RESEARCH ARTICLE

River-specific macrogenomic diversity in *Simulium guianense* s. *l.* (Diptera: Simuliidae), a complex of tropical American vectors associated with human onchocerciasis

Peter H. Adler¹ *, Neusa Hamada², Jeane Marcelle Cavalcante do Nascimento², Maria Eugenia Grillet³

¹ Department of Plant and Environmental Sciences, Clemson University, Clemson, South Carolina, United States of America, ² Instituto Nacional de Pesquisas da Amazônia, Coordenação de Biodiversidade, Curso de pós-graduação em Entomologia, Manaus, Amazonas, Brazil, ³ Laboratorio de Biología de Vectores y Parásitos, Instituto de Zoología y Ecología Tropical, Facultad de Ciencias, Universidad Central de Venezuela, Caracas, Venezuela

*padler@clemson.edu

Abstract

*Simulium guianense* Wise is a Latin American vector complex of black flies associated with transmission of the causal agent of human onchocerciasis (river blindness). An analysis of the chromosomal banding patterns of 607 larvae of *S. guianense* s. *l.* revealed a high level of variation involving 83 macrogenomic rearrangements across 25 populations in Brazil, French Guiana, and Venezuela. The 25 populations were assigned to 13 cytoforms (A1, A2, B1–B4, C, D, E1–E4, and F), some of which are probably valid species. Based on geographical proximity, a member of the B group of cytoforms probably represents the name-bearing type specimen of *S. guianense* and the primary vector in the last-remaining onchocerciasis foci in the Western Hemisphere. Cytoform B3 in Amapá State is implicated as an anthropophilic simuliid in an area currently and historically free of onchocerciasis. Distributions of cytoforms are associated with geography, elevation, and drainage basin, and are largely congruent with ecoregions. Despite extraordinarily large larval populations of *S. guianense* s. *l.* in big rivers and consequent production of female flies for dispersal, the cytoforms maintain their chromosomal distinction within individual rivers, suggesting a high degree of fidelity to the specialized breeding habitats—rocky shoals—of the natal rivers.

Introduction

Cryptic species and genetically differentiated forms are common in medically important Diptera [1–4]. The largest aggregates of cryptic species among blood-feeding arthropods are in the family Simuliidae, and include species complexes responsible for vector-borne diseases, such as human onchocerciasis or river blindness [5]. The discovery that simuliid vectors are complexes of multiple, structurally cryptic species ranks among the significant advancements in
the battle against onchocerciasis [6]. Cryptic taxa typically differ in breeding habitats, geographical distributions, vectorial capacity, and other traits of significance for ecology, epidemiology, and vector control [1,7,8].

Human onchocerciasis was introduced into the New World via the slave trade, eventually becoming established in six countries [9]. In the Americas, this parasitic disease was formerly prevalent in 13 endemic foci in Brazil, Colombia, Ecuador, Guatemala, Mexico, and Venezuela, where more than half a million people were considered at risk of infection [10]. By 2014, a relentless mass-distribution program of ivermectin had interrupted or eliminated transmission in 11 of the 13 Latin American foci [11]. Challenges remain in the two Amazonian foci straddling Brazil and Venezuela, where more than 29,500 people, mostly of the semi-nomadic indigenous Yanomami population, live in an active transmission zone [10,12]. Transmission of the disease agent, *Onchocerca volvulus*, occurs in the lowlands (0–500 m above sea level, asl) and uplands (500–1200 m asl) of the Amazonian region in Brazil and Venezuela [12,13], where *Simulium guianense* Wise is the predominant human-biting black fly and main vector of *O. volvulus* in the hyperendemic and mesoendemic areas [12,14,15]. The immature stages of *S. guianense* inhabit primarily large, swift, open-canopy rivers [13,15].

Geographical variation in the epidemiological importance and biting behavior of *S. guianense*, such as the degree of anthropophily versus zoophily [14,16], provided early hints of a species complex. A chromosomal analysis of *S. guianense* provided additional evidence of hidden diversity, revealing four cytological forms, based on five collections [17]. The nearly one-to-one correspondence between the number of cytologically distinct populations and the number of collections suggested additional forms across the distribution [18] of *S. guianense* in Brazil and other northern South American countries. A subsequent chromosomal study of three more populations, coupled with a molecular barcoding analysis of eight populations, provided further evidence of diversity within *S. guianense* [19], as did additional barcoding studies [20,21].

To determine the extent and patterns of differentiation within the *S. guianense* complex, we conducted a geographically broad macrogenomic study, based on rearrangements of the polytene chromosomes in the larval silk glands. Our study complements that of Crainey et al. [22], who used a molecular approach to examine genetic structure of *S. guianense* at eight sites, most of which correspond to sites in our study area.

Materials and methods

Ethics statement

In Brazil, black fly collections were made on public or owner-permitted private lands. A permit to collect zoological specimens was provided by SISBIO (permanent permit number 10873–1 to NH). In French Guiana, collecting was arranged by Institut Pasteur. In Venezuela, a special collection permit from INPARQUES (National Institute of Natural Parks) allowed us to sample black flies in the Gran Sabana region of southern Venezuela. No collections involved endangered or protected species.

Study area

In Brazil, *S. guianense s.l. (sensu lato)* was collected in 12 states representing four geographic regions: four in the North Region, three in the Northeast, two in the Central-West, and three in the Southeast (Table 1; Figs 1–3). Although *S. guianense s.l.* has been reported in Parana and Santa Catarina states in the South region [18], we did not find it despite our collecting efforts in the three states of this region. Sites with *S. guianense s.l.* were located in the Amazonia, Atlantic Forest (Mata Atlântica), Caatinga, and Cerrado biomes (Table 1). In French
Table 1. Collection sites for larvae of *Simulium guianense* s.l. analyzed chromosomally.

| Country, Region, State | Drainage Basin | Location | River Width (m) | Water Temp. (°C) | Elevation (m) | Collection Date | Cytoform (n) |
|------------------------|----------------|----------|-----------------|------------------|--------------|----------------|--------------|
| Brazil                 |                |          |                 |                  |              |                |              |
| Amapá                  | Oiapoque       | Oiapoque, Rio Oiapoque, Cachoeira Grande, 03°48'13"N 51°52'31"W | 700 | 27.8 | 7 | 9/VIII/2011 | B3 (63) |
| Amapá                  | Araguari       | Serra do Navio, Rio Água Fria, 00°48'34"N 51°58'45"W | 20 | 24.8 | 78 | 3/VII/2011 | B2 (8) |
| Amapá                  | Araguari       | Amapá, Ferreira Gomes, Rio Tracajatuba, 01°01'22"N 51°05'35"W | 10 | 26.3 | 14 | 11/VIII/2011 | B3 (18) |
| Amazonas               | Amazonas       | Presidente Figueiredo, below dam in Rio Pitinga, Cachoeira 40 ilhas, 00°53'39"S 59°37'00"W | 50 | 29.0 | 66 | 7/II/2006 | B2 (31) |
| Pará                   | Xingu          | Altamira, Rio Xingu, Quebra Canela, downstream of Altamira, 03°18'06"S 52°02'34"W | 4900 | 30.4 | 86 | 26/X/2007 | D (42) |
| Pará                   | Xingu          | Altamira, Rio Iriê, 03°49'16"S 52°38'16"W | 1000 | 31.5 | 122 | 25/X/2007 | D (9) |
| Roraima                | Amazonas       | Caroebe, Ramal 37, Rio Caroebe, Fazenda Cachoeirinha, 00°57'09"N 59°37'00"W | 20 | 28.2 | 146 | 23/III/2012 | B2 (25) |
| Roraima                | Branco         | Amajari, Fazenda São Sebastião, Rio Ereú, 04°02'00"N 61°23'10"W | 30 | 31.3 | 143 | 26/III/2012 | B2 (24) |
| Roraima                | Branco         | Caracarai, Rio Branco, Cachoeira Bem Querer, 01°55'07"N 61°00'01"W | 600 | 29.0 | 48 | 20/III/2013 | B2 (7) |
| Roraima                | Branco         | Cantá, Rio Cachorrro, bridge, 02°15'19"S 57°40'02"W | 20 | 28.0 | 66 | 28/III/2012 | B2 (27) |
| Northeast              |                |          |                 |                  |              |                |              |
| Ceará                  | Parnaiba       | Rio Pirangi, waterfall Pirapora, 03°33'32"S 41°21'57"W | 20 | 26.1 | 355 | 6/II/2011 | F (10) |
| Maranhão               | Tocantins      | Carolina, Rio Farinha, 06°51'49"S 47°28'08"W | 450 | 25.0 | 168 | 16/II/2000, 11/II/2001 | C (32) |
| Piauí                  | Pamaiba        | Barras, Rio Longá, near bridge, 04°12'16"S 42°14'21"W | 170 | 29.6 | 75 | 27, 28/V/2011 | F (29) |
| Piauí                  | Pamaiba        | Piracurucu, Rio Jacareí, BR 343, bridge, 03°43'59"S 41°40'54"W | 20 | 28.4 | 74 | 2/VI/2011 | F (11) |
| Central-West           |                |          |                 |                  |              |                |              |
| Góias                  | Tocantins      | Rio Tocantins and Rio Mucambã, near Minaçu | - | - | 305 | 11, 14/XI/1991 | A1 (36) |
| Góias                  | Paraná         | Rio Verde, Rio Verdão, 17°32'34"S 30°33'33"W | 150 | 20.0 | 523 | 20/V/2006 | E4 (43) |
| Mato Grosso            | Araguaia       | Nova Xavantina, Travessão das facas, Rio das Mortes, 14°48'03"S 52°38'38"W | 200 | 22.9 | 276 | 7/II/2012 | E3 (15) |
| Mato Grosso            | Araguaia       | Barra do Garça, Rio Araguaia, Travessão da Pitomba, 15°53'07"S 52°06'10"W | 210 | 24.0 | 293 | 13/II/2012 | E2 (45) |
| Mato Grosso            | Tapajós        | Santa Rita do Trivelato, Rio Teles Pires, Salto Magessi, 13°34'34"S 55°16'00"W | 160 | 29.2 | 356 | 2/XI/2012 | B1 (23) |
| Southeast              |                |          |                 |                  |              |                |              |
| Espírito Santo         | Doce           | Sooretama, Rio São José, 19°07'33"S 40°14'26"W | 60 | - | 36 | 3/V/2013 | E1 (30) |
| Minas Gerais           | São Paulo      | Januaria, Rio Pandeiros (cachoeira), 15°30'42"S 44°45'11"W | 32 | 23.5 | 500 | 2/VI/2014 | A2 (21) |
| Minas Gerais           | São Paulo      | Terra Roxa, Rio Pardo, 20°48'20"S 48°15'22"W | 200 | 26.0 | 477 | 17/X/2005 | E3 (20) |
| French Guiana          | Maroni         | Mariposoulá, Rio Maroni, 03°27'53"N 54°00'15"W | 130 | 26.0 | 106 | 17/V/1999 | B2 (22) |

(Continued)
Guiana, *S. guianense* was collected from the Maroni River, which originates on the northern slope of the Tumucumaque Mountains near the Brazilian border and runs through a dense tropical rain forest to the Atlantic Ocean [23]. In Venezuela, *S. guianense s.l.* was collected in Canaima National Park of the Gran Sabana region in the southeastern corner of Bolívar state [24]. Most of the Gran Sabana uplands lie between 500 and 1500 m above sea level. The area is covered mainly by treeless savannas interspersed with montane and gallery forests.

To provide context for interpreting our results, we use the freshwater ecoregions based on the composition and distribution of freshwater fish species [25]. The ecoregions in which our collections are represented are briefly characterized as follows [26]:

**Amazoneas Estuary & Coastal Drainages.** This ecoregion, in the Amazônia biome, lies mostly below 100 m asl and includes large river deltas in the lower Amazon River basin and adjacent coastal drainages that enter the Atlantic Ocean.

**Amazones Guiana Shield.** Located in the Amazônia biome, this ecoregion includes the river basins that drain much of the southern slopes of the Guiana Shield and flow into the Amazon River. The rivers are black, clear, or whitewater, with elevations reaching 2100 m asl.

**Guianas.** Included in the Amazônia biome, this ecoregion comprises the predominantly flat coastal lowlands of the Guiana Shield, reaching elevations of 1286 m asl. It includes rivers that flow into the Atlantic Ocean from the northern and eastern slopes of the Guiana Shield.

**Northeastern Mata Atlantica.** Located in the Atlantic Forest biome, this ecoregion comprises all coastal drainages in eastern Brazil, with the rivers flowing into the Atlantic Ocean. The area has a diverse landscape, such as plateaus, valleys, and crystalline mountains reaching elevations of 2890 m asl.

**Orinoco Guiana Shield.** Located in the Amazônia biome, this area includes the lowlands and highlands of the Guiana Shield, with forested lowlands, rolling high plains, rounded hills, and tepuis. The streams and rivers are typically clear or blackwater and often have rapids and waterfalls; they flow into the Rio Orinoco and Atlantic Ocean. Elevations reach 2800 m asl.

**Parnaiba.** Located in the Caatinga and Cerrado biomes, this ecoregion covers the drainage basin of the Rio Parnaiba and coastal drainages, with elevations ranging from sea level to more than 900 m asl. Its rivers flow into the Atlantic Ocean.

**São Francisco.** Occupying areas in three biomes—Cerrado, Caatinga, and Atlantic Forest (the smallest portion)—this ecoregion encompasses all drainages of the São Francisco River basin [27], from sea level to 1000 m asl.

**Tapajós—Juruena.** This ecoregion includes areas in the Amazônia biome in the north and the Cerrado in the south. The basin of the Rio Tapajós and its tributaries constitute the freshwater environment of this area, with drainages flowing into the Amazon River. Elevations range from 28 m to 873 m asl.

---

**Table 1.** (Continued)

| Country, Region, State | Drainage Basin | Location | River Width (m) | Water Temp. (˚C) | Elevation (m) | Collection Date | Cytoform (n) |
|------------------------|----------------|----------|-----------------|-----------------|---------------|----------------|--------------|
| Venezuela              | Caroni         | Grand Savana National Park, Rio Parupa, 05˚40'38"N 61˚32'43"W | 10              | 22.9            | 1270          | 15/III/2007     | B4 (38)     |
| Bolivar                | Caroni         | Grand Savana National Park, Rio Yuruani, 05˚05'16"N 61˚05'56"W | 30              | 24.5            | 870           | 12/III/2007     | B4 (14)     |

1 *n* = Number of larvae completely analyzed chromosomally.

2 The two Minaçu (Goiás state) sites of Charalambo et al. [17] were not sampled in the present study, but are included as a single entry for completeness; river width and temperature were not stated.

https://doi.org/10.1371/journal.pone.0181679#t001
Tocantins—Araguaia. This ecoregion includes the Tocantins and Araguaia river basins in the Amazônia and Cerrado biomes. The drainages flow into the Rio Pará, Amazon estuary, or Atlantic Ocean. The heterogeneous landscape ranges from 25 m to 1600 m asl.

Upper Paraná. This ecoregion covers areas in the Atlantic Forest and Cerrado biomes from about 120 m to more than 1500 m asl. The basin of the upper Rio Paraná and its tributaries constitute the freshwater environment of this ecoregion. The drainages flow into the Lower Paraná River.
Fig 2. Sampling sites for *Simulium guianense* cytoforms A and B. A. Pandeiros River, Minas Gerais state. B. Teles Pires River, Mato Grosso state. C. Pitinga River, Amazonas state. D. Branco River, Roraima state. E. Oiapoque River, Amapá state. F. Yuruani River, Bolivar state.

https://doi.org/10.1371/journal.pone.0181679.g002
Fig 3. Sampling sites for *Simulium guianense* cytoforms D, E, and F. A. Xingu River, Pará state. B. São José River, Espírito Santo state. C. Araguaia River, Mato Grosso state. D. Pardo River, São Paulo state. E. Mortes River, Mato Grosso state. F. Longá River, Piauí state.

https://doi.org/10.1371/journal.pone.0181679.g003
Xingu. Located in the Amazônia (about 90% of the total area) and Cerrado biomes, the Xingu ecoregion includes the basins of the Rio Xingu, with drainages flowing into the Amazon River. Elevations reach 800 m asl.

Collection of larvae
Larvae of *S. guianense s. l.* were collected at 25 sites in Brazil, French Guiana, and Venezuela, representing 11 ecoregions and a range of elevations (7–1270 m asl), river widths (10–4900 m), and water temperatures (20.0˚–31.5˚C) (Table 1, Fig 1). Samples were taken with forceps from the leaves of the Podostemaceae or, where the plants were scarce, from rocks (Longá River), sticks (Yuruani River), or rocks and leaves (Pirangi River). The samples were fixed in 3 changes of 1:3 acetic ethanol and held at -4˚C until processing.

Chromosome preparation and interpretation
Larvae were split posteroventrally and Feulgen-stained following the procedures of Charalambous et al. [17]. Silk glands with stained polytene chromosomes, plus one gonad for gender determination [28], were placed in a drop of 50% acetic acid and flattened under a coverslip by thumb pressure. Representative larvae from each collection were deposited in the Invertebrate Collection of the Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil, and the Invertebrate Collection of Laboratorio de Biologia de Vectores y Parásitos, Instituto de Zoología y Ecología Tropical, Caracas, Venezuela.

Photographs of high-quality chromosome preparations were taken under oil immersion on an Olympus BX40 or BX51 compound microscope. Photographic negatives were scanned with a Nikon Coolscan V and imported into Adobe® Photoshop® Elements 8 to prepare chromosomal maps. Photographic negatives of chromosomes were deposited in the Clemson University Arthropod Collection, Clemson, SC.

As customary [29], we based our standard map on the predominant and most central sequence for the short (S) and long (L) arms of each chromosome (I, II, and III) in the *S. guianense* complex. Although we followed the conventions of Charalambous et al. [17] for constructing the standard map, the newly discovered diversity of band sequences in our samples required modification of the original standard map to represent the central and most predominant sequences. III-L-4, III-L-6, and IIIIL-3 were shown as homozygous standard by Charalambous et al. [17] in all larvae of their two Toncantins (Minaçu) populations (cytoform A1) and in their corresponding figures (Figures 5 and 6 in [17]). However, only the Minaçu populations and one other similar population (Pandeiros River) had these sequence polarities among all studied populations. We, therefore, adopted an opposite interpretation and considered the Minaçu (and Rio Pandeiros) populations as inverted for III-L-4, III-L-6, and IIIIL-3, rendering all other populations standard for these sequences. The section numbers within these inversions were renumbered to provide consecutive sequences for the revised standard map.

Selected landmarks labeled on our maps follow the nomenclature of Rothfels et al. [29]. IIS, for instance, includes the trapezoidal, Ring of Balbiani, bulge, and shoestring markers. All rearrangements that were found in our samples are indicated on our chromosomal maps by brackets or arrows. Inversions fixed in a cytoform are italicized in the text and underlined on our maps. Numbering of inversions follows that used by Charalambous et al. [17]; newly discovered inversions continue the numbering sequence in each arm. Polymorphic (floating) rearrangements, including those linked to sex, are in Roman type. Polymorphisms with a minimum of four larvae in each of three band-sequence categories (ss, si, ii, where s = standard, i = inverted) were tested for linkage to gender; if sex linkage was not significant (P > 0.05), the polymorphisms were tested for Hardy-Weinberg equilibrium.
We use “cytoform” as a neutral term for a chromosomally cohesive and distinguishable entity based on one or more chromosomal features; no statement about reproductive isolation is implied. We designate cytoforms with letters, according to the precedent established by Charalambous et al. [17]. To encode additional information regarding relationships, we also designated some cytoforms with a number following a letter (e.g., B1, B2, B3, and B4); thus, for example, each numbered member of the B group is chromosomally distinct but shares unique chromosomal characters with other B group members.

Relationships among cytoforms
A cladistic approach was used to infer relationships among cytoforms. We used shared chromosomal inversions, relative to the standard sequence, as evidence of common ancestry [30]. As a caveat, we reiterate that the choice of the standard sequence was based on the centrality and overall predominance of the sequence in each arm of the S. guianense complex, rather than on synapomorphic rearrangements for which polarity will need to be determined once appropriate outgroups are examined [31].

We used all inversions shared between two or more populations to produce a character (inversion) matrix for the 25 populations. However, III-14, which was found in a single larva in each of two geographically distant populations (Branco and Jacareí) is considered a misread in one of the populations and was not used in the character matrix for our analysis. Four character states were proposed for the inversions: 0 = absent, 1 = fixed, 2 = autosomally polymorphic, and 3 = X-linked.

The matrix was constructed using the software WinClada [32] and was exported to TNT [33,34], where searches were conducted under implied weights with different values of k (i.e., 2, 3, 4, 5, 7, and 10). This procedure was performed to evaluate potential topological changes under different k values [35]. Searches were conducted using the traditional search command (under Analyze), with 10,000 replications of random addition sequences, followed by tree bisection and reconnection, saving 100 optimal trees per replication. Relative Bremer support [36] was calculated as a measure of group support, using 1000 suboptimal trees up to five steps longer than the shortest tree (obtained using a traditional search, under k = 5). The trees were rooted using cytoform C, a monomorphic segregate with a banding sequence identical to the standard sequence for S. guianense s.l.

Results
Chromosomal generalities
Of 616 larvae of S. guianense s.l. prepared for analysis, 607 (98.5%) were scored for the entire banding sequence; the 9 partially scored or unscored larvae were not used in any analyses. The typical haploid complement of three submetacentric chromosomes was preserved across all populations. The nucleolar organizer was in the base of IL (section 24/25 junction). A chromocenter and supernumerary (B) chromosomes were absent.

Chromosomal groups and cytoforms
We recorded 83 chromosomal rearrangements, including 81 paracentric inversions, 1 hetero-band, and 1 secondary nucleolar organizer, among 607 larvae in 25 populations of S. guianense s.l. Inversions (fixed and polymorphic) were distributed in approximately equal numbers in IS and the 3 long arms, but were scarce in IIS and IIIS: 23 inversions in IS, 16 in IL, 3 in IIS, 20 in III, 1 in IIIS, and 18 in IIIL. Average inversion heterozygosity per larva ranged from 0.00 in the monomorphic Farinha River and Teles Pires River populations to 2.92 in the Ereú River.
We recognize the following three chromosomal groups and 13 cytoforms whose diagnostic chromosomal rearrangements are summarized in Table 2 and Figs 4–14:

**Group A**

Two cytoforms, one in the Central-West Region and the other in the Southeast Region comprised the A group. Our reinterpretation of the chromosomal photomaps of Charalambous et al. [17] depicted the A group as uniquely defined by *IIL*-4 and *IIL*-6 (Figs 9–11).

**Cytoform A1.** We had no samples of cytoform A1, as described by Charalambous et al. [17] from two sites in the Toncantins-Araguaia ecoregion of Brazil. Our data were drawn from our reinterpretation of the information provided by Charalambous et al. [17]. A1 was fixed for *IIIL*-3 (Figs 13 and 14). Five low-frequency (< 0.16) polymorphic inversions and cytologically undifferentiated sex chromosomes were recorded by Charalambous et al. [17] (Table 3).

**Cytoform A2.** Our population of this cytoform from the state of Minas Gerais, Brazil, in the São Francisco ecoregion differed from A1 by having *IL*-8 fixed (Fig 7), which it shared with some members of the B group, and *IIIL*-3 as an autosomal polymorphism (frequency = 0.43). It had two additional, unique polymorphisms (IS-23 and IL-16; Figs 4 and 7) with frequencies less than 0.15 (Table 3). The homologues were loosely paired, and the sex chromosomes were cytologically undifferentiated.

**Group B**

The B group, a cluster of four cytoforms, was uniquely defined by *IIL*-2, *IIL*-3, and *IIIL*-9 (Table 4; Figs 9, 11 and 14). All but B1 also shared IS-3 (Figs 4 and 5) and IL-6 (Fig 7). B2, B3, and B4 represented all of our populations in Brazil’s North Region, French Guiana, and southeastern Venezuela. B1 in the Teles Pires River of the Central-West Region, was the most geographically remote member of the B group, almost 1500 km from the nearest other population (Pitinga River) in the B group. *IIIL*-7 (Fig 14) was fixed in all B group members except B4 and the Oiapoque and Maroni populations of B2.

**Cytoform B1.** Though lacking IS-3, cytoform B1 from Brazil’s Teles Pires River in the Tapajós-Juruena ecoregion joined the B group by sharing *IIL*-2, *IIL*-3, and *IIIL*-9 (Table 4;
Figs 11 and 14). It was fixed for IIIIL-7 and uniquely fixed for IIL-13 (Figs 9 and 14). The only polymorphisms were a secondary nucleolar organizer heterozygously expressed in section 12 of one female larva and a heteroband in section 18 of two male larvae (Fig 4). Sex chromosomes were cytologically undifferentiated.

Cytoform B2. This cytoform included six populations from the Amazonas Guiana Shield ecoregion and one from the adjacent Guianas ecoregion, all of which shared IS-13 (Table 4; Fig 4), in addition to IS-3, IL-6, IIL-2, IIL-3, IIIIL-7, and IIIIL-9. IS-13, however, was rare (frequency = 0.02) in the Maroni River population, and, if missed, might have led to assignment of this population to cytoform B3. Four of the seven populations of cytoform B2 had at least partial linkage of IS-13 to the X chromosome (Table 5). Larger samples from the Branco and Água Fria rivers might have revealed partial X linkage of IS-13. If, however, IS-13 is strictly autosomal in the Branco and Água Fria populations, they could join B3, the implication being that IS-13 was lost in the B3 populations of the Oiapoque and Tracajatuba rivers. Alternatively, the apparent linkage of IL-14 (Fig 7) to the X in the Branco River population, if upheld by larger samples, would suggest another cytoform in the B group. Of 24 different autosomal polymorphisms in populations of B2, half had frequencies greater than 0.20 in at least one population (Table 4, Figs 4, 5, 7, 9, 11 and 14): IS-13, IS-15, and IL-6 in the Branco River; IS-13 and IL-8 in the Água Fria River; IL-8 and IIIIL-14 in the Pitinga River; IL-8 in the Caroeobe River; IL-12, IL-13, IIL-14, IIL-16, IIIIL-17, and IIIIL-13 in the Ereu River; and IL-8, IIL-20, and IIIIL-7 in the Maroni River. The centromeres paired ectopically in at least some nuclei of 12%
**Fig 5.** IS distal half of cytoform B3, showing a partial IS-3 chromosomal sequence. Breakpoints of floating inversions IS-15, IS-18, and IS-22 are indicated by brackets. Male larva (Oiapoque River).

https://doi.org/10.1371/journal.pone.0181679.g005

**Fig 6.** IL base (above) and sections 28–41 of cytoform F, showing IL-5 chromosomal sequence. Breakpoints of IL-7 and IL-11 are indicated by brackets. Male larva (Longá River). C = centromere, N.O. = nucleolar organizer.

https://doi.org/10.1371/journal.pone.0181679.g006
of the Ereu population. The chromosomes of the Branco population were more condensed than those of any other population. IL-8 in the Pitinga River was in Hardy Weinberg equilibrium (12 ss, 12 si, 7 ii; df = 1, $\chi^2 = 1.30$, $P > 0.05$), as were the following four inversions in the Ereu River (df = 1, $P > 0.05$): IIL-14 (4 ss, 13 si, 7 ii; $\chi^2 = 0.24$), IIL-16 (5 ss, 13 si, 6 ii; $\chi^2 = 0.17$), IIL-17 (5 ss, 14 si, 5 ii; $\chi^2 = 0.67$), and IIIL-13 (5 ss, 14 si, 5 ii; $\chi^2 = 0.67$) (Table 4).

**Cytoform B3.** This cytoform was represented by one population from the Oiapoque Basin (Guianas ecoregion) and one from the Araguari Basin (Amazonas Estuary & Coastal Drainages ecoregion) in Brazil. In addition to IS-3, IL-6, IL-8, IIL-2, IIL-3, IIIL-7, and IIIL-9, the two populations were weakly united by the absence of IS-13 (Table 4). IL-8 (Fig 7) was in
high frequency (> 0.84), and the sex chromosomes were cytologically undifferentiated. IS-5, IS-11, and III-10 were rare (0.01) polymorphisms in the Oiapoque population (Figs 4 and 11). Lack of a uniquely shared chromosomal character prohibited us from including the Oiapoque and Tracajatuba populations in any other cytoform in the B group. We did not find 3 low-frequency (< 0.10) inversions (IS-6, IL-2, and IIII-2) discovered in the Oiapoque population by Charalambous et al. [17]. The absence of IL-8 and IIII-7 in the Oiapoque population sampled 20 years earlier about 3 km from our collection site and studied by Charalambous et al. [17] could not be reconciled with the high frequency of these inversions (0.85 and 0.91, respectively) in our material.

Fig 9. IIL standard chromosomal sequence of cytoform F. Breakpoints of 12 inversions of various cytoforms are indicated by brackets. Letters a—h, when assembled alphabetically, produce the IIL-2, 3, 13 sequence of cytoform B1 from the standard sequence. Male larva (Longá River). C = centromere, gB = gray band, J = jagged, P = puffing band, Pb = parabalbiani.

Fig 10. IIL of cytoform E4, showing the IIL-11, 12 chromosomal sequence. Breakpoints of IIL-4, IIL-6, and IIL-8 are indicated by brackets. Female larva (Verdão River). C = centromere, gB = gray band, J = jagged, Pb = parabalbiani.
The two Venezuelan populations from the Orinoco Guiana Shield ecoregion had IS-3, IL-6, III-2, IL-3, and IIIIL-9. They were united, albeit weakly, by floating inversions IL-4 and IIIS-1 (Figs 7 and 8) and absence of IIIIL-7, which was fixed, or nearly so, in all other members of the B group. IS-8 possibly was linked to the X chromosome in the Yuruani River population (Table 4, Fig 4). Of 12 additional autosomal polymorphisms, only IIIIL-8 and IIIIL-9 (Fig 9) in the Parupa River population and IL-4, IIIS-1, and IIIIL-5 (Figs 7, 8 and 13) in the Yuruani population had frequencies of 0.20 or higher (Table 4). IIIIL-9 (12 ss, 18 si, 8 ii) in the Parupa population was in Hardy–Weinberg equilibrium ($df = 1, \chi^2 = 0.07, P > 0.05$).

**Cytoform B4.** The two Venezuelan populations from the Orinoco Guiana Shield ecoregion had IS-3, IL-6, III-2, IL-3, and IIIIL-9. They were united, albeit weakly, by floating inversions IL-4 and IIIS-1 (Figs 7 and 8) and absence of IIIIL-7, which was fixed, or nearly so, in all other members of the B group. IS-8 possibly was linked to the X chromosome in the Yuruani River population (Table 4, Fig 4). Of 12 additional autosomal polymorphisms, only IIIIL-8 and IIIIL-9 (Fig 9) in the Parupa River population and IL-4, IIIS-1, and IIIIL-5 (Figs 7, 8 and 13) in the Yuruani population had frequencies of 0.20 or higher (Table 4). IIIIL-9 (12 ss, 18 si, 8 ii) in the Parupa population was in Hardy–Weinberg equilibrium ($df = 1, \chi^2 = 0.07, P > 0.05$).
Cytoform C

A population from the Farinha River in the Toncantins-Araguaia ecoregion of Brazil represented cytoform C, a monomorphic segregate in our study (Table 3). Its banding sequence was

Fig 13. III distal to section 88 of cytoform F, showing standard chromosomal sequence. Breakpoints of 13 inversions of various cytoforms are indicated by brackets. Male larva (Longá River).

https://doi.org/10.1371/journal.pone.0181679.g013

Fig 14. III of cytoform B3, showing III-7, 9 chromosomal sequence. Breakpoints of 4 additional inversions of various cytoforms are indicated by brackets. Female larva (Oiapoque River). C = centromere.

https://doi.org/10.1371/journal.pone.0181679.g014
identical to the standard for *Simulium guianense*. Our results agreed with those of Charalambous et al. [17], who found that the Toncantins River population about 30 km downriver from our site, a tributary of the Tocantins River, was monomorphic.

*IIL-4* and *IIL-3* of Charalambous et al. [17] are actually standard in this cytoform, as depicted on our modified standard map (Figs 9 and 13). Sex chromosomes were microscopically undifferentiated.

**Cytoform D**

Two populations in Brazil’s Xingu ecoregion represented cytoform D. They uniquely shared fixed inversions *IS-16*, *IS-17*, *IL-3*, and *IIL-5*, plus *IIL-18* (Figs 4, 7 and 9), which was fixed in the Xingu River and in high frequency (0.89) in its tributary, the Iriri River (Table 3). The only other rearrangements were *IIL-15* and *IIL-18* (Fig 13), which appeared heterozygously in

Table 3. Frequency of inverted chromosomal constituents for *Simulium guianense* cytoforms A1, A2, C, D, and F.

| Cytoform | A1 | A2 | C  | D  | D  | F  | F  | F  |
|----------|----|----|----|----|----|----|----|----|
| River    |    |    |    |    |    |    |    |    |
| Toncantins |    |    |    |    |    |    |    |    |
| Pandeiros |    |    |    |    |    |    |    |    |
| Farinha² |    |    |    |    |    |    |    |    |
| Xingu    |    |    |    |    |    |    |    |    |
| Iriri    |    |    |    |    |    |    |    |    |
| Jacareí  |    |    |    |    |    |    |    |    |
| Pirangi  |    |    |    |    |    |    |    |    |
| Longá    |    |    |    |    |    |    |    |    |
| ♀:♂     | NA | 8:13 | 22:10 | 29:13 | 3:6 | 6:5 | 4:6 | 21:8 |
| IS-1     | 0.09 | | | | | | | |
| IS-2     | 0.06 | | | | | | | |
| IS-10    | | | | | | | | 0.02 |
| IS-16    | | 1.00 | 1.00 | | | | | |
| IS-17    | | 1.00 | 1.00 | | | | | |
| IS-23    | | 0.10 | | | | | | |
| IL-1     | 0.16 | | | | | | | |
| IL-3     | | 1.00 | 1.00 | | | | | |
| IL-4     | | | | | | | | |
| IL-5     | | | | | | | | 0.73 |
| IL-8     | | 1.00 | | | | | | |
| IL-16    | | 0.14 | | | | | | |
| IIL-1    | 0.04 | | | | | | | |
| IIL-4³   | 1.00 | 1.00 | | | | | | |
| IIL-5    | | 1.00 | 1.00 | | | | | |
| IIL-6²   | 1.00 | 1.00 | | | | | | |
| IIL-18   | | 1.00 | 0.89 | | | | | |
| Heter.⁴  | NA | 1.24 | 0.00 | 0.05 | 0.22 | 0.73 | 0.50 | 0.52 |

1 All data from Charalambous et al. [17] for two populations combined (Toncantins and Mucambão rivers); sex ratio and number of heterozygotes per larva not given (NA), but 34–36 larvae were scored for the listed inversions.

² Years 2000 (*n* = 23) and 2001 (*n* = 9) combined.

³ *IIL-4*, *IIL-6*, and *IIL-3* are shown as homozygous standard by Charalambous et al. [17] in their figures (Figures 5 and 6 in [17]) and as standard in all larvae in their Minaçu (cytoform A1) populations. However, we consider these sequences to be inverted in the Minaçu (cytoform A1) populations because they have the opposite polarity, i.e., the inverted sequence *sensu* Charalambous et al. [17], in all other studied populations (except the closely related cytoform A2).

⁴ Average number of heterozygous autosomal inversions per larva.

https://doi.org/10.1371/journal.pone.0181679.t003
Table 4. Frequency of inverted chromosomal constituents for group B of *Simulium guianense* s. l.

| Cytoterm | B1   | B2   | B2   | B2   | B2   | B2   | B2   | B2   | B3   | B3   | B4   | B4   |
|----------|------|------|------|------|------|------|------|------|------|------|------|------|
| River    | Teles Pires | Branco | Água Fria | Pitinga | Caroebe | Ereu | Cachorro | Maroni | Tracajatuba | Oiapoque | Parupa | Yuruani |
| ♀:♂     | 12:11 | 4:3   | 3:5   | 17:14 | 16:9  | 11:13 | 18:9  | 10:12 | 9:9   | 28:35 | 22:16 | 7:7   |
| IS-3     | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| IS-4     |      |      |      |      |      |      |      |      |      |      |      |      |
| IS-5     |      |      |      |      |      |      |      |      |      |      |      | 0.04 |
| IS-6     |      |      |      |      |      |      |      |      |      |      |      |      |
| IS-7     |      |      |      |      |      |      |      |      |      |      |      | 0.04 |
| IS-8     | 0.07 |      |      |      |      |      |      |      |      |      |      |      |
| IS-11    |      |      |      |      |      |      |      |      |      |      |      | 0.18 |
| IS-13    | 0.50 | 0.31 |      |      |      |      |      |      |      |      |      |      |
| IS-14    |      |      |      |      |      |      |      |      |      |      |      | 0.04 |
| IS-15    | 0.21 |      |      |      |      |      |      |      |      |      |      |      |
| IS-16    |      |      |      |      |      |      |      |      |      |      |      |      |
| IS-17    |      |      |      |      |      |      |      |      |      |      |      |      |
| IS-18    |      |      |      |      |      |      |      |      |      |      |      |      |
| IS-19    |      |      |      |      |      |      |      |      |      |      |      |      |
| IS-20    |      |      |      |      |      |      |      |      |      |      |      | 0.02 |
| IS-21    |      |      |      |      |      |      |      |      |      |      |      | 0.02 |
| IS-22    |      |      |      |      |      |      |      |      |      |      |      | 0.02 |
| IL-2     |      |      |      |      |      |      |      |      |      |      |      |      |
| IL-4     |      |      |      |      |      |      |      |      |      |      | 0.01 | 0.25 |
| IL-5     |      |      |      |      |      |      |      |      |      |      |      |      |
| IL-6     | 0.21 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| IL-7     |      |      |      |      |      |      |      |      |      |      |      |      |
| IL-8     | 0.75 | 0.64 | 0.24 | 0.24 | 0.15 | 0.62 | 0.97 | 0.85 |      |      |      |      |
| IL-12    |      |      |      |      |      |      |      |      |      |      | 0.98 |      |
| IL-13    |      |      |      |      |      |      |      |      |      |      | 0.96 |      |
| IL-14    |      |      |      |      |      |      |      |      |      |      |      | 0.98 |
| IL-15    |      |      |      |      |      |      |      |      |      |      | 0.98 |      |
| IL-16    |      |      |      |      |      |      |      |      |      |      | 0.98 |      |
| IIS-1    |      |      |      |      |      |      |      |      |      |      |      | 0.98 |
| IIS-3    |      |      |      |      |      | 0.18 | 0.04 |      |      |      |      |      |
| IIL-2    | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| IIL-3    | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| IIL-7    |      |      |      |      |      |      |      |      |      |      |      | 0.07 |
| IIL-8    |      |      |      |      |      |      |      |      |      |      |      | 0.20 |
| IIL-9    |      |      |      |      |      |      |      |      |      |      | 0.45 | 0.04 |
| IIL-10   |      |      |      |      |      |      |      |      |      |      | 0.02 | 0.01 |
| IIL-13   | 1.00 |      |      |      |      |      |      |      |      |      |      |      |
| IIL-14   | 0.31 | 0.12 | 0.56 |      |      |      |      |      |      |      |      |      |
| IIL-15   |      |      |      |      |      |      |      |      |      |      |      |      |
| IIL-16   |      |      |      |      |      |      |      |      |      |      |      |      |
| IIL-17   |      |      |      |      |      |      |      |      |      |      |      |      |
| IIL-19   |      |      |      |      |      |      |      |      |      |      |      | 0.12 |
| IIL-20   |      |      |      |      |      |      |      |      |      |      |      | 0.26 |
| IIL-3    | 0.19 |      |      |      |      |      |      |      |      |      |      |      |
| IIL-2    |      |      |      |      |      |      |      |      |      |      |      |      |
| IIL-4    |      |      |      |      |      |      |      |      |      |      |      |      |
| IIL-5    |      |      |      |      |      |      |      |      |      |      |      |      |
| IIL-7    | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.88 | 1.00 | 0.91 |      |      |

(Continued)
one larva each in the Xingu River. Sex chromosomes were cytologically undifferentiated. The chromosomal homologues of both populations were the most loosely paired among all of our samples. IIIL-18 appears to be present on the map of Charalambous et al. (Figure 19 in [17]), though overlooked. The putative absence of IS-16 and IS-17 reported for the Xingu population by Charalambous et al. [17] could not be reconciled with our population analysis. We note, however, that the two populations were sampled about 14 years and 20 km (straight line) apart.

Group E

Five populations along the southern margin of the distribution of S. guianense s. l., in the Central-West and Southeast regions of Brazil, were characterized by a high frequency (0.29–1.00) of IIS-2 (Fig 8). Sex chromosomes were cytologically undifferentiated for all group members. The presence of three inversions (IL-6, IL-8, and IIIIL-7) in at least one member of the E group (Table 6), which also were shared with the B group, suggested a relationship between the B and E groups; the inference is that these inversions were polymorphic in a hypothetical ancestor of the two groups.

Cytoform E1. Fixation of IIS-2 (Fig 8) characterized this cytoform from Brazil’s São José River in the Doce Basin of Espírito Santo state (Northeastern Mata Atlântica ecoregion). Cytoform E1 otherwise carried two unique polymorphisms, IL-7 and IIIIL-11 in the Pardo River population (Upper Paraná ecoregion) and IL-8, IIS-2,
and III-11 in the Mortes River population (Tocantins-Araguaia ecoregion) (Table 6; Figs 7, 8 and 13).

Cytoform E4. The Verdão River population (Upper Paraná ecoregion) was defined by two unique fixed inversions (III-11 and III-12, Fig 10) and three unique polymorphisms, viz. IL-9, IL-10, and IIIL-8 (Table 6; Figs 7 and 13). IL-9 (7 ss, 23 si, 13 ii; $\chi^2 = 0.36$) and IL-10 (29 ss, 10 si, 4 ii; $\chi^2 = 3.80$) were in Hardy-Weinberg equilibrium (df = 1, $P > 0.05$). IS-12 (Fig 4) was shared with E2, and IIIL-7 (Fig 14) was shared with the B group.

Table 5. Sex chromosomes in populations of Simulium guianense cytotoform B2.

| Population | Sex Chromosomes $^1$ |
|------------|----------------------|
|            | $X_0X_0$ | $X_0X_1$ | $X_1X_0$ | $X_1X_1$ | $X_2X_2$ | $X_3X_3$ | $X_0Y_0$ | $X_1Y_0$ | $X_1Y_1$ | $X_3Y_0$ |
| Agua Fria  | 3         | 0        | 0        | 0        | 5        | 0        | 0        | 0        | 2         |
| Branco     | 1         | 0        | 0        | 0        | 3        | 0        | 0        | 0        | 2         |
| Cachoro    | 0         | 0        | 18       | 0        | 0        | 9        | 0        | 0        | 2         |
| Caroebe    | 0         | 0        | 16       | 0        | 0        | 9        | 0        | 0        | 2         |
| Eru         | 0         | 0        | 11       | 0        | 1        | 7$^2$    | 5        | 0        | 2         |
| Maroni     | 10        | 6        | 9        | 2        | 0        | 1        | 12       | 0        | 0         |
| Pitinga    | 0         | 6        | 9        | 2        | 0        | 1        | 12       | 0        | 0         |

$^1$ $X_0$ and $Y_0$ = microscopically undifferentiated, $X_1 = IS-13$, $X_2 = IS-13+14$, $X_3 = IL-14$, $Y_1 = IS-13$.

$^2$ 1 $X_1Y_0$ male was also heterozygous for IS-15, but whether IS-15 was associated with the X or the Y could not be determined.

https://doi.org/10.1371/journal.pone.0181679.t005

Table 6. Frequency of inverted chromosomal constituents for group E of Simulium guianense s. l.

| Cytoform | E1 | E2 | E3 | E3 | E3 | E4 |
|----------|----|----|----|----|----|----|
| River    | Sao Jose | Araguaia | Mortes | Pardo | Verdao |
| 9:3:7    | 19:11 | 22:23 | 6:9 | 14:6 | 25:18 |
| IS-12    | 1.00 | 0.02 | 0.12 | 0.13 |
| IS-19$^1$| 0.40$^2$ | 0.27 | 0.05 |
| IL-9     | 0.57$^2$ | 0.21$^2$ |
| IL-10    | 0.22$^2$ |
| IL-11    | 0.02 |
| III-12   | 1.00 | 1.00 |
| III-7    | 0.12 | 0.15 |
| III-10   | 1.00 | 0.14 |
| III-11   | 0.30 | 0.65 |
| III-16   | 0.02 |
| Heter.$^3$| 0.23 | 1.27 | 1.53 | 1.40 | 1.81 |

$^1$ IS-19 was represented by a knot with exact breakpoints unresolvable but near those of IS-12.

$^2$ All inversions tested were in Hardy-Weinberg equilibrium (df = 1, $P > 0.05$).

$^3$ Average number of heterozygous autosomal inversions per larva.

https://doi.org/10.1371/journal.pone.0181679.t006
Cytoform F

Three populations in the Northeast Region, all geographically within 120 km of one another in the Parnaiba ecoregion, were united by the unique floating inversions IL-5 (Fig 6) and IIIL-6 (Fig 13). The sex chromosomes were microscopically undifferentiated. Two rare polymorphisms, IS-10 and IIIL-14 (Figs 4 and 13), were found heterozygously in one Longá and one Jacareí larva, respectively (Table 3). We suspect that IIIL-14 in the Jacareí larva was a misread of a mimic inversion in the Branco River population, given the geographic distance (ca. 2,200 km) and absence of other shared rearrangements between the two populations. Cytoform F was superficially similar to cytoform C, the geographically nearest cytoform, about 650 km northeast in the Farinha River; both cytoforms lacked fixed inversions. The absence of IL-5 and IIIL-6 in cytoform C might reflect clinal variation. Treating C and F as a single cytoform, however, forms a potentially unnatural group without a uniquely shared rearrangement.

Evolutionary relationships

Our analysis of evolutionary relationships, based on 29 characters (inversions, Table 7), recovered three most parsimonious trees of 72 steps, with consistency and retention indices of 0.69 and 0.85, respectively. The same topologies were achieved for each value of \( k \) (2, 3, 4, 5, 7, and 10) used in the implied weighted analysis. The basic structure of the three trees was highly similar. All recovered the same major clades (Group A, Group B, Cytoform D, Group E, and Cytoform F) as monophyletic, but in an unresolved polytomy. In addition to the strict consensus tree (Fig 15), we show one of the three trees that provides the greatest resolution of relationships (Fig 16). This tree differed from the other two trees only by 1) grouping the B3 populations of Tracajatuba and Oiapoque rather than placing them separately either in a trichotomy with the B2 cluster or in a polytomy with B4 and members of the B2 cluster, and 2) resolving the placement of Pitinga rather than placing it in a trichotomy with Branco + Ereu and all other B2 populations.

Our hypothesis of relationships showed that the two populations of cytoform D were supported as monophyletic by inversions IS-16, IS-17, II-3, III-5, and III-18. The A group, composed of two clades (A1 and A2), was supported by inversions III-4, III-6, and IIII-3. Cytoform F, which included three populations, was recovered as a monophyletic group, supported by two autosomal polymorphisms (IL-5 and IIIL-6). The E group, composed of four cytoforms, was supported as monophyletic by autosomal polymorphism IIIS-2. Within this group, E1 and E4 were monophyletic clades, whereas E2 and E3 were not recovered as distinct lineages, but instead nested in a clade supported by inversions IL-8 and IIIL-11. IL-8 is hypothesized to have been an ancestral polymorphism at a deeper node that included members of the A, B, and E groups. As such, it persisted as a polymorphism in some lineages, was fixed in A2, and was lost in A1, B1, B2 (Branco and Ereu populations), B4, E1, and E4. The B group was recovered as monophyletic, supported by inversions III-2, IIII-3, IIII-9, and IIII-7, which was lost in B4. Within this group, each cytoform (B1, B2, B3, and B4) was recovered as monophyletic. B1 was sister to the other cytoforms of the B group. The clade composed of B2+B3+B4 was supported by fixed inversion IS-3 and IL-6, which was fixed except in the Branco population of B2, in which it was an autosomal polymorphism. Two groups were recovered in B2: one formed by the Branco + Ereu populations, characterized by IS-15, and the other including the remaining five B2 populations, supported by autosomal polymorphism IL-8.

Distributions of cytoforms

Related cytoforms were clustered geographically, and each cytoform showed fidelity to a particular freshwater ecoregion (Fig 17). Only three of the 11 ecoregions represented by our
collections harbored more than one cytoform: Guianas had two (B2 and B3), Upper Parana had two (E3 and E4), and Toncantins-Araguaia had four (A1, C, E2, and E3). The B group occupied the northwestern region of Brazil and adjacent areas related to the Guiana Shield: Orinoco Guiana Shield, Amazonas Guiana Shield, and Guianas. Only the populations of the A`gua Fria and Tracajatuba rivers (Amazonas Estuary & Coastal Drainages) and the geographically remote Teles Pires River (Tapajos-Juruena) were outside the Guiana Shield ecoregions. Although the B1 population was 300 km from the nearest other sampled population (cytoform E3 in the Mortes River), versus 1500 km from the nearest group B population (Pitinga River), it shared no inversions with cytoform E3.

**Discussion**

**Interspecific relationships**

*Simulium guianense s. l.* is currently placed in the *S. orbitale* group of the subgenus *Trichodagmia*, based on morphological criteria [15,37]. A molecular (COI) barcoding analysis of nine of the 18 nominal species in the *S. orbitale* group, including *S. guianense s. l.*, however, did not
recover this group [21]. We note, however, that the current concept [15] of the *S. orbitale* group includes taxa at one time contained in two separate subgenera, *Thyrsopelma* and *Greneriella* [38].

Our unpublished chromosomal analysis of eight other members of the Brazilian *S. orbitale* group indicates that *S. guianense s. l.* can be derived from the group standard by at least 12 inversions and that at least eight of these inversions are uniquely shared by all cytoforms of the *S. guianense* complex. An earlier molecular (COI) analysis suggested that one member of the *S. orbitale* group, the morphologically similar *S. litobranchium* of Hamada et al. [20], clustered...
within *S. guianense s. l.* [22]. Our chromosomal analysis, however, unequivocally shows that *S. guianense s. l.* is monophyletic and that *S. litobranchium*, based on material from the type locality (Ponte de Pedra, Goiás State), is not a member of the *S. guianense* complex. Rather, it is removed from the standard banding sequence of the *S. guianense* complex by at least 10
fixed inversions. The chromosomal results find support in a morphological analysis showing *S. litobranchium* as the sister species of *S. perplexum* Shelley et al. and these two species as the sister group of *S. guianense s. l.* [37].

**Taxonomic status of cytoforms**

Four of the 13 cytoforms (B1, D, E2, and E4) are each fixed for one or more unique inversions. The entire B group shares two unique fixed inversions. The most chromosomally differentiated cytoforms, B3 and D, are separated by nine fixed inversions, three nearly fixed inversions, and a geographic distance of less than 500 km. The geographically nearest cytoforms with at least one fixed-inversion difference are E2 (Araguaia River) and E3 (Mortes River), approximately 130 km apart, with no obvious topographical barriers to gene exchange. Although the chromosomal data suggest multiple species within *S. guianense s. l.*, no opportunities were presented for using chromosomal features (e.g., fixed differences) in strict sympathy as the...
criterion for inferring reproductive isolation between any of the cytoforms. *Simulium metallicum* s. l., another Latin American vector associated with onchocerciasis, consists of 17 cytoforms [39–41]. About half of the cytoforms of *S. metallicum* s. l., however, have been found in sympathy with one another and confirmed as reproductively isolated species [39,41]. We suspect that, like *S. metallicum* and other Neotropical vector complexes such as *S. ochraceum* [42], *S. guianense* consists of multiple, reproductively isolated species. However, additional sampling of *S. guianense* s. l. is needed to minimize geographic gaps between cytoforms and allow rigorous tests of reproductive isolation.

The presence of shared inversions (IL-6, IL-8, and IIIL-7) between some members of the well-defined B and E groups suggests either gene exchange (introgression) or common ancestry. If the inversions reflect common ancestry, as we suggest, they exhibit the typical pattern in the Simuliidae: the ability of a single inversion to assume different states (e.g., fixation, loss, or polymorphism) in separate evolutionary lineages [29,39,43,44].

A previous molecular analysis of material from six of our sites, representing cytoforms B2, B3, C, E3, and E4, showed no pattern of clustering, with one exception [22]. The Farinha River population (cytoform C) clustered as a distinct group in a maximum likelihood tree based on the COI sequence. Of the five cytoforms molecularly evaluated [22], C is chromosomally the most remote. Thus, the molecular and chromosomal data are congruent for the most chromosomally divergent cytoform among those evaluated, but the chromosomal data provide finer resolution of the genetic structure of the other populations.

A few taxonomically critical collections are lacking. We do not have material from the type locality of *S. guianense* on the upper Essequibo River in Guyana to fix the name with the relevant cytoform. The type locality is within about 200 km of the Cachorro and Caroebe populations of cytoform B2, but it is in the Essequibo ecoregion, separate from all other ecoregions of our sampled populations. Although the type is probably a member of the B group, we hesitate to assign it to a particular cytoform, given the influence of ecoregion on simuliid distributions [45]. We also lack material from the type localities associated with the two names currently held in synonymy with *S. guianense*. The type specimen of *S. pintoi* from Piracicaba in São Paulo State is about 220 km from our nearest sampling site in the Pardo River, which supports cytoform E3. *Simulium ortizi* is based on material from San Felix in Bolivar State, Venezuela, roughly 315 km from our nearest sampling site in the Parupa River, which supports cytoform B4.

**Association of cytoforms with onchocerciasis**

Only the B group has been found in Venezuela and the North Region of Brazil, the general area of the last-remaining Latin American onchocerciasis foci, suggesting that one (or more) of its members is the vector. Although we did not sample rivers within the foci, they are within a few hundred kilometers of the Ereú (cytoform B2) and Parupa (cytoform B4) populations, B2 having been collected only from the lowlands and B4 only from the uplands. A caveat is that elevation, in addition to river system, can influence gene flow in simuliids, even over elevational distances as little as several hundred meters [46]. Caution, therefore, is needed in assigning vector status to any known member(s) of the B group.

Shelley et al. [47] suggested that cytoform A1 was predominantly zoophilic and, therefore, not a vector in the Minacuá area of Brazil historically afflicted with onchocerciasis. We recorded *S. guianense* s. l. biting humans along the Tracajatuba River in the state of Amapá where onchocerciasis has never been a problem; the corresponding larval population was cytoform B3. The cytoform identity is unknown for populations of *S. guianense* s. l. in areas currently or historically associated with onchocerciasis. Relevant material, therefore, is critically needed
from the New World’s last-remaining onchocerciasis foci along the border of Brazil and Venezuela. In other areas of Latin America, *S. metallicum* s.l. includes six cytoforms implicated as vectors through their geographic association with onchocerciasis foci, whereas the remaining cytoforms have been found only in onchocerciasis-free areas [39, 41]. The pattern is repeated in *S. ochraceum* s.l., another Latin American vector complex in which only a subset of cytoforms is associated with onchocerciasis [42].

**Spatial distribution patterns of cytoforms**

The discrete distributions of the 13 cytoforms suggest an association with geographic factors that might govern the distributions. The Amazon River separates the B group, except B1, from all other cytoforms. A COI analysis of eight populations of *S. guianense* s.l. also indicated genetic differences between opposite sides of the Amazon River [19]. The role of the Amazon River as a barrier to dispersal is well documented for numerous taxa, including winged animals such as birds [48]. Our populations of *S. guianense* s.l. south of the Amazon River are chromosomally more divergent (i.e., more cytoforms) than those on the northern side, although the chromosomal diversity (i.e., number of different rearrangements) is nearly the same (45 south versus 49 north of the Amazon). The greater diversity of cytoforms (9 south versus 2 north) perhaps reflects greater habitat diversity, expressed as different ecoregions. Ecological diversity among closely related species of black flies has been suggested as an indication that ecological adaptation has been important in driving evolution in the Simuliidae [46,49].

Cytoforms in the Northeast Region of Brazil align more with areas of rainforest endemism [50] or with geography than with drainage basin. Cytoform F, for example, is represented by a geographic cluster of populations in an area of endemism in a small independent drainage (Parnaiba) flowing directly to the ocean. If drainage basin or ecoregion were universally important for *S. guianense* s.l., however, the Pardo and Verdão rivers should support similar chromosomal profiles, and the Araguaia, Farinha, Mortes, and Tocantins populations should group together. Based on drainage basin alone, the grouping of the Pardo and Mortes populations (cytoform E3) is puzzling. These populations, however, are weakly grouped based on an absence of diagnostic chromosomal rearrangements; additional (molecular?) study might reveal greater divergence.

The influence of physicochemical properties of rivers, such as pH and conductivity, on cytoform distributions is unknown, although these properties differ across the distribution of *S. guianense* s.l., with different cytoforms experiencing different physicochemical environments [51,52]. Environmental conditions also can influence some aspects of chromosomal expression. The degree of pairing of the chromosomal homologues, for example, is positively related to water temperature in at least some species of black flies in temperate regions [53]. Whether this scenario applies to *S. guianense* s.l. or is cytoform specific is not known. However, we observed the greatest degree of nonpairing in cytoform D, which was collected in the warmest waters (> 30˚C)—a finding opposite our expectation, based on data [53] for temperate species.

The four cytoforms in the B group reflect a distributional series of nonoverlapping elevations: B3 at 7–14 m asl, B2 at 48–146 m, B1 at 356 m, and B4 at 870–1270 m. The cytoforms of *S. ochraceum* s.l., a vector complex involved with onchocerciasis in Central America, also exhibit distinct elevational distributions [42]. Typically, as elevation decreases, rivers become larger. River size is a strong predictor of distributions of many species [54], and cryptic species in a complex are often distributed along and among streams and rivers by size of the watercourse [55]. For *S. guianense* s.l., however, this association is weak, especially in the B group, which exhibits no correlation between river width and elevation (Spearman’s rho -0.070,
P = 0.838). B2, for example, is found in the Caroebe River, about 20 m wide at 146 m asl, and in the Branco River, roughly 600 m wide at 48 m asl.

Differential microdistribution patterns have been documented for larvae and pupae of isomorphic simuliid species [56]. However, different substrates used by the larvae of S. guianense s. l. in our study—leaves of Podostemaceae in the Jacareí River, rocks and leaves of Podostomaceae in the Pirangi River, and bedrock in the Longá River—cannot be attributed to cytoform; larvae in these three rivers were chromosomally homogeneous (cytoform F). Rather, we agree with Santos-Neto et al. [52] who suggested that the differences in substrate use are attributable to scarcity of the typical substrate, the leaves of Podostemaceae.

A striking pattern reflected in the chromosomal inversion profiles is the remarkable river specificity of the populations. Given the enormous populations [52] produced over multiple generations, potential dispersal capability, and apparent lack of topographical barriers to gene exchange, S. guianense s. l. might be expected to be genetically homogeneous across rivers over a wide geographic area. Yet, we find the opposite: populations of S. guianense s. l. are typically river specific. In one of the main onchocerciasis areas in southern Venezuela, for example, the immature stages of S. guianense s. l. inhabit a particular site (“Raudal of Goaharibos”) in the Orinoco River [57], even though adults of S. guianense s. l. are found in most Yanomami villages throughout the area [58]. What drives this specificity? Adler et al. [59] have argued that as watercourses become larger, they become less common and, therefore, less likely for females to find. Selection, therefore, should favor females that return to oviposit in natal habitats where larvae and pupae are adapted to the particular riverine conditions. The return would be especially advantageous to S. guianense s. l., which inhabits not only large rivers, but more specifically, the sporadically distributed rocky shoals of large rivers. Site fidelity would build site-specific genetic profiles, potentially leading to population differentiation, including speciation. As a corollary, within-river genetic diversity for S. guianense s. l. would be expected to be low [59]. The extreme examples of this prediction are found in cytoforms B1, C, and D, which express virtually no polymorphism.

Given the high degree of river specificity, environmental perturbations are likely to differentially influence the cytoforms of the S. guianense complex, potentially putting some cytoforms (species?) at risk. For a multivoltine complex of cytoforms, the consequences of natural fluctuations in water levels that might inundate or leave appropriate habitat dry raise questions about the responses of ovipositing females and other life stages over time. For example, does oviposition occur in the natal rivers during droughts and floods, and, if so, are the eggs able to survive these events? Studies of larval populations and their chromosomal inversion profiles within rivers over time could provide insights into the responses to these events and the degree of dispersal. The construction of dams on large rivers in Brazil also could have profound effects on populations of S. guianense s. l. as rocky shoals and riffle areas—prime breeding habitats—become permanently inundated. Substrate switching by the larvae of S. guianense s. l., for example, has been attributed to the construction of a dam in Amazonas [51]. Extinction is the extreme consequence of river specificity in the face of habitat alteration. Environmental disturbances, thus, carry greater risks for biodiversity than previously appreciated, particularly in light of the pattern that is coming to light in the Simuliidae: many so-called species represent taxonomic mosaics of forms and species ranging from pests and vectors to endangered species [59, 60].

Acknowledgments
We thank all individuals and institutions who provided field assistance and permits to collect on their land and to Florence Fouque and Institut Pasteur (French Guiana) to provide the
conditions for collecting in the Maroni River. We also thank Vivian C. Oliveira for help preparing the map (Fig 1), and W. S. Procunier for useful comments on the manuscript. This research was part of the project “Diversidade criptica em Simulium (Thysopelma) (Diptera: Simuliidae) e relações entre suas espécies”.

Author Contributions

Conceptualization: Peter H. Adler, Neusa Hamada.
Data curation: Peter H. Adler, Neusa Hamada.
Formal analysis: Peter H. Adler, Neusa Hamada, Jeane Marcelle Cavalcante do Nascimento.
Funding acquisition: Neusa Hamada, Maria Eugenia Grillet.
Investigation: Peter H. Adler, Neusa Hamada, Jeane Marcelle Cavalcante do Nascimento, Maria Eugenia Grillet.
Methodology: Peter H. Adler, Neusa Hamada, Jeane Marcelle Cavalcante do Nascimento.
Project administration: Peter H. Adler, Neusa Hamada, Maria Eugenia Grillet.
Resources: Peter H. Adler, Neusa Hamada, Jeane Marcelle Cavalcante do Nascimento, Maria Eugenia Grillet.
Supervision: Peter H. Adler, Neusa Hamada.
Validation: Peter H. Adler, Neusa Hamada, Jeane Marcelle Cavalcante do Nascimento, Maria Eugenia Grillet.
Visualization: Peter H. Adler, Neusa Hamada, Jeane Marcelle Cavalcante do Nascimento.
Writing – original draft: Peter H. Adler.
Writing – review & editing: Peter H. Adler, Neusa Hamada, Jeane Marcelle Cavalcante do Nascimento, Maria Eugenia Grillet.

References

1. Procunier WS (1989) Cytologic al approaches to simuliid biosystematics in relation to the epidemiology and control of human onchocerciasis. Genome 32: 559–569. https://doi.org/10.1139/g89-483 PMID: 2680764
2. Adler PH (2017) Biodiversity of biting flies: implications for humanity. In: Footitt RG, Adler PH (eds.), Insect biodiversity: science and society. Volume 1, 2nd edition. Chichester: John Wiley & Sons, pp. 713–745.
3. Pramual P, Wongpakarn K, Adler PH (2011) Cryptic biodiversity and phylogenetic relationships revealed by DNA barcoding of Oriental black flies in the subgenus Gomphostilbia (Diptera: Simuliidae). Genome 54: 1–9. https://doi.org/10.1139/G10-100 PMID: 21217800
4. Hernández-Triana LM, Chaverri LG, Rodríguez-Pérez MA, Prosser SWJ, Hebert PDN, Gregory TR, et al. (2015) DNA barcoding of Neotropical black flies (Diptera: Simuliidae): species identification and discovery of cryptic diversity in Mesoamerica. Zootaxa 3936: 93–114. https://doi.org/10.11646/zootaxa.3936.1.5 PMID: 25947423
5. Post RJ, Mustapha M, Krueger A (2007) Taxonomy and inventory of the cytospecies and cytotypes of the Simulium damnosum complex (Diptera: Simuliidae) in relation to onchocerciasis. Trop Med Internat Health 12: 1342–1353. https://doi.org/10.1111/j.1365-3156.2007.01921.x PMID: 18045261
6. Meredith SEO (1988) [1987] The role of systematics in black fly population management. In: Kim KC, Merritt RW (eds.), Black flies: ecology, population management, and annotated world list. University Park: Pennsylvania State University Press, pp. 53–61.
7. Basañez MG, Churcher TS, Grillet ME (2009) Onchocerca-Simulium interactions and the population and evolutionary biology of Onchocerca volvulus. Adv Parasitol 68: 263–313. https://doi.org/10.1016/S0065-308X(08)00611-6 PMID: 19289198
8. Adler PH, Cheke RA, Post RJ (2010) Evolution, epidemiology, and population genetics of black flies (Diptera: Simuliidae). Infect Gen Evol 10: 846–865. https://doi.org/10.1016/j.meegid.2010.07.003
PMID: 20624485

9. Procunier WS, Hirai H (1986) The chromosomes of Onchocerca volvulus. Parasitol Today 2: 307–309.
PMID: 15462744

10. WHO (2016) Progress toward eliminating onchocerciasis in the WHO Region of the Americas: verification of elimination of transmission in Guatemala. Weekly Epidemiol Rec 91 (43): 501–516.

11. WHO (2017). Onchocerciasis. Fact Sheet 374. http://www.who.int/mediacentre/factsheets/fs374/en/

12. Botto C, Basáñez MG, Escalonada M, Vivás-Martínez S, Villamizar N, Noya-Alarcón O, et al. (2016) Evidence of suppression of onchocerciasis transmission in the Venezuelan Amazonian focus. Parasites & Vectors 9 (1): 1. https://doi.org/10.1186/s13071-016-1313-z
PMID: 26813296

13. Botto C, Villamizar N, Jokić Ž, Noya-Alarcón O, Cortéz J, Escalonada M, et al. (2013) Landscape epidemiology of human onchocerciasis in southern Venezuela. Ref Module Earth Sys Environ Sci. 14 pp.

14. Basáñez MG, Yarzabal L, Takaoka H, Suzuki H, Noda S, Tada I (1988) The vectorial role of several blackfly species (Diptera: Simuliidae) in relation to human onchocerciasis in the Sierra Parima and Upper Orinoco regions of Venezuela. Ann Trop Med Parasitol 82: 597–611.
PMID: 3256278

15. Shelley AJ, Hernández LM, Maia-Herzog M, Luna Dias APA, Garritano PR (2010) The blackflies (Diptera: Simuliidae) of Brazil. In: Arias JR, Golovach S, Wantzen KM, Domínguez E (eds.) Aquatic Biodiversity in Latin America. Volume 6. Sofia, Bulgaria: Pensoft.

16. Shelley AJ, Lowry CA, Maia-Herzog M, Luna Dias APA, Moraes MAP (1997) Biosystematic studies on the Simuliidae (Diptera) of the Amazonia onchocerciasis focus. Bull Brit Mus Nat Hist (Entomol) 96: 1–121.

17. Charalambous M, Shelley AJ, Maia Herzog M, Luna Dias APA (1996) Four new cytotypes of the onchocerciasis vector blackfly Simulium guianense in Brazil. Med Vet Entomol 1: 111–120. https://doi.org/10.1111/j.1365-2915.1996.tb00716.x

18. Adler PH, Crosskey RW (2017) World blackflies (Diptera: Simuliidae): a comprehensive revision of the taxonomic and geographical inventory [2017]. http://www.clemson.edu/cafls/biomia/pdfs/blackflyinventory.pdf [Accessed 15 June 2017].

19. Mattos AA (2007) Caracterização molecular e citológica de diferentes populaces geográficas de Simulium guianense Wise s. I., no Brasil. Master’s thesis, Instituto Nacional de Pesquisas da Amazônia and Universidade Federal do Amazonas, Manaus, Brazil.

20. Hamada N, Pepinelli M, Mattos-Glória A, Luz SLB (2010) A new black fly species from Brazil, closely related to Simulium guianense Wise (Diptera, Simuliidae), revealed by morphology and DNA barcoding. Zootaxa 2428: 22–36. https://doi.org/10.5281/zenodo.194650

21. Hernández-Triana LM, Crainey JL, Hall A, Faith F, MacKenzie-Dodds J, Shelley AJ, et al. (2012) DNA barcodes reveal cryptic genetic diversity within the blackfly subgenus Trichodagmia Enderlein (Diptera: Simuliidae) and related taxa in the New World. Zootaxa 3514: 43–69.

22. Crainey JL, Mattos-Glória A, Hamada N, Luz SLB (2014) New tools and insights to assist with the molecular identification of Simulium guianense s.l., main Onchocerca volvulus vector within the highland areas of the Amazonian onchocerciasis focus. Acta Tropic 131: 47–55. https://doi.org/10.1016/j.actatropica.2013.10.019
PMID: 24200838

23. Négrel P, Lachassagne P (2000) Geochemistry of the Maroni River (French Guiana) during the low water stage: implications for water-rock interaction and groundwater characteristics. J Hydrol 237: 212–233. https://doi.org/10.1016/S0022-1694(00)00308-5

24. Hamada N, Grillet ME (2001) Black flies (Diptera: Simuliidae) of the Gran Sabana (Venezuela) and Pacaraima Region (Brazil): distributional data and identification keys for larvae and pupae. Entomotrópica 16: 29–49.

25. Abell R, Thieme ML, Reverga C, Bryer M, Kottelat M, Bogutskaya N, et al. (2008) Freshwater ecoregions of the world: a new map of biogeographic units for freshwater biodiversity conservation. BioScience 58: 403–414. https://doi.org/10.1641/0006-3568(2008)58[403:FEOTW]2.0.CO;2

26. FEOW (2015) Freshwater Ecoregions of the World. The Nature Conservancy and World Wildlife Fund. www.feow.org [Accessed 7 May 2017].

27. Ministério do Meio Ambiente (2006) Caderno da Região Hidrográfica do São Francisco/Ministério do Meio Ambiente, Secretaria de Recursos Hídricos. Brasília, MMA.

28. Adler PH, Currie DC, Wood DM (2004) The black flies (Simuliidae) of North America. Ithaca, NY: Cornell University Press.

29. Rothfels K, Feraday R, Kaneps A (1978) A cytological description of sibling species of Simulium venustum and S. verecundum with standard maps for the subgenus Simulium Davies [sic] (Diptera). Can J Zool 56: 1110–1128. https://doi.org/10.1139/z78-155
30. Rothfels KH (1979) Cytotaxonomy of black flies (Simuliidae). Annu Rev Entomol 24: 507–539. https://doi.org/10.1146/annurev.en.24.010179.002451

31. Adler PH, Huang YT (2011) Integrated systematics of the Simuliidae (Diptera): evolutionary relationships of the little-known Palearctic black fly Simulium acrotrichum. Can Entomol 143: 612–628.

32. Nixon KC (2002) WinClada, version 1.00.08. Published by the author, Cornell University, Ithaca, NY.

33. Goloboff PA, Catalano SA (2016) TNT, a free program for phylogenetic analysis. Cladistics 32: 221–238. https://doi.org/10.1111/cla.12160

34. Goloboff PA, Farris JS, Nixon K (2008) TNT, a free program for phylogenetic analysis. Cladistics 24: 774–786. https://doi.org/10.1111/j.1096-0031.2008.00217.x

35. Prendini L (2000) Phylogeny and classification of the superfamily Scorpionoidea Latreille 1802 (Chelicerata, Scorpiones): an exemplar approach. Cladistics 16: 1–78. https://doi.org/10.1111/j.1096-0031.2000.tb00348.x

36. Conn J, Rothfels KH, Procunier WS, Hirai H (1989) The Simulium metallicum complex (Diptera: Simuliidae) in Latin America: a cytological study. Can J Zool 67: 1217–1245.

37. Adler PH, Borkent A, Hamada N, McCreadie JW (2017) Biodiversity of Simulium metallicum sensu lato (Diptera: Simuliidae), a complex of Neotropical vectors associated with human onchocerciasis. Acta Tropica 173: 171–179. https://doi.org/10.1016/j.actatropica.2017.06.017 PMID: 28624513

38. Tangkawanit U, Kuvangkadilok C, Baimai V, Adler PH (2009) Cytosystematics of the blackfly subgenus Trichodagmia Enderlein (Diptera: Simuliidae) in Thailand. Zool J Linn Soc 155: 289–315. https://doi.org/10.1111/j.1096-3642.2008.00433.x

39. Pramual P, Kuvangkadilok C, Baimai V, Walton C (2005) Phylogeography of the black fly Simulium tani (Diptera: Simuliidae) from Thailand as inferred from mtDNA sequences. Mol Ecol 14: 3989–4001. https://doi.org/10.1111/j.1365-294X.2005.02639.x PMID: 16262854

40. Amorim DS (2012) Biogeografia da Região Neotropical. In: Rafael JA, Melo GAR, de Carvalho CJB, Casari SA, Constantino R (eds.), Insetos do Brasil: diversidade e taxonomia. Ribeirão Preto, Brasil: Holos, Editora, pp. 112–132.

41. Gomes SAG, Py-Daniel V (2002) Caracterização fisicoquímica de dois criadouros de larvas de Thyrso-pelma guianense (Wise, 1911) (Diptera, Culicomorpha, Simuliidae) da Amazônia brasileira. Entomol Vectores 9: 443–446.
52. Santos-Neto CR, Hamada N, Couceiro SRM (2015) Bionomics of the black fly *Simulium guianense* (Diptera: Simuliidae) in northeast Brazil. Florida Entomol 98: 446–450. https://doi.org/10.1653/024.096.0209

53. Rothfels K, Featherston D (1981) The population structure of *Simulium vittatum* (Zett.): the IIIL-1 and IS-7 sibling species. Can J Zool 59: 1857–1883. https://doi.org/10.1139/z81-255

54. McCreadie JW, Adler PH (1998) Scale, time, space, and predictability: species distributions of preimaginal black flies (Diptera: Simuliidae). Oecologia 114: 79–92. https://doi.org/10.1007/s004420050423 PMID: 28307561

55. Adler PH (1988) Ecology of black fly sibling species. In: Kim KC, Merritt RW (eds.), Black flies: ecology, population management and annotated world list. University Park: Pennsylvania State University Press, pp. 63–76.

56. McCreadie JW, Colbo MH (1993) Larval and pupal microhabitat selection by *Simulium truncatum* Lundström, *S. rostratum* Lundström, and *S. verecundum* AA (Diptera: Simuliidae). Can J Zool 71: 358–367. https://doi.org/10.1139/z93-050

57. Villamizar N, Cortez J, Noya-Alarcón O, Escalona M, Botto C, Grillet ME (2011) Primera descripción del hábitat acuícola de *Simulium guianense* s.l. (Diptera: Simuliidae) en el área endémica de oncocercosis, al sur de Venezuela. Bol Malaria Salud Ambiental 51: 97–100.

58. Grillet ME, Basañez MG, Vivas-Martínez S, Villamizar N, Frontado H, Cortez J, et al. (2001) Human onchocerciasis in the Amazonian area of southern Venezuela: spatial and temporal variations in biting and parity rates of black fly (Diptera: Simuliidae) vectors. J Med Entomol 38: 520–530. https://doi.org/10.1603/0022-2585-38.4.520 PMID: 11476332

59. Adler PH, Kudelová T, Kudela M, Seitz G, Ignjatović-Čupina A (2016b) Cryptic biodiversity and the origins of pest status revealed in the macrogenome of *Simulium colombaschense* (Diptera: Simuliidae), history's most destructive black fly. PLoS ONE 11 (1): e0147673. PMID: 26808274

60. Adler PH (2016) Pathways to becoming pests and vectors: lessons from the Simuliidae. Nova Acta Leopoldina (Neue Folge) 411: 23–32.