotic filariasis caused by *O. dewitteijaponica* has been described in a resident of Hiroshima Prefecture, Honshu, Japan and *Onchocerca lupi* has been reported to infect dogs, cats, and humans [33]. It is against this background that this study is proposing further investigation on the threat and potentials of emerging zoonotic onchocerciasis (*O. ochengi*) due to cattle, *Simulium* vectors, and humans overlap in the study area.

Conclusion

The study revealed a novel incidence of *O. ochengi* (G44_O.o. Gashaka) in human populations. Two samples (G49_O. v. Gashaka and Y02_O.v. Yorro) have distinct variation (diversity) from those of other regions, suggesting an emergence of new polymorphic strains of *O. volvulus* associated with the persistent transmission of onchocerciasis in long term treatment with ivermectin. The study is relevant in future application of the drug regime and dynamics of the epidemiology of the disease. The application of molecular tools in this study has shown their potential in the detection during mapping and other dynamics epidemiology and control strategies.

List Of Abbreviations

rRNA	ribosomal Ribonucleic Acid
DNA	Deoxyribonucleic Acid
PCR	Polymerase Chain Reaction
NADH	Nicotinamide adenine dinucleotide Hydrogen
CDTI	Community Directed Treatment with Ivermectin
MDA	Mass Drug Administration
ABU	Ahmadu Bello University
ABUCUHSR	Ahmadu Bello University Committee for Human Subject Research
REA	Rapid Epidemiological Assessment
TBE	Tris/Borate/EDTA
BLAST	Basic Local Alignment Search Tools
MEGA X	Molecular Evolutionary Genetics Analysis
G44 O.o	Gashaka sample number 44 of <i>Onchocerca ochengi</i>

G49 O.v Gashaka sample number 49 of *Onchocerca volvulus*

Y02 O.v Yorro sample number 02 of *Onchocerca volvulus*

Y07 O.v Yorro sample number 07 of *Onchocerca volvulus*

Declarations

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Author Contributions

Conceived and designed the experiments: DEA ISN IHN GC, Performed the experiments: DEA GC, Analyzed the data: DEA ISN, Contributed reagents/materials/analysis tools: AR GC DEA ISN IMM, Wrote the paper: DEA AR ISN ISH IMM.

Declarations

Ethics approval and Consent to participate: Approval was obtained from the ethics committee from Ahmadu Bello University Zaria, Committee for Human Subject Research (ABUCUHSR/Zoology/2020/006). Informed consent was obtained from all individual participants included in the study.

Consent for publication: All participants were informed and consented to the publication of this article

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

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Tables

Table 1. Pairwise nucleotide distance in a p-distance model of 16S *Onchocerca volvulus* sequences between this study and sequences from other regions

	1	2	3	4	5	6	7	8
1.AY462903.1_O._volvulus_Mali								
2. DQ523754.1_O._volvulus_Yemen	0.000							
3.KC167346.1_O._volvulus_Cameroon	0.000	0.000						
4. DQ523754.1_O._volvulus_Ghana	0.000	0.000	0.000					
5. DQ523754.1_O._volvulus_Uganda	0.000	0.000	0.000	0.000				
6. Y02_O._volvulus_Nigeria (this study)	0.003	0.003	0.003	0.003	0.003			
7. Y07_O. volvulus Nigeria (this study)	0.000	0.000	0.000	0.000	0.000	0.021		
8. G49_O. volvulus Nigeria (this study)	0.007	0.007	0.007	0.007	0.007	0.003	0.007	

Numbers 1–5 are the reference sequences (16S) derived from Mali, Yemen, Cameroon, Ghana and Uganda (GenBank: AY462902; DQ523754; KC167346.1; DQ523754.1 and DQ523754.1). Number 6-8 are the samples from the study area. The values are pairwise nucleotide differences (Substitute) in the p-distance model.

Table 2. Pairwise nucleotide distance in a p-distance model of 16S *Onchocerca volvulus* sequences between this study and sequences of extant *Onchocerca* species

	1	2	3	4	5	6	7	8	9
Samples									
1. G49_O.v._Gashaka (this study)									
2. Y07_O.v. Yorro (this study)	0.004								
3. Y02_O.v. Yorro (this study)	0.000	0.004							
4. DQ523753.1 <i>O. ochengi</i>	0.022	0.018	0.022						
5. AY462894.1 <i>O. gibsoni</i>	0.064	0.060	0.064	0.060					
6. DQ523748.1 <i>O. ramachandrini</i>	0.117	0.112	0.117	0.101	0.101				
7. AY462904.1 <i>O. gutturosa</i>	0.064	0.060	0.064	0.055	0.055	0.055			
8. DQ523749.1 <i>O. Siisa</i>	0.022	0.018	0.022	0.014	0.014	0.014	0.014		
9. AY462902 <i>O. volvulus</i>	0.004	0.000	0.004	0.018	0.018	0.018	0.018	0.060	

Numbers 1–3 are the samples from the study area. Number **4–9** are the published reference sequences of some *Onchocerca* species from the GenBank (DQ523753.1 *O. ochengi*, AY462894.1 *O. gibsoni*, DQ523748.1 *O. ramachandrini*, AY462904.1 *O. gutturosa*, DQ523749.1 *O. Siisa*, AY462902 *O. volvulus*). The values are pairwise nucleotide differences (Substitute) in the p-distance model.

Figures

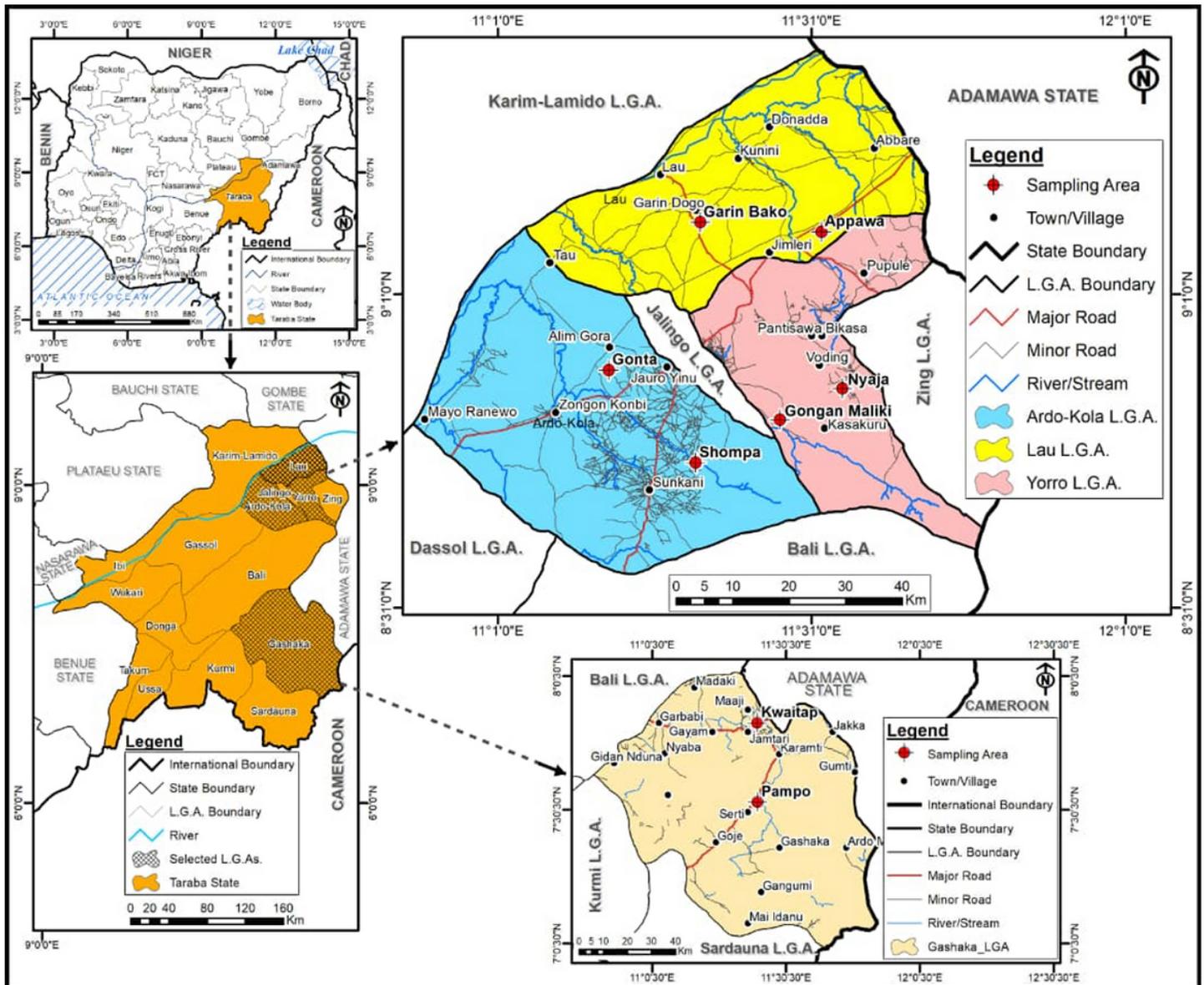


Figure 1

Map of sampling sites in Taraba State, Nigeria Prepared by Map Gallery, Geography Department, Ahmadu Bello University (ABU) Zaria, Nigeria (2020). Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

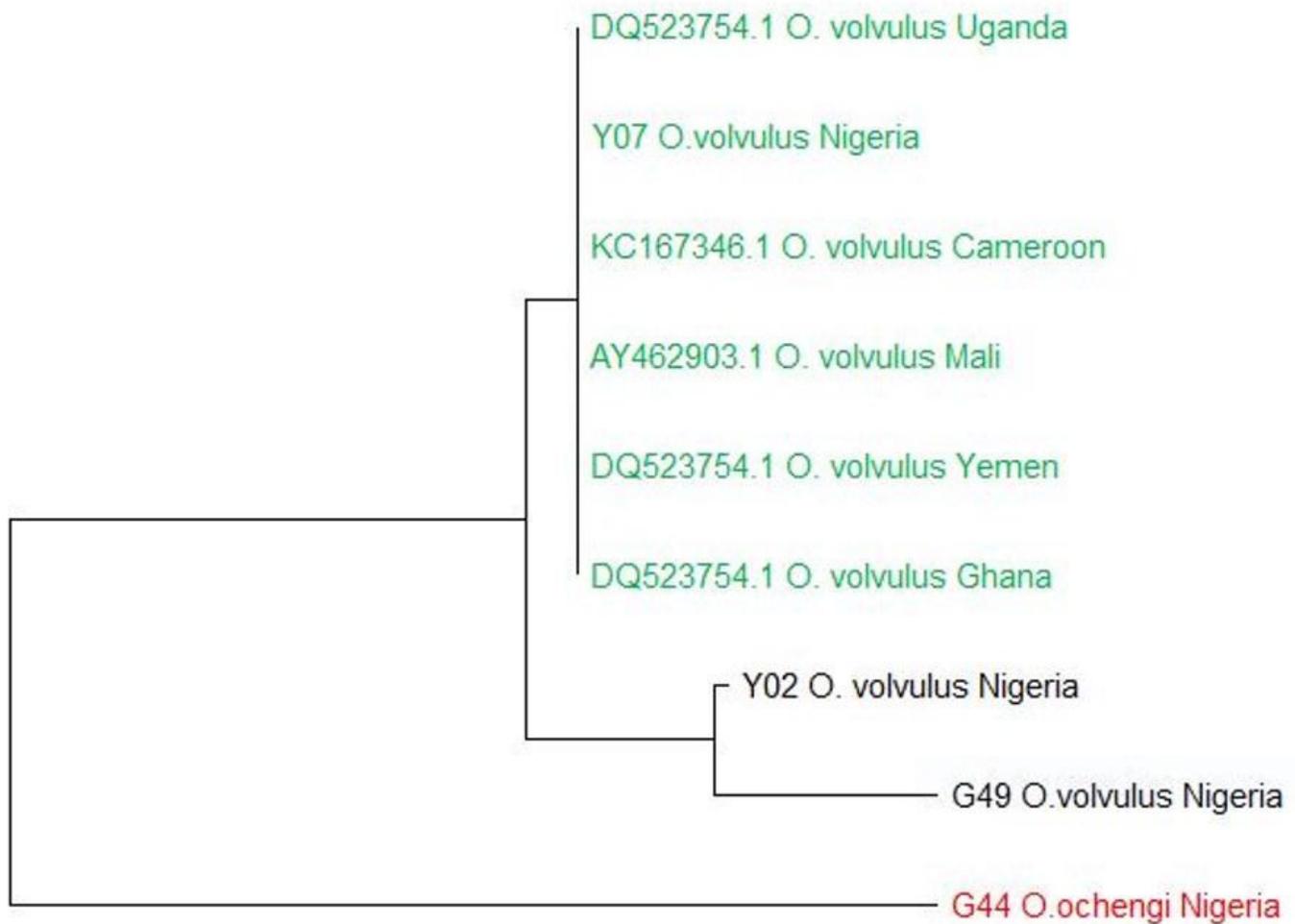


Figure 3

16S rRNA gene sequences phylogenetic tree constructed using the Neighbor-Joining method. The tree show the *Onchocerca* evolutionary diversity in this study and other members of in-group species of some countries from the GenBank. Clusters: 1 in green colour, 2 in black colour and 3 in red colour.

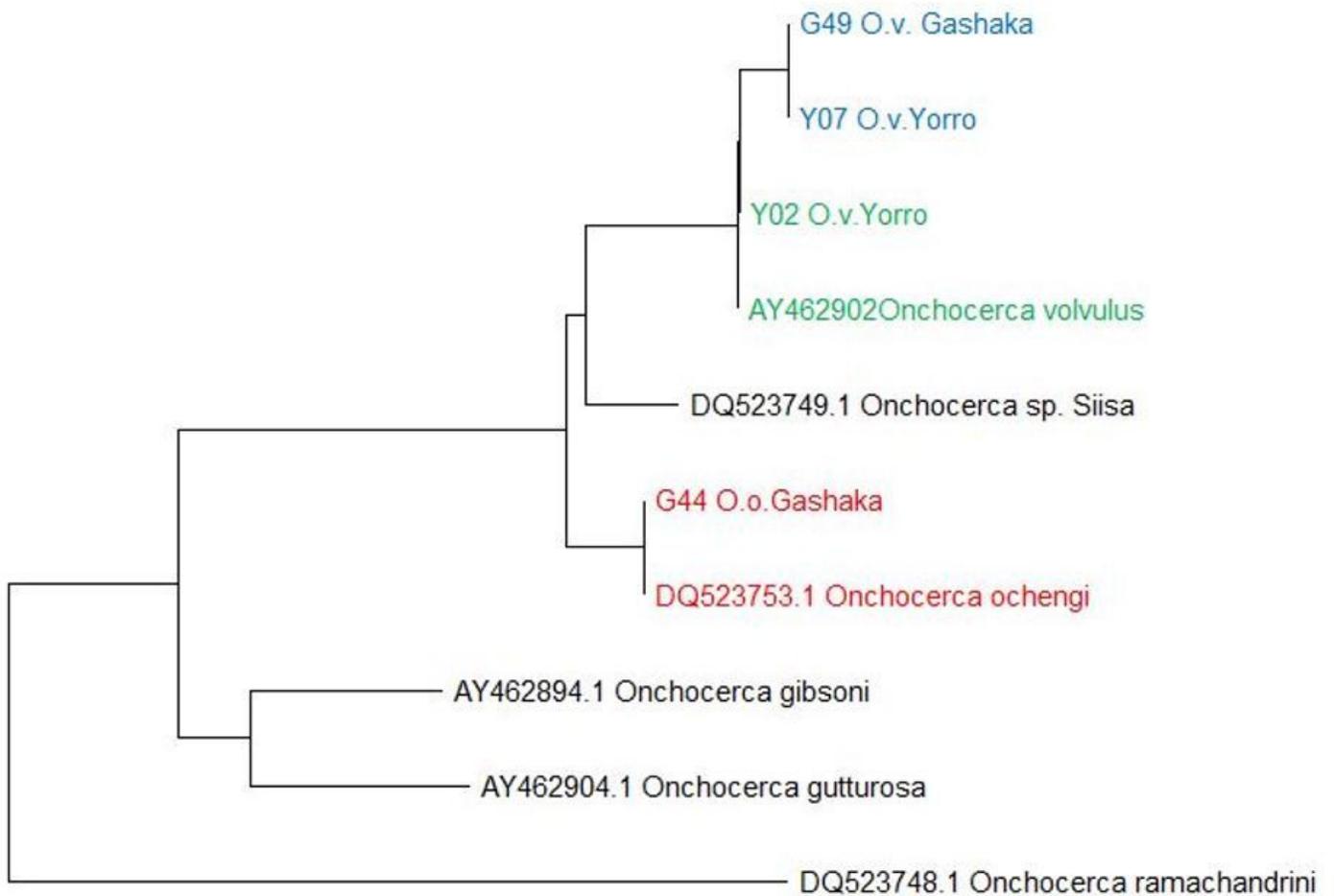


Figure 4

A representative phylogenetic 16S rRNA gene tree constructed using the Neighbor-Joining method. The tree showed the *Onchocerca* evolutionary diversity in this study and some in-group species sequences obtained from the GenBank indication in colours: blue = *O. volvulus* species diversity identified; green = similarity of sample of study site and *O. volvulus* obtained from GenBank; red = similarity of sample of study site and *O. ochengi* obtained from GenBank; black = other in-group species obtained from GenBank.

Maker	Genotype	Gene Accession number	Consensus sequence/Sample sequence	Single nucleotide polymorphism
16s	<i>O. Volvulus</i> n= 3	MN717178 – MN717179	<pre> ATTCCCAATACVGGTGATTTGTTTTTATGGTTTCAGTATGAATGAAGTTTTTAGC AGCTTTTACTTTTGTGTTGAATTAATTTTAAATTAATAAATAATTAGTTAAGST ATTACAAGAAAGTCTTCGGAAATTTGTTTGAATTTGAAATTTTATTTTAAATTT TTCTTGGGGATGGATTTAAGAAAGTTTATACTATTGTTATTATAAATAAATTACTC CGGAGTTAACAGGGTTGTAGACATAAATAAGGTTTTATATTAGTGTGCTGCGCTA CATCGATGTTGATATTTTTTGATAAATGGAGAGGTTTTTTTGTATTTAATCTGTTTC T>>TTKATAAAAAAATTGACTTGAATTATTWATTTCGAAAACRGKT>RTCKTKAAA> AAMGCKGTTYM>CTKRRADMK>TKRCWYCTGA>G>GGT>STGCWGGGT>TG CCGA>CGTYKCTTGGRRMRSTCYCWMTGR>SGARW>GCTCCTTCGGAGGGCC CTCAGGGAAA </pre>	
	<i>O. Ochengi</i> n= 1	MN717151	<pre> TTGTTTTTATGGTTTCAAGTATGAATGAAGTTTTTAGCAGTTTT TTTATTACTTTTTTATTTGAATTAATTTTTTGAATTAATAAATAATTAGT TAAGGTATTACAAGATAAAGTCTTCGGAAATTTGTTTGAATTTGAAATTTTATTTTAAATTT TGAAATTTTGTGTTTTAATTTTCTTGGGGATGGATTTAAGAA AGTTTTATACTATTGTTATTATAAATAAATACTCCGGAGTTAACAG GGTTGTAGACATAAATAAGATTTTATATTAGTGTGCTGCGCTAC ATCGATGTTGATATTTTTTTGATAATGGAGAGGTTTTTTTTTATT TTGAGACTGTTCTTGTATAAATAAATTGACTTGAATTAAGTTTA GTTCTGCTGAGACANAGCGGTTTATCTNAATTC </pre>	

Figure 5

The summary of sequence data obtained *Onchocerca volvulus* and *Onchocerca ochengi* in this study a: ambiguity codes at variable positions are in colour-coded as follows: C or T – yellow; A or C – purple; A or G – red; A or T – blue; G or C – turquoise; G or T – green. b: The bases present at the variable positions are listed in the order of occurrence.

Supplementary Files

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- [GraphicAbstractfinal2.jpg](#)