Discrimination between *Onchocerca volvulus* and *O. ochengi* filarial larvae in *Simulium damnosum s.l.* and their distribution throughout central Ghana using a 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.

Keywords:

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

68 **Background**

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

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

100 invasive source of parasite material. Genetic analysis of entomological derived
101 parasites will ultimately require dissection of infected blackflies to obtain individual
102 parasite larvae, and subsequently, an efficient and sensitive means to validate the
103 species of parasite collected.
104 Species characterisation of dissected larvae is challenging (although not
105 impossible) based on morphological characteristics [5, 7, 8]; it requires significant
106 time and expertise, and is therefore relatively low-throughput. Some studies report
107 using O-150-based diagnostic assays [9, 10], whilst others describe the prevalence of
108 *O. volvulus* without molecular confirmation [11-14]. Given that a number of
109 *Onchocerca* parasites, including the common cattle parasite *O. ochengi*, can be
110 transmitted by the same intermediate vector [5], it is likely that *O. volvulus* larval
111 stages may often be overestimated, and in turn, negatively influence the predictions
112 of disease transmission. In this study, we report the development of a versatile
113 molecular tool to discriminate the onchocerciasis parasite *O. volvulus* from other
114 *Onchocerca* species such as *O. ochengi* based on mitochondrial DNA (mtDNA)
115 sequence variation. This approach was validated using *Onchocerca* larvae dissected
116 from blackflies that were collected from three distinct regions in Ghana.
117
118 **Methods**
119 Blackflies were sampled from 14 communities in 5 river basins in central Ghana
120 during 2011 (number of blackflies (n) = 12031), 2012 (n = 9706) and 2013 (n = 9138)
121 using a human landing collection protocol as described previously [15]. Ethical
122 approval was obtained for the use of human vector collectors from the Institutional
123 Review Board of the Council for Scientific and Industrial Research, Ghana. All
124 blackflies caught were dissected to collect *Onchocerca* larvae (129 from the head, 51
125 from the thorax, and 5 from the abdomen used here), after which all larvae collected
126 were dried on microscope slides for preservation. In total, 160 infective (L3),
127 together with 21 pre-infective L2 and 4 L1 larvae were analysed (Supplementary
128 Table 1) using the molecular techniques described, which included 14 larvae
129 collected from 8 infected flies in the 2011 cohort, 24 larvae from 11 flies collected in
130 2012, and 147 larvae from 109 flies collected in the 2013 cohort. Individual larva
131 were recovered from slides by applying 2 µl of HPLC-grade water (Sigma) directly

132 onto the specimen and allowing it to rehydrate, so that the larva would detatch from
133 the slide or could be dislodged by a gentle scrape with a pipette tip. Each larva was
134 individually transferred to 20 µl of sample lysis solution (DirectPCR Lysis reagent
135 [MouseTail; Viagen Biotech] supplemented with 0.3 mg/ml (w/v) proteinase K
136 [Roche]), and incubated for 18 h at 55°C followed by 85°C for 1 h. Larval lysates were
137 diluted with HPLC-grade water 1/10 prior to PCR. Adult *O. volvulus* samples were
138 obtained as previously described [16]. Adult *O. ochengi* were collected from infected
139 cattle udders obtained from an abattoir in Ngaoundéré, Cameroon. Nodules were
140 collected from the skin, from which worms were extracted using forceps via a small
141 incision before being fixed in 95% ethanol. DNA was extracted from individual adult
142 *O. volvulus* and *O. ochengi* samples using an Isolate II Genomic DNA extraction kit
143 (Bioline, Australia) following the manufacturers' instructions.
144 High resolution melt analysis was performed after real time PCR amplification
145 of a 79-bp product using the Bio-Rad CFX system (Bio-Rad laboratories). A 10 µl
146 reaction mix was prepared by combining 5 µl of 2X SsoAdvanced Universal SYBR
147 Green qPCR Supermix (Bio-Rad Laboratories), 0.5 µl of each of SP_OVOO_79bp (5'-
148 GTTTGGTTCTTGTTGATTTG-3') and ASP_OVOO_79bp (5'-ACATTAAACCCCCTTACC-3')
149 primers (0.5 µM working concentration), 2 µl of HPLC grade water, and 2 µl of the
150 diluted larva lysate. The amplification protocol was performed as follows: 98°C for 3
151 min, followed by 45 cycles of 98°C for 10 sec, 59.5°C for 10 sec and 72°C for 10 sec.
152 The melt protocol was performed immediately after the amplification, and was as
153 follows: 95°C for 10 sec, followed by a 65°C annealing step from which the
154 temperature was increased by 0.2°C/plate read until a final temperature of 95°C.
155 PCR products generated from adult *O. volvulus* and *O. ochengi* samples were cloned
156 into the plasmid pGem-T-Easy (Promega) and sequenced to confirm the nucleotide
157 variants. All lysates, together with positive (cloned products and adult *O. volvulus*
158 and *O. ochengi* genomic DNA samples) and no-template controls (NTC), were
159 analysed in duplicate. Raw melt data was analysed using Precision Melt software
160 (version 1.2; Bio-Rad Laboratories), from which the data was exported into Microsoft
161 Excel for further quantitative analysis. Spatial (within flies i.e., head, thorax or
162 abdomen, and between river basins), temporal (sampling year) and larval stage (L1,
163 L2 or L3) differences between *O. volvulus* and *O. ochengi* were each examined using

164 a 2 × 3 chi-square (χ^2) test with 2 degrees of freedom, and a p-value < 0.05 was
165 considered to represent significant deviation from the null hypothesis.
166 A subset of post-PCR and HRM products were analysed by ApaI restriction
167 digest to confirm the *O. volvulus* specific digestion. A 20 µl reaction consisting of 10
168 µl PCR product together with 0.5 µl ApaI (50,000 U/ml; New England Biolabs), 2 µl of
169 CloneSmart buffer and 7.5 µl of HPLC-grade water was incubated for 3 h at 25°C,
170 after which the products were run on a 2% agarose gel at 100 V for 50 mins and
171 visualised using GelRed DNA stain (Biotum).
172
173 **Results**
174 Whole mitochondrial genome alignments of the *O. volvulus* (NC_001861; [17]) and
175 *O. ochengi* (obtained from genomic sequence available at
176 http://www.nematodes.org/genomes/onchocerca_ochengi/ ; Blaxter Lab, University
177 of Edinburgh) sequences was performed to identify a PCR compatible region that
178 contained (i) a restriction site that was unique to one species, and (ii) additional
179 polymorphism(s) that would result in a difference in the melting temperature
180 between amplicons generated for each of the two species. A 79-bp region spanning
181 8654- to 8733-bp of the *O. volvulus* mitochondrial genome was chosen that
182 contained three nucleotide T > C transitions (*O. volvulus*: C,C,C; *O. ochengi*: T,T,T)
183 between the mtDNA alignment of the two species, one of which was found in an
184 ApaI restriction site that is present in *O. volvulus* but absent in *O. ochengi* (Figure
185 1A). Melt curve analysis demonstrated a shift in Tm between the two sequences,
186 with a mean Tm of 79.8°C and 78.6°C for the *O. volvulus*- and *O. ochengi*-derived
187 sequences, respectively (Figure 1B). This translated into a significant and consistent
188 deviation in melt curves as depicted in the fluorescence difference plots (Figure 1C).
189 To confirm that the difference in melt curves was consistent with the prediction that
190 the ApaI restriction site was present in the *O. volvulus* sequences but not in the *O.*
191 *ochengi* sequences, 42 larvae-derived, 4 adult-derived and 2 cloned PCR products
192 were analysed by restriction digest, of which a representative gel is shown in Figure
193 1D. The melt curve and restriction digest data were 100% concordant in the samples
194 analysed, demonstrating that both approaches were equally predictive of the
195 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 that *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]).

227 The distribution of both species throughout the sampling region was not
228 uniform (Figure 2). Although no temporal differences between sampling years for
229 each species was seen (χ^2 = 1.075; df = 2, p = 0.292), a significant spatial trend was
230 observed that suggested that the western communities sampled had a lower
231 prevalence of *O. volvulus* (24.4%, n = 136; Black Volta, Tain and Tombe river basins),
232 a roughly equal prevalence of *O. volvulus* and *O. ochengi* in the central Pru river
233 basin (47.2% *O. volvulus*; n = 36), and a higher prevalence of *O. volvulus* (92.9%, n =
234 14) in the eastern-most Daka river basin (χ^2 = 29.888; df = 2, p = 1.618E-7). These
235 results are not necessarily in contrast to a recent investigation of persistent *O.*
236 *volvulus* with negligible *O. ochengi* transmission in Ghana [9]. Much of that study
237 was focused on two southern Ghanaian communities, and very few or no larvae
238 were found in the two communities that were shared with this study (Asubende &
239 Agborlekame, respectively); moreover, 3 of the 7 communities used by Lamberton *et*
240 *al.* were east of the Black Volta Lake, which would be consistent with high *O.*
241 *volvulus* prevalence observed in the eastern-most community (Wiae) sampled here.
242 We speculate that the high infection rate reported and difference in prevalence of *O.*
243 *ochengi* in a number of study regions presented here is correlated with the high
244 numbers of cattle in the north-western river basins, particularly during the dry
245 season (December to April). However, given that the sample size of larvae for many
246 communities was low (median = 6.5, range = 1-55 larvae/community; limited by the
247 number of larvae present in the blackfly populations and therefore by the number of
248 blackflies screened), further sampling is required to support these findings,
249 particularly in the Daka river basin where *Onchocerca* larvae were obtained from
250 only a single community out of the five communities in which flies were sampled.
251
252 **Conclusions**
253 A simple but versatile tool that discriminates between *O. volvulus* and *O. ochengi*
254 single larvae obtained by dissection of the blackfly intermediate host is described,
255 and offers a number of advantages over traditional O-150-based speciation assays.
256 By exploiting the multicopy nature of the mitochondrial genome, this assay is very
257 sensitive, requiring only 1/100th of a single larva, thus leaving sufficient material for
258 further genetic analysis. Although both *O. volvulus* and *O. ochengi* samples could be

259 distinguished easily via HRM or RFLP assays, no effort was made to identify other
260 described *Onchocerca* species that are potentially present [18-20]; further work is
261 required to characterise melt profiles and/or restriction sites from additional
262 *Onchocerca spp.* voucher specimens as they become available. In the context of
263 onchocerciasis entomological surveillance, which aims to detect transmission of the
264 human pathogen, this study demonstrates the need to accurately and efficiently
265 determine the identity of larvae in the vector in order to correctly estimate parasite
266 transmission. In Ghana, nationwide sampling of blackfly populations is necessary to
267 determine the distribution of *Onchocerca* spps. Implementation of genetic tools such
268 as those described here will inform the appropriate control strategy needed by
269 onchocerciais control programmes as they strive towards onchocerciasis elimination
270 in Ghana.
271

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.

Acknowledgments:

We thank Mr. Francis Veriegh for contributing to the blackfly dissection, and PD Dr Adrian Streit, Dr Annette Kuesel and Dr Aime Adjami for helpful suggestions toward the manuscript. This investigation received financial support from TDR, the Special Programme for Research and Training in Tropical Diseases, co-sponsored by UNICEF, UNDP, The World Bank and WHO, the German Research Foundation (DFG Re 1536/5-1) and the European Foundation Initiative for Neglected Tropical Diseases. We thank Dr Mark Blaxter and Dr Benjamin Makepeace for access to the unpublished *O. ochengi* genome sequence, which was generated with support of the EU-funded programme "Enhancing Protective Immunity Against Filariasis".

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)		
				O. volvulus	*O. ochengi*	*O. volvulus*	*O. ochengi*	*O. volvulus*	*O. ochengi*	
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

297 **Figure 1.** Genetic discrimination of *O. volvulus* and *O. ochengi*. (A) Sequence of the
298 PCR 79-bp amplicon used to discriminate between *O. volvulus* and *O. ochengi*.
299 Nucleotide differences between the two species are highlighted in bold, and the
300 ApaI restriction site present in the *O. volvulus* sequence is underlined. (B)
301 Normalised HRM data depicting melt curves from individual *O. volvulus* (green) and
302 *O. ochengi* (red) samples. Data presented includes cloned positive control 79-bp
303 products (pGem_Ov and pGem_Oo), adult DNA, and 4 larvae samples from both
304 species, each in duplicate. (C) The same samples are presented as in (B), showing the
305 difference curves normalised to the *O. ochengi* group. (D) Representative RFLP
306 analysis of amplicons generated in the HRM assay for each species, showing
307 digestion of the *O. volvulus* sequence, but not the *O. ochengi* sequence, with ApaI.
308
309 **Figure 2.** Distribution of *O. volvulus* and *O. ochengi* throughout Ghana. Pie charts
310 depict relative prevalence of *O. volvulus* (orange) and *O. ochengi* (purple) in each
311 community. Communities sampled in central Ghana (see insert) and the number of
312 samples analysed are as follows: Abua (ABA; n = 1), Agbelekame I (AB; n = 54),
313 Akanyakrom (AKA; n = 5), Asubende (ASU; n = 9), Baaya (BAY; n = 15), Beposo (BEP, n
314 = 1), Fawoman (FAW; n = 3), Fawoman-Banda (FAB, n = 8), Kyingakrom (KYN; n = 3),
315 Mantukwa (MAN; n = 4), New Longoro (NLG; n = 11), Senyase (SEN; n = 3), Tainso
316 (TAN; n = 55), Wiae (WIA; n = 14). The colour coding of communities reflect the river
317 basin in which they lie; Black Volta (black), Tain (dark blue), Tombe (light blue) Pru
318 (green) and Daka (red).
319

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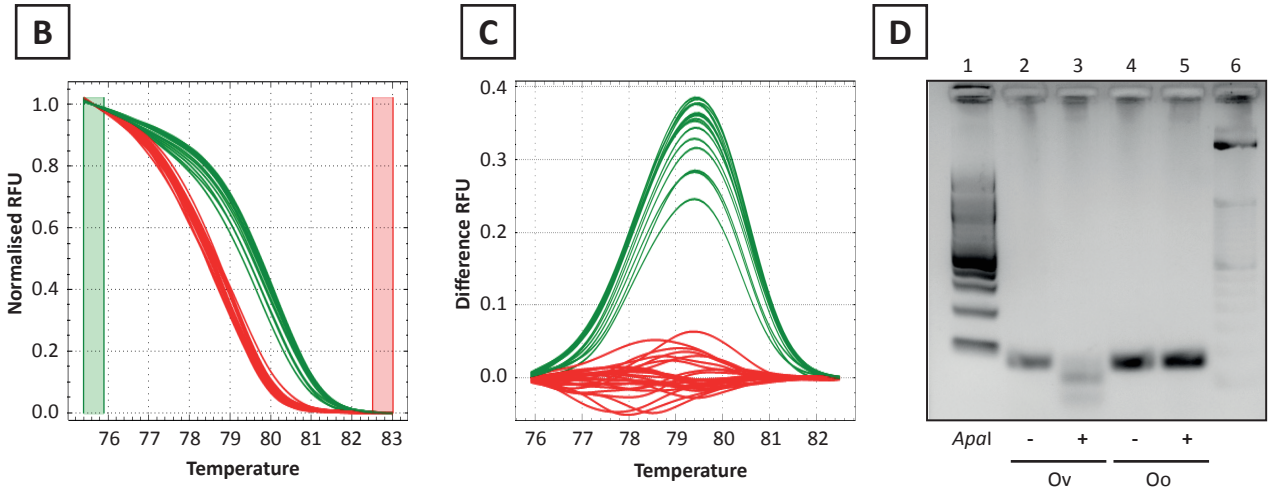
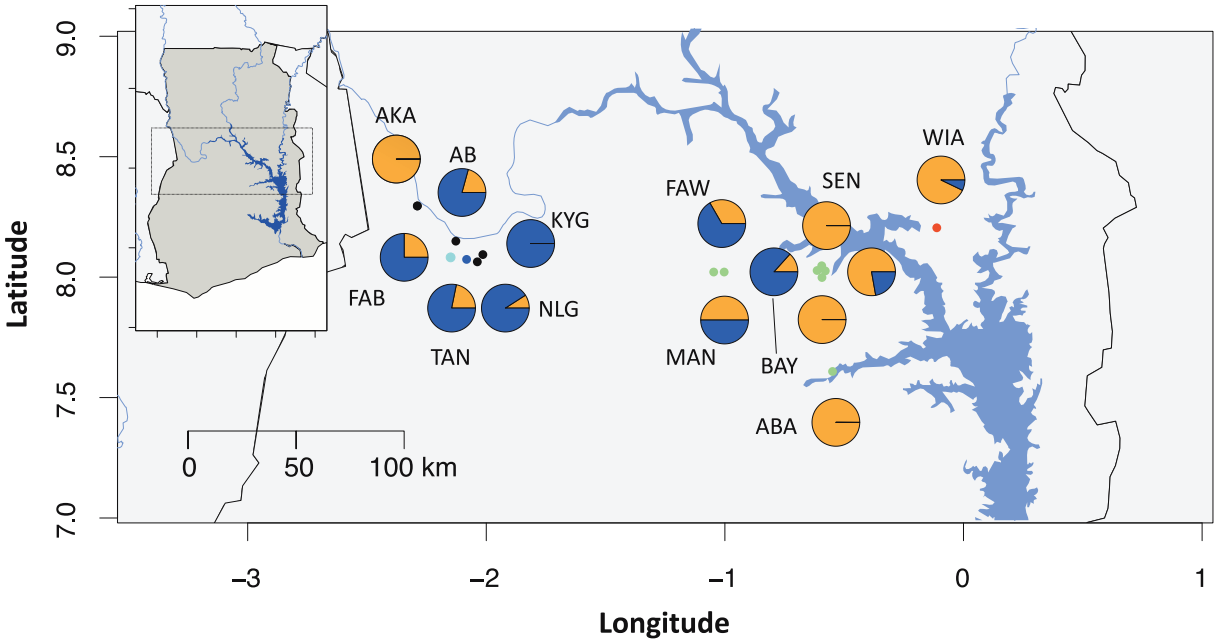
406

A

O. ochengi GTTTAGTTCTTGTTGATTTGCATCGGGC**T**CCTTTTGAT**T**TTTCTGAGTGTGAAAGTGAG**T**TGGTAAGGGGGTTTAATGT

O. volvulus GTTTGGTTCTTGTTGATTTGCATCGGGC**C**CCTTTTGAT**C**TTTCTGAGTGTGAAAGTGAG**C**TGGTAAGGGGGTTTAATGT

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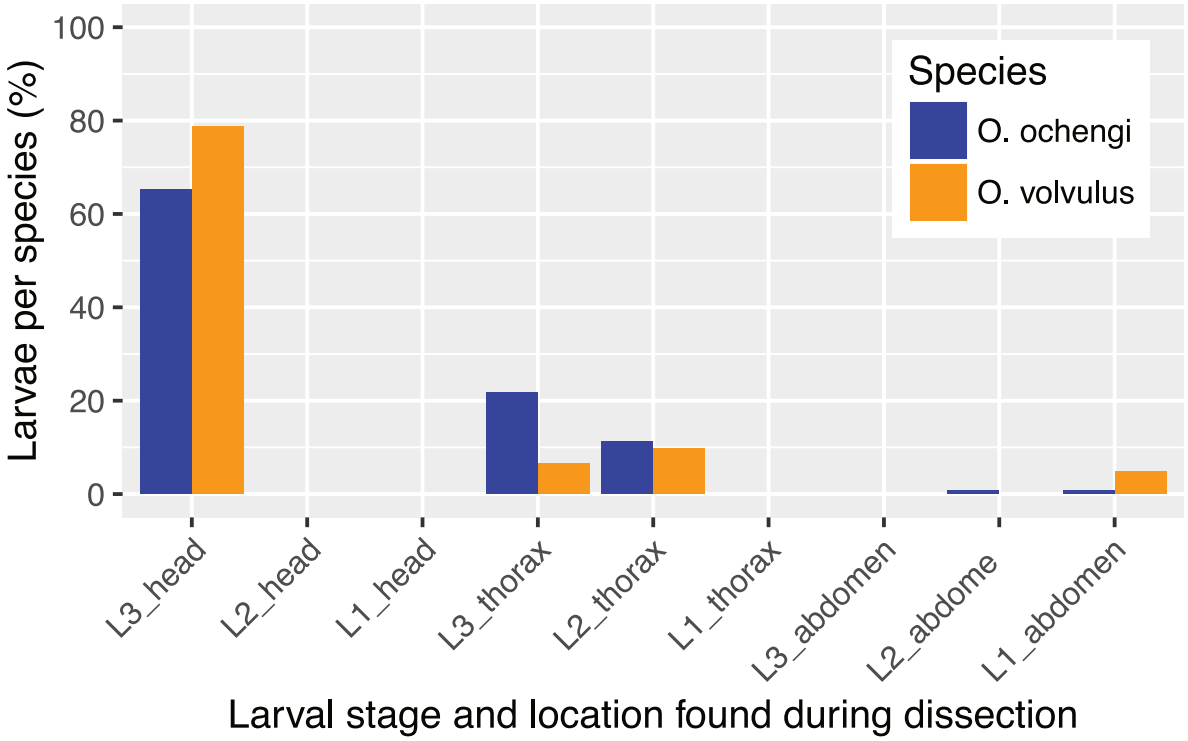

Supplementary Figure 1. Proportion of *O. volvulus* (orange) and *O. ochengi* (purple) larval life stages (L3, L2, & L1) found during dissection of the blackfly head, thorax and abdomen. Values reported are a percentage of the total larvae found within each species.