

1 Discrimination between *Onchocerca volvulus* and *O. ochengi* filarial larvae in
2 *Simulium damnosum* s.l. and their distribution throughout central Ghana using a
3 versatile high-resolution speciation assay

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Abstract

Background

Genetic surveillance of the human filarial parasite, *Onchocerca volvulus*, from onchocerciasis endemic regions will ideally focus on genotyping individual infective larval stages collected from their intermediate host, Simuliid blackflies. However, blackflies also transmit other *Onchocerca* species, including the cattle parasite *O. ochengi*, which are difficult to distinguish from the human parasite based on morphological characteristics alone. This study describes a versatile approach to discriminate between *O. volvulus* and *O. ochengi* that is demonstrated using parasite infective larvae dissected from blackflies.

Results

A speciation assay was designed based on genetic differentiation between *O. volvulus* and *O. ochengi* mitochondrial genome sequences that can be performed in high-throughput high-resolution melt- or lower throughput conventional RFLP analyses. This assay was validated on 185 *Onchocerca* larvae dissected from blackflies captured from 14 communities in Ghana throughout 2011-13. The frequency of *O. ochengi* was approximately 67% of all larvae analysed, which is significantly higher than previously reported in this region. Furthermore, the species distribution was not uniform throughout the study region, with 25%, 47%, and 93% of *O. volvulus* being found in the western-most (Black Volta, Tain and Tombe), the central (Pru) and eastern-most (Daka) river basins, respectively.

Conclusions

This tool provides a simple and cost-effective approach to determine the identity and distribution of two *Onchocerca* species, and will be valuable for future genetic studies that focus on parasites collected from blackflies. The results presented highlight the need to discriminate *Onchocerca* species in transmission studies, as the frequency of each species varied significantly between the communities studied.

64 **Keywords:**

65 *Onchocerciasis*, *Onchocerca volvulus*, *Onchocerca ochengi*, high-resolution melt
66 analysis (HRM), restriction fragment length polymorphism (RFLP), speciation, Ghana

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Background

Onchocerca volvulus is a human filarial parasite responsible for the disease onchocerciasis, and is transmitted between hosts by blackflies of the genus *Simulium*. Collection of blackflies throughout disease endemic regions forms a significant part of onchocerciasis surveillance campaigns to determine the prevalence and transmission of the parasite. Two approaches are commonly used; the first, pool-screening, has been extensively used to determine the prevalence of a single parasite in a pool of low hundreds of blackfly heads [1], which can be detected using an *Onchocerca*-specific O-150 polymerase chain reaction (PCR) assay together with an *O. volvulus*-specific DNA probe. Pool-screening has been the predominant tool used for parasite surveillance by the African Programme for Onchocerciasis Control (APOC, Burkina Faso), and is suitable particularly when the prevalence of *O. volvulus* is low in areas where widespread use of ivermectin has taken place. Adaptation of the pool-screening approach to detect *O. volvulus* has recently been demonstrated using loop-mediated isothermal amplification (LAMP) targeting either the *O. volvulus* glutathione S-transferase 1a gene [2], or the O-150 repeat (Nankoberanyi *et al.*, in prep), which promises to improve the throughput, cost and sensitivity of the conventional approach. Although pool-screening has the capacity to examine large numbers of flies, the primary objective of the assay is simply to determine the presence or absence of the parasite in a defined number of vectors. The second approach focuses on the isolation of parasites by the dissection of individual blackflies, and pre-dates the use of pool-screening (having been in use since the initial Onchocerciasis Control Programme (OCP) in West Africa). Dissection has the advantage that it allows the developmental stage and location of the parasite(s) within the blackfly to be determined [3]. Data on larval prevalence in the vector are used together with the biting density of the fly to calculate the transmission potential of the parasite [4, 5].

Genetic diagnostic tools are under development by us and others that aim to genetically define parasite populations, characterise transmission zones, and screen parasite populations for polymorphisms associated with variation in response to ivermectin [6]. Such analyses could be integrated with and complement existing entomological surveillance practices using the vector stages of the parasite as a non-

invasive source of parasite material. Genetic analysis of entomological derived parasites will ultimately require dissection of infected blackflies to obtain individual parasite larvae, and subsequently, an efficient and sensitive means to validate the species of parasite collected.

Species characterisation of dissected larvae is challenging (although not impossible) based on morphological characteristics [5, 7, 8]; it requires significant time and expertise, and is therefore relatively low-throughput. Some studies report using O-150-based diagnostic assays [9, 10], whilst others describe the prevalence of *O. volvulus* without molecular confirmation [11-14]. Given that a number of *Onchocerca* parasites, including the common cattle parasite *O. ochengi*, can be transmitted by the same intermediate vector [5], it is likely that *O. volvulus* larval stages may often be overestimated, and in turn, negatively influence the predictions of disease transmission. In this study, we report the development of a versatile molecular tool to discriminate the onchocerciasis parasite *O. volvulus* from other *Onchocerca* species such as *O. ochengi* based on mitochondrial DNA (mtDNA) sequence variation. This approach was validated using *Onchocerca* larvae dissected from blackflies that were collected from three distinct regions in Ghana.

Methods

Blackflies were sampled from 14 communities in 5 river basins in central Ghana during 2011 (number of blackflies (n) = 12031), 2012 (n = 9706) and 2013 (n = 9138) using a human landing collection protocol as described previously [15]. Ethical approval was obtained for the use of human vector collectors from the Institutional Review Board of the Council for Scientific and Industrial Research, Ghana. All blackflies caught were dissected to collect *Onchocerca* larvae (129 from the head, 51 from the thorax, and 5 from the abdomen used here), after which all larvae collected were dried on microscope slides for preservation. In total, 160 infective (L3), together with 21 pre-infective L2 and 4 L1 larvae were analysed (Supplementary Table 1) using the molecular techniques described, which included 14 larvae collected from 8 infected flies in the 2011 cohort, 24 larvae from 11 flies collected in 2012, and 147 larvae from 109 flies collected in the 2013 cohort. Individual larva were recovered from slides by applying 2 µl of HPLC-grade water (Sigma) directly

onto the specimen and allowing it to rehydrate, so that the larva would detach from the slide or could be dislodged by a gentle scrape with a pipette tip. Each larva was individually transferred to 20 µl of sample lysis solution (DirectPCR Lysis reagent [MouseTail; Viagen Biotech] supplemented with 0.3 mg/ml (w/v) proteinase K [Roche]), and incubated for 18 h at 55°C followed by 85°C for 1 h. Larval lysates were diluted with HPLC-grade water 1/10 prior to PCR. Adult *O. volvulus* samples were obtained as previously described [16]. Adult *O. ochengi* were collected from infected cattle udders obtained from an abattoir in Ngaoundéré, Cameroon. Nodules were collected from the skin, from which worms were extracted using forceps via a small incision before being fixed in 95% ethanol. DNA was extracted from individual adult *O. volvulus* and *O. ochengi* samples using an Isolate II Genomic DNA extraction kit (Bioline, Australia) following the manufacturers' instructions.

High resolution melt analysis was performed after real time PCR amplification of a 79-bp product using the Bio-Rad CFX system (Bio-Rad laboratories). A 10 µl reaction mix was prepared by combining 5 µl of 2X SsoAdvanced Universal SYBR Green qPCR Supermix (Bio-Rad Laboratories), 0.5 µl of each of SP_OVOO_79bp (5'-GTTTGTTCTTGTTGATTG-3') and ASP_OVOO_79bp (5'-ACATTAAACCCCTTACC-3') primers (0.5 µM working concentration), 2 µl of HPLC grade water, and 2 µl of the diluted larva lysate. The amplification protocol was performed as follows: 98°C for 3 min, followed by 45 cycles of 98°C for 10 sec, 59.5°C for 10 sec and 72°C for 10 sec. The melt protocol was performed immediately after the amplification, and was as follows: 95°C for 10 sec, followed by a 65°C annealing step from which the temperature was increased by 0.2°C/plate read until a final temperature of 95°C. PCR products generated from adult *O. volvulus* and *O. ochengi* samples were cloned into the plasmid pGem-T-Easy (Promega) and sequenced to confirm the nucleotide variants. All lysates, together with positive (cloned products and adult *O. volvulus* and *O. ochengi* genomic DNA samples) and no-template controls (NTC), were analysed in duplicate. Raw melt data was analysed using Precision Melt software (version 1.2; Bio-Rad Laboratories), from which the data was exported into Microsoft Excel for further quantitative analysis. Spatial (within flies i.e., head, thorax or abdomen, and between river basins), temporal (sampling year) and larval stage (L1, L2 or L3) differences between *O. volvulus* and *O. ochengi* were each examined using

a 2×3 chi-square (χ^2) test with 2 degrees of freedom, and a p-value < 0.05 was considered to represent significant deviation from the null hypothesis.

A subset of post-PCR and HRM products were analysed by Apal restriction digest to confirm the *O. volvulus* specific digestion. A 20 µl reaction consisting of 10 µl PCR product together with 0.5 µl Apal (50,000 U/ml; New England Biolabs), 2 µl of CloneSmart buffer and 7.5 µl of HPLC-grade water was incubated for 3 h at 25°C, after which the products were run on a 2% agarose gel at 100 V for 50 mins and visualised using GelRed DNA stain (Biotum).

Results

Whole mitochondrial genome alignments of the *O. volvulus* (NC_001861; [17]) and *O. ochengi* (obtained from genomic sequence available at http://www.nematodes.org/genomes/onchocerca_ochengi/; Blaxter Lab, University of Edinburgh) sequences was performed to identify a PCR compatible region that contained (i) a restriction site that was unique to one species, and (ii) additional polymorphism(s) that would result in a difference in the melting temperature between amplicons generated for each of the two species. A 79-bp region spanning 8654- to 8733-bp of the *O. volvulus* mitochondrial genome was chosen that contained three nucleotide T > C transitions (*O. volvulus*: C,C,C; *O. ochengi*: T,T,T) between the mtDNA alignment of the two species, one of which was found in an Apal restriction site that is present in *O. volvulus* but absent in *O. ochengi* (Figure 1A). Melt curve analysis demonstrated a shift in T_m between the two sequences, with a mean T_m of 79.8°C and 78.6°C for the *O. volvulus*- and *O. ochengi*-derived sequences, respectively (Figure 1B). This translated into a significant and consistent deviation in melt curves as depicted in the fluorescence difference plots (Figure 1C). To confirm that the difference in melt curves was consistent with the prediction that the Apal restriction site was present in the *O. volvulus* sequences but not in the *O. ochengi* sequences, 42 larvae-derived, 4 adult-derived and 2 cloned PCR products were analysed by restriction digest, of which a representative gel is shown in Figure 1D. The melt curve and restriction digest data were 100% concordant in the samples analysed, demonstrating that both approaches were equally predictive of the species in question. This result does not, however, exclude the possibility that

genetic variation may exist within either or both species of *Onchocerca* examined (or other potential *Onchocerca* species endemic to onchocerciasis regions [18-20]) that will result in melt curves that deviate from the *O. volvulus* and *O. ochengi* control sequences described here. For example, an analysis of the mitochondrial sequence of the *O. ochengi* Siisa variant [18] revealed a T,T,C haplotype that differed from the T,T,T haplotype of the adult *O. ochengi* presented (Dr Adrian Streit, personal communication). Although a single base change is unlikely to confound the interpretation between the melt profiles of the *O. volvulus* and *O. ochengi* control samples described here, this platform does offer further opportunity to explore genetic variation among and within species. Therefore, amplicons giving rise to melt curves that do not match controls should be sequenced.

Of the 141 flies from which *Onchocerca* larvae were recovered, 43 flies contained more than a single larva (range: 2-6). Interestingly, 4 of the 43 flies (9.3% of flies with multiple larva, 2.8% of total) contained both *O. volvulus* and *O. ochengi* larvae, which must represent larval uptake by the blackfly from two blood meals from different hosts at different times. This is not surprising, given the prevalence of cattle and human cohabitation, and that blackflies will seek a blood meal from either host [21, 22]. The overall proportion of each larval stage was similar between both species ($\chi^2 = 3.397$; df = 2; p = 0.091), however, the spatial distribution of each stage within the fly was not equal; although a greater proportion of L3 were found in the head relative to the thorax in both species, the difference between both head and thorax was greatest in *O. volvulus* with a higher proportion of L3 collected from the head (Supplementary Figure; $\chi^2 = 6.822$; df = 2; p = 0.016), which suggests that while the rate of development within the fly may be similar between the species, *O. volvulus* may migrate towards the head of the fly earlier than *O. ochengi*. These observations (co-infection and likely equivalent transmission potential (based on prevalence of L3)), do suggest that the human and bovine hosts are constantly exposed to both parasites which, in turn, raises an interesting question in regard to the number of times host-switching may have occurred between human and cattle (a speciation hypothesis whereby the most recent common ancestor of *O. onchengi* and *O. volvulus* was a cattle parasite that established in the human host [23]).

The distribution of both species throughout the sampling region was not uniform (Figure 2). Although no temporal differences between sampling years for each species was seen ($\chi^2 = 1.075$; $df = 2$, $p = 0.292$), a significant spatial trend was observed that suggested that the western communities sampled had a lower prevalence of *O. volvulus* (24.4%, $n = 136$; Black Volta, Tain and Tombe river basins), a roughly equal prevalence of *O. volvulus* and *O. ochengi* in the central Pru river basin (47.2% *O. volvulus*; $n = 36$), and a higher prevalence of *O. volvulus* (92.9%, $n = 14$) in the eastern-most Daka river basin ($\chi^2 = 29.888$; $df = 2$, $p = 1.618E-7$). These results are not necessarily in contrast to a recent investigation of persistent *O. volvulus* with negligible *O. ochengi* transmission in Ghana [9]. Much of that study was focused on two southern Ghanaian communities, and very few or no larvae were found in the two communities that were shared with this study (Asubende & Agborlekame, respectively); moreover, 3 of the 7 communities used by Lamberton *et al.* were east of the Black Volta Lake, which would be consistent with high *O. volvulus* prevalence observed in the eastern-most community (Wiae) sampled here. We speculate that the high infection rate reported and difference in prevalence of *O. ochengi* in a number of study regions presented here is correlated with the high numbers of cattle in the north-western river basins, particularly during the dry season (December to April). However, given that the sample size of larvae for many communities was low (median = 6.5, range = 1-55 larvae/community; limited by the number of larvae present in the blackfly populations and therefore by the number of blackflies screened), further sampling is required to support these findings, particularly in the Daka river basin where *Onchocerca* larvae were obtained from only a single community out of the five communities in which flies were sampled.

Conclusions

A simple but versatile tool that discriminates between *O. volvulus* and *O. ochengi* single larvae obtained by dissection of the blackfly intermediate host is described, and offers a number of advantages over traditional O-150-based speciation assays. By exploiting the multicopy nature of the mitochondrial genome, this assay is very sensitive, requiring only 1/100th of a single larva, thus leaving sufficient material for further genetic analysis. Although both *O. volvulus* and *O. ochengi* samples could be

distinguished easily via HRM or RFLP assays, no effort was made to identify other described *Onchocerca* species that are potentially present [18-20]; further work is required to characterise melt profiles and/or restriction sites from additional *Onchocerca spp.* voucher specimens as they become available. In the context of onchocerciasis entomological surveillance, which aims to detect transmission of the human pathogen, this study demonstrates the need to accurately and efficiently determine the identity of larvae in the vector in order to correctly estimate parasite transmission. In Ghana, nationwide sampling of blackfly populations is necessary to determine the distribution of *Onchocerca* spp. Implementation of genetic tools such as those described here will inform the appropriate control strategy needed by onchocerciasis control programmes as they strive towards onchocerciasis elimination in Ghana.

Competing Interests:

The authors have no competing interests to declare.

Author's Contributions:

SRD designed the study, performed the molecular biology and data analysis, and drafted the manuscript. MYO-A, MT and SA designed the entomological study for transmission assessment, SA was involved in the collection and dissection of blackflies, and preparation of larvae samples. MYO-A and MT was involved in the coordination of sample collection. AR provided samples. WG participated in the analysis and helped draft the manuscript. All authors read and approved the final version of the manuscript.

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295 **Supplementary Table 1.** Distribution of communities and samples used in this study

Community	River Basin	Geographic coordinates		Larvae processed and species identified by HRM per sample collection year ¹						Total larvae per community
		Latitude	Longitude	2011 (12,031 flies dissected)		2012 (9,706 flies dissected)		2013 (9,138 flies dissected)		
				<i>O. volvulus</i>	<i>O. ochengi</i>	<i>O. volvulus</i>	<i>O. ochengi</i>	<i>O. volvulus</i>	<i>O. ochengi</i>	
Abua (ABA)	Pru	7.58587	-0.53485	1	-	-	-	-	-	1
Agbelekame I (AB)	Black Volta	8.14018	-2.12185	2	2	1	4	8	9 L2; 27 L3 ;	53
Akanyakrom (AKA)	Black Volta	8.289	-2.277	-	-	5	-	-	-	5
Asubende (ASU)	Pru	8.01085	-0.58528	-	-	2	-	5	2	9
Baaya (BAY)	Pru	8.01119	-0.59444	-	-	-	-	2	1 L2; 12 L3	15
Beposo (BEP)	Pru	8.0026	-0.57395	-	-	-	-	1	-	1
Fawoman (FAW)	Pru	8.0111	-1.01303	-	-	1	2	-	-	3
Fawoman-Banda (FAB)	Tombe	8.07108	-2.1441	-	-	-	6	2	-	8
Kyingakrom (KYN)	Black Volta	8.05578	-2.03266	-	3	-	-	-	-	3
Mantukwa (MAN)	Pru	8.01064	-1.0014	-	-	-	-	2	2	4
New Longoro (NLG)	Black Volta	8.08158	-2.02096	-	1	-	-	1	1 L2; 8 L3	11
Senyase (SEN)	Pru	8.01247	-0.59251	-	-	-	-	1 L1;1 L2;1 L3	-	3
Tainso (TAN)	Tain	8.0603	-2.06538	-	5	-	3	1 L1; 4 L2; 7 L3	1 L1; 5 L2; 29 L3	55
Wiae (WIA)	Daka	8.19219	-0.09429	-	-	-	-	1 L1; 12 L3	1	14
Total larvae processed per species per year				3	11	3	15	49 (3 L1; 5 L2; 41 L3)	98 (1 L1; 16 L2; 81 L3)	185

296 ¹ L3 larvae unless otherwise stated

Figure 1. Genetic discrimination of *O. volvulus* and *O. ochengi*. (A) Sequence of the PCR 79-bp amplicon used to discriminate between *O. volvulus* and *O. ochengi*. Nucleotide differences between the two species are highlighted in bold, and the Apal restriction site present in the *O. volvulus* sequence is underlined. (B) Normalised HRM data depicting melt curves from individual *O. volvulus* (green) and *O. ochengi* (red) samples. Data presented includes cloned positive control 79-bp products (pGem_Ov and pGem_Oo), adult DNA, and 4 larvae samples from both species, each in duplicate. (C) The same samples are presented as in (B), showing the difference curves normalised to the *O. ochengi* group. (D) Representative RFLP analysis of amplicons generated in the HRM assay for each species, showing digestion of the *O. volvulus* sequence, but not the *O. ochengi* sequence, with Apal.

Figure 2. Distribution of *O. volvulus* and *O. ochengi* throughout Ghana. Pie charts depict relative prevalence of *O. volvulus* (orange) and *O. ochengi* (purple) in each community. Communities sampled in central Ghana (see insert) and the number of samples analysed are as follows: Abua (ABA; n = 1), Agbelekame I (AB; n = 54), Akanyakrom (AKA; n = 5), Asubende (ASU; n = 9), Baaya (BAY; n = 15), Beposo (BEP, n = 1), Fawoman (FAW; n = 3), Fawoman-Banda (FAB, n = 8), Kyingakrom (KYN; n = 3), Mantukwa (MAN; n = 4), New Longoro (NLG; n = 11), Senyase (SEN; n = 3), Tainso (TAN; n = 55), Wiae (WIA; n = 14). The colour coding of communities reflect the river basin in which they lie; Black Volta (black), Tain (dark blue), Tombe (light blue) Pru (green) and Daka (red).

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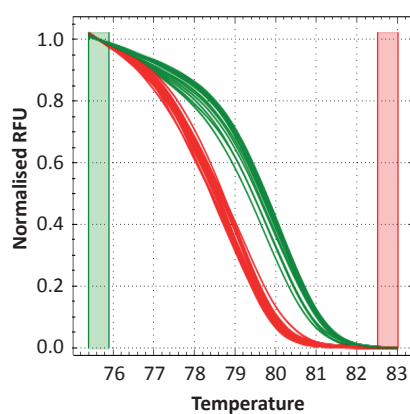
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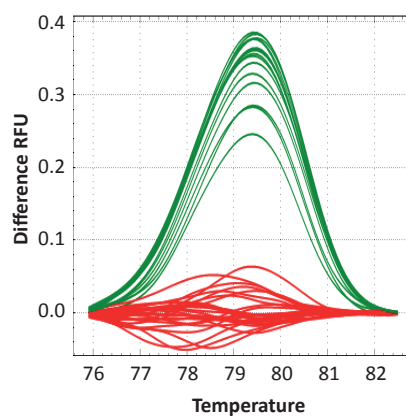
A

O. ochengi GTTTAGTTCTTGTGATTGTCATCGGGCTCCTTTTGATTCTTCTGAGTGTGAAAGTGAGTTGGTAAGGGGGTTTAATGT
O. volvulus GTTTGGTTCTTGTGATTGTCATCGGGCCCTTTTGATCTTCTGAGTGTGAAAGTGAGCTGGTAAGGGGGTTTAATGT

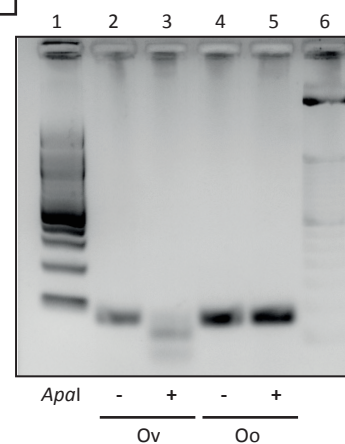
B

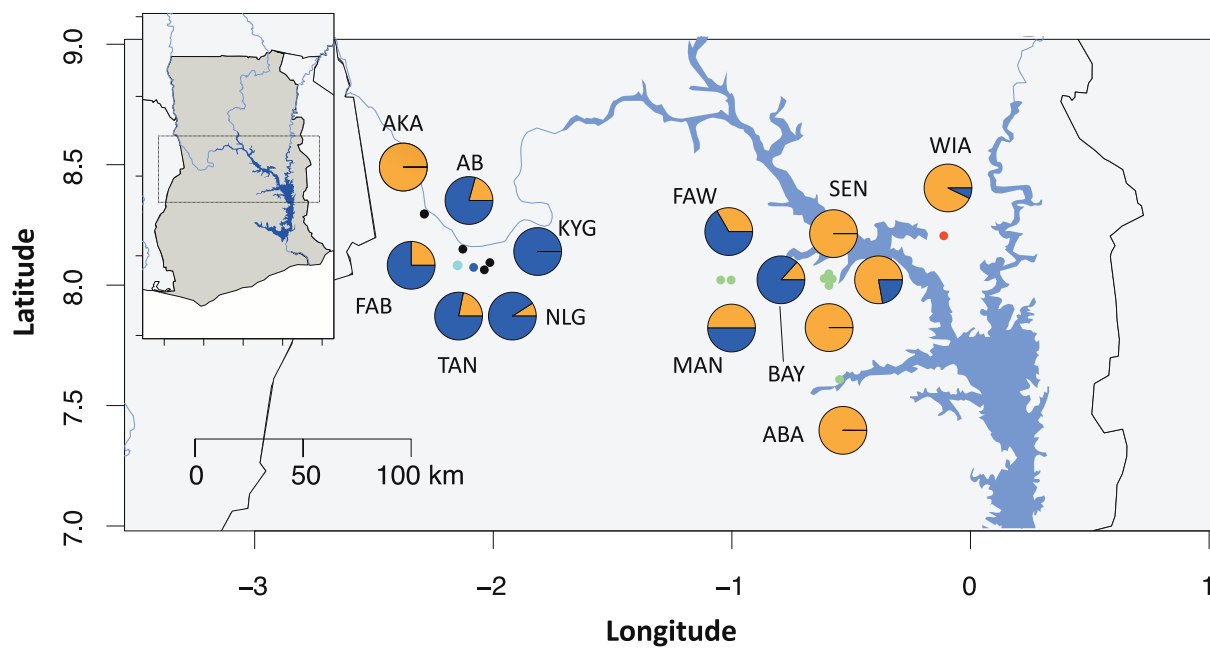


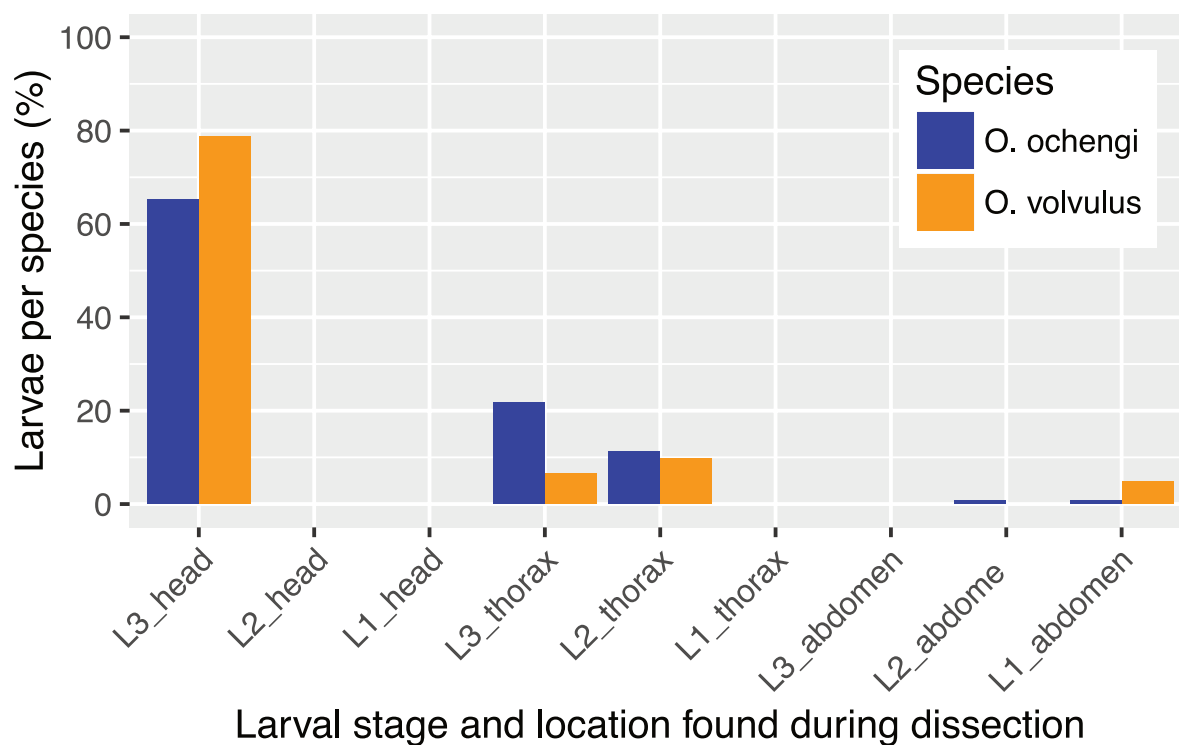
C



D







Supplementary Figure 1. Proportion of *O. volvulus* (orange) and *O. ochengi* (purple) larval life stages (L3, L2, & L1) found during dissection of the blackfly head, thorax and abdomen. Values reported are a percentage of the total larvae found within each species.