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Biodiversity of *Simulium metallicum sensu lato* (Diptera: Simuliidae), a complex of Neotropical vectors associated with human onchocerciasis

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ABSTRACT

The polytene chromosomes of 130 larvae of the Neotropical *Simulium metallicum* complex from Brazil, Costa Rica, and Ecuador revealed five cytoforms, including three ('M', 'N', and 'O') that are new and two ('B' and 'J') that represent range extensions of up to 850 km. The discovery of three new cytoforms brings the total number in the complex to 17. Cytoforms 'B', 'J', and 'N' are reproductively isolated from one another, and their species status is corroborated by morphological evidence. None of the three new cytoforms is known from current or historical onchocerciasis foci, although 'M' inhabits the periphery of the former Ecuadorian Santiago onchocerciasis focus a mere 30 km to the west. The number of fixed chromosomal differences, as many as 24, separating some members of the *S. metallicum* complex far exceeds that known between members of any other simuliid species complex. Two distinct groupings can be diagnosed within the *S. metallicum* complex, based on at least eight fixed chromosomal rearrangements and structural characters in the larval stage. Consequently, a recommendation is made to recognize the *S. horacioi* complex and the *S. metallicum* complex *sensu stricto*. Recognition of two separate complexes provides potential phylogenetic content with predictive power for understanding biological phenomena such as vector potential.

1. Introduction

One of the preeminent recent achievements in medical entomology was the elimination or interruption of the transmission of human onchocerciasis in 11 of the 13 foci in the New World, largely via a relentless ivermectin program; the only remaining foci are along the Amazonian border area of Brazil and Venezuela (WHO, 2016). Most evidence suggests that onchocerciasis entered the New World from Africa via the slave trade (Procunier and Hirai, 1986; Morales-Hojas et al., 2007). Although the precise time of the introduction(s) has not been established, onchocerciasis was first brought to light in the Western Hemisphere—in the Guatemalan highlands—in 1915 by the physician Rodolfo Robles (1916).

A strong foundation in the systematics of insect vectors, such as black flies, is at the core of epidemiological understanding and control of vector-borne diseases (Procunier, 1989). At least nine species or species complexes of Simuliidae in the New World have been incriminated as vectors of *Onchocerca volvulus* (Leuckhart), the causal agent of human onchocerciasis (summarized by Adler and McCreadie, 2009). One of the premiere historical vectors from Mexico to northern Venezuela was *Simulium metallicum* Bellardi, established chromosomally in 1982 to be a species complex (Mantel, 1982). Subsequent chromosomal studies through 1999 recognized 14 cytoforms, at least seven of which were considered valid species (Hirai 1983, 1985; Conn et al., 1989; Conn, 1990; Millest, 1990; Arteaga and Muñoz de Hoyos, 1999). The cytoforms are ecologically differentiated (Millest et al., 1999), and seven of them occur in former onchocerciasis foci (Conn et al., 1989; Millest, 1990; Grillet et al., 1995). Morphological differences in larvae of the complex often correspond with cytoforms (Conn et al., 1989; Millest, 1990; Hamada and Fouque, 2001). COI barcoding of *S. metallicum s. l.* from six Neotropical countries produced five distinct clusters, reinforcing the chromosomal and ecological evidence of a diverse species complex (Hernández-Triana et al., 2014, 2015).

The *S. metallicum* complex has a broad geographic distribution, ranging from the Mexican border with the United States, into the Caribbean (Jamaica), and south to southern Brazil and northern Peru (Adler and Crosskey, 2016). Members of the complex are common, nearly isomorphic simuliids recognized by the small, subtriangular postgenal cleft of the larvae; long, six-filamented pupal gill; rounded basal protuberance on the elongated male gonostylus; and iridescent

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Table 1
Collections of larvae of the *Simulium metallicum* complex from Brazil, Costa Rica, and Ecuador used in chromosomal analyses.

Site	Location	Latitude, longitude	Elevation (m asl)	Date	Cytoform (*n*)
1	Brazil, São Paulo State, Botucatu municipality, Indiana farm	22°54′36”S 48°24′00”W	679 m	11 May 2004	O (31)
2	Costa Rica, San José Province, Moravia, Zurqui de Moravia Creek[a] [ZADBI-1224–1227]	10°02′50”N 84°00′30”W	1586 m	5, 9 Aug 2013	B (12), J (34), N (15)
3	Ecuador, Esmeraldas/Carchi Province, E10 Hwy.	00°51′44”N 78°27′11”W	570 m	21 May 2014	M (6)
4	Ecuador, Esmeraldas Province, trickle paralleling road	00°48′47”N 78°29′29”W	1287 m	22 May 2014	M (32)

[a] Site details are given by Borkent and Brown (2015).

Table 2
Frequency of rearranged chromosomal homologues in larval samples of the *Simulium metallicum* complex from Brazil, Costa Rica, and Ecuador.

	Brazil		Costa Rica		Ecuador	
Site	1	2	2	2	3	4
Cytoform	‘O’	‘B’	‘J’	‘N’	‘M’	‘M’
Females:Males	17:14	8:4[a]	18:16[a]	6:9	4:2	16:16[b]
IS − 1[c]			1.00	1.00		
IS-4			*[d]			
IS N.O. suppression		*[e]				
IS-5 + hb		*[e]				
IL-1			1.00	1.00		
IL-2			1.00	1.00		
IL-4			1.00			
IL-15						0.02
IIS-1			1.00	1.00		
IIS-3			1.00	1.00		
IIS-4			1.00	1.00		
IIL-1		1.00				
IIL-2		1.00				
IIL-3		1.00				
IIL-4			1.00	1.00		
IIL-5			1.00	1.00		
IIL-6			1.00	1.00		
IIL-7			1.00	1.00		
IIL-8				1.00		
IIL-22					*[f]	*[f]
IIL-25					*[f]	*[f]
IIL-26			0.01			
IIL-27				0.20		
IIL-28				1.00		
IIL-29	1.00					
IIL eb				0.03		
IIL tb				0.03		
IIIL-1		1.00				
IIIL-2		1.00				
IIIL-3		1.00				
IIIL-4		1.00				
IIIL-15			0.03			
IIIL-17				1.00		
IIIL-18			1.00	1.00		
IIIL-19				1.00		
IIIL-20			1.00	0.30		
IIIL-21			1.00			
IIIL-22	1.00					
Heterozygotes[g]	0.00	0.08	0.09	1.00	0.00	0.03

[a] 1 female and 1 male of ‘B’ and 1 male of ‘J’ were infected with an unidentified mermithid nematode.
[b] 1 female and 1 male of ‘M’ were infected with an unidentified microsporidium.
[c] Italicized inversions were fixed.
[d] IS-4 was absolutely linked to the X chromosome.
[e] Heterozygous suppression of the nucleolar organizer occurred in 2 of 4 males (but in 0 of 8 females). Nucleolar suppression linked to the Y chromosome is known in all other previously analyzed populations of ‘B’ (Conn et al., 1989). If nucleolar suppression was Y-linked in our sample, IS-5 + hb (heterozygous) in 2 female larvae represented an alternative X chromosome.
[f] IIL-22 and IIL-25 were absolutely linked to the X chromosome; the Y chromosome was always standard for IIL-25 and infrequently (11% of male larvae) for IIL-22.
[g] Mean number of heterozygous autosomal inversions per larva.

longitudinal vittae on the shiny black female scutum (Shelley et al., 2002, 2010).

Our objective was to provide an expanded geographic analysis of the cytogenetics of the *S. metallicum* complex, including material from Brazil and Ecuador, representing the most southern populations studied to date, and from Costa Rica additional to the one small population previously analyzed by Mantel (1982) and Conn et al. (1989). Our study increases the number of cytoforms from 14 to 17.

2. Material and methods

Larvae of the *S. metallicum* complex were hand collected with forceps from small (≤ 1 m wide) streams, permanent in Brazil and Costa Rica, and temporary in Ecuador (Table 1); they were fixed in three changes of 1:3 acetic ethanol. Larvae (penultimate and ultimate instars) for chromosomal preparation were presorted according to head-spot pattern: negative or positive (*sensu* Millest, 1990). After chromosomal analysis, one gill histoblast from each mature larva was dissected to link its configuration with cytoform.

Chromosome squashes from larval silk glands, plus gonads, were prepared by routine Feulgen methods (Adler et al., 2016a). Larval gender was determined by gonadal shape (elongated in females, subspherical in males) and confirmed on slide preparations by the presence of sporadically distributed (female) or clustered (male) meiotic chromosomes.

Selected chromosomes were photographed under oil immersion on an Olympus BX40 compound microscope, and photographic negatives were scanned, or chromosomes were photographed on a BH-2 Olympus microscope fitted with a Jenoptik ProgRes® SpeedXT Core 5 digital camera; images from both methods were imported into Adobe® PhotoShop® Elements 8 to construct chromosomal maps. Larval carcasses were transferred to 80% ethanol and deposited in the Clemson University Arthropod Collection, Clemson, SC, along with 1 pupal exuviae of cytoform ‘J’, 11 pupae of cytoform ‘M’ (80% ethanol), 5 females and 7 males reared from pupae of cytoform ‘M’ (pinned with exuviae in glycerin microvials), and photographic negatives of representative chromosomes.

Section numbering (1–100) follows that of the standard map of Conn et al. (1989), beginning with the short arm (S) of chromosome I and continuing through chromosome II to the end of the long arm (L) of chromosome III. We use the numbering of the standard map because the section numbering differs for some rearranged sequences presented as photomaps by Conn et al. (1989); for instance the IIIL sequence of cytoform ‘J’ in Fig. 13b of Conn et al. (1989) shows correct breakpoints for IIIL-15, but their numbered section limits within the inversion differ from those of the IIIL standard (Fig. 9 of Conn et al., 1989). Rearrangements are indicated by brackets or arrows on our photographic maps. Terminology of landmarks follows that of Rothfels et al. (1978). Inversions fixed across or within cytoforms are italicized in the text and on our photomaps. Previously known inversions are designated with the same numbers used by Conn et al. (1989) and Arteaga and Muñoz de Hoyos (1999); novel inversions are designated based on the last-