

Cytotaxonomy of *Simulium cauchense* Floch & Abonnenc and *Simulium quadrifidum* Lutz (Diptera: Simuliidae) in Brazilian Amazonia

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Simulium cauchense Floch & Abonnenc and *Simulium quadrifidum* Lutz are widely distributed in the Amazon region and are morphologically similar at the larval and pupal stages. Chromosomally, these species are readily distinguished by the position of the nucleolar organizer, which is in the short arm of chromosome I in *S. cauchense* and in the long arm of chromosomes III in *S. quadrifidum*. They also differ by three fixed inversions. Sex chromosomes are undifferentiated in both species. Chromosomal resolution of the two species allowed us to evaluate four structural features previously used as diagnostic aids at the larval stage. Characters that distinguish larvae of the two species are the number of branches and branching patterns of the dorsal abdominal setae and the dark band on each primary fan. Branching patterns of the gill histoblasts were often diagnostic, with *S. quadrifidum* exhibiting more proximal branching and *S. cauchense* more distal branching. Sites where both species occurred sometimes had larvae with one petiole branching proximally and the other distally; in these cases examination of the chromosomes permitted assignment of the specimen to species. Pigmentation patterns of larvae, on the other hand, are highly variable. Color typically is sex linked in both species.

Key words: *Simulium* (*Psaroniocompsa*) - polytene chromosomes - cytotaxonomy - Brazilian Amazon

Cytotaxonomic studies of black flies have repeatedly demonstrated the value of chromosomal characters in elucidating phylogenetic relationships, revealing sibling species and providing diagnostic aids for species identification (Rothfels 1988, Adler et al. 2004). In Brazil, numerous black flies have been investigated cytotaxonomically (e.g., Campos et al. 1996, 2001, Charalambous et al. 1996, Hamada & Adler 1999, Luz 1999, Ríos-Velásquez et al. 2002, Pereira 2004). While chromosomal studies are often essential in revealing sibling species and resolving relationships, the strongest taxonomic and phylogenetic resolution of black flies comes from a combined chromosomal-morphological approach. This approach has permitted an analysis of species diversity in the Amazon Basin (Hamada et al. 2002) that is more critical than has been possible using the conventional morphotaxonomic approach alone.

Various subgeneric classifications have been used for Neotropical black flies. Crosskey and Howard (1997) and Crosskey (2002), for example, recognize the Neotropical subgenus *Psaroniocompsa*, with 38 species and 5 species groups. Py-Daniel (1983) and Coscarón (1987) consider the *S. amazonicum* and *S. quadrifidum* species groups of Crosskey and Howard (1997) to represent their

subgenera *Cerqueirellum* and *Coscaroniellum*, respectively. Py-Daniel and Sampaio (1995) ranked these two subgenera as genera. Cytogenetic techniques can provide independent assessments of these phylogenetic hypotheses and yield insight into classification issues.

The objective of the present study is to resolve the chromosomal differences between *Simulium cauchense* Floch & Abonnenc and *Simulium quadrifidum* Lutz, two members of the subgenus *Psaroniocompsa* (Crosskey & Howard 1997), and to evaluate the usefulness of morphological discriminators previously used for the larvae. *S. cauchense* is known from Brazil, French Guiana, Guyana, and Venezuela, whereas *S. quadrifidum*, with a slightly broader distribution, is known from Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Suriname, and Venezuela.

MATERIALS AND METHODS

Larvae were collected from 15 streams in the states of Amapá, Amazonas, Rondônia, and Roraima (Fig. 1). *S. quadrifidum* was collected at 13 sites and *S. cauchense* at 6; the latter species was not collected in the state of Rondônia. Most collections were made in 2000 and 2001, although two collections were made in 1997 and one each was made in 1996, 1999, 2002, and 2003 (Table I).

Larvae were hand collected from all available substrates and fixed in Carnoy's solution (1 part glacial acetic acid: 3 parts absolute ethanol); the fixative was changed 3 or 4 times in the field and the samples were maintained on ice. In the laboratory, the fixative was changed once more and the samples were held at 4°C, pending chromosomal analysis.

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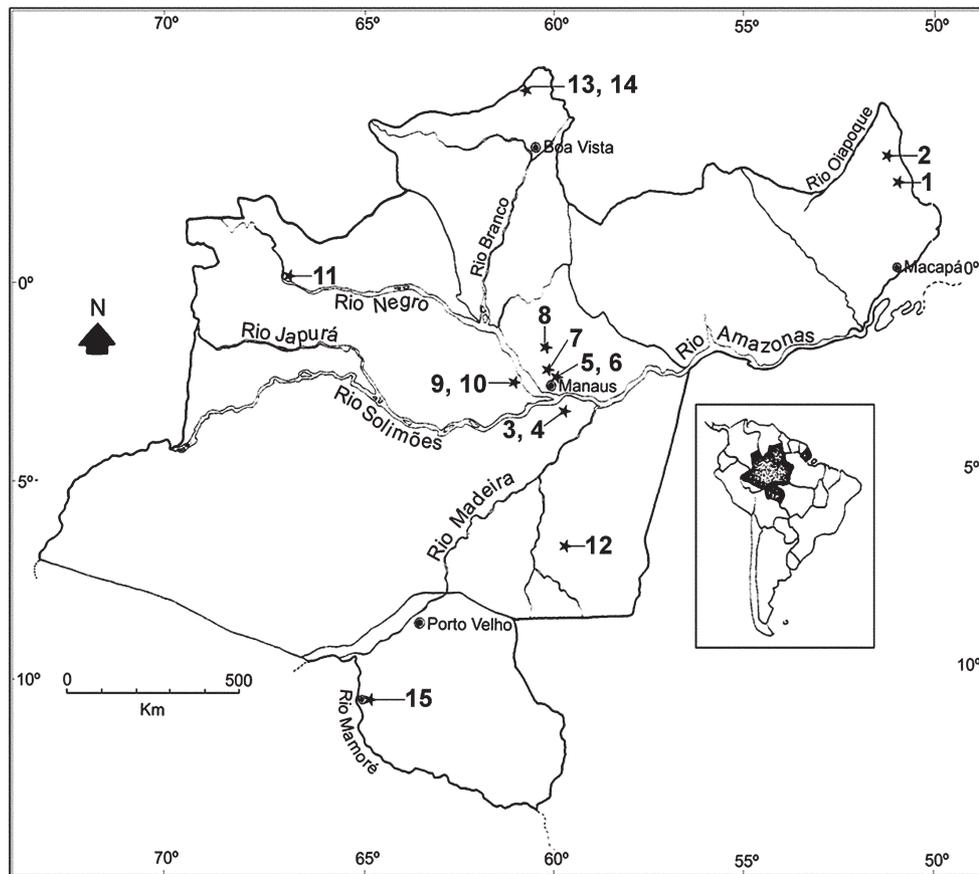


Fig. 1: map of the Brazilian Amazon region, showing collection sites of *Simulium quadrifidum* and *Simulium cauchense* (Diptera: Simuliidae).

The following morphological characters of final-instar larvae were evaluated for their utility in species identification: presence of dark spots on the cephalic rays (Py-Daniel 1983), branching pattern of the dorsal abdominal setae (Hamada et al. 2003), body pigmentation pattern, and branching pattern of the gill histoblast (Shelley et al. 1997, Hamada & Grillet 2001, Hamada et al. 2003).

The Feulgen technique (Rothfels & Dunbar 1953) was used to stain the polytene chromosomes in the silk glands of last-instar larvae. This technique also stained the larval gonads, allowing gender identification *in situ* based on gonad shape (elongate in females, oval to round in males). The sex chromosomes then can be identified *a posteriori* by association of rearrangements with gender. Chromosomal nomenclature follows that of Rothfels (1988) and Rothfels et al. (1978). Fixed inversions are italicized in the text and underlined on the figures; floating inversions appear in Roman type.

The banding sequence of *S. quadrifidum* was used as the standard sequence against which the chromosomes of *S. cauchense* were compared, primarily because *S. quadrifidum* had better chromosomal quality. The chromosomes of a male larva from Amazonas (site 5, Table I) were photographed under oil immersion, and maps were constructed for intraspecific and interspecific comparisons.

Larval specimens are deposited in the Clemson University Arthropod Collection (Clemson, South Carolina, US) and the Invertebrate Collection of the Instituto Nacional de Pesquisas da Amazônia (Inpa) (Manaus, AM, Brazil). Photographic negatives of chromosomes are in the Clemson University Arthropod Collection.

RESULTS

Larval morphology - The most consistent structural features for distinguishing larvae of the two species were the dark spots on the primary rays of the cephalic fan, which typically appeared as a dark band on the fans in *S. quadrifidum* but were absent in *S. cauchense* (Fig. 2), the number of branches of the dorsal abdominal setae [*S. quadrifidum*, mean = 4.6 branches ($n = 6$, $SD = 0.5$), *S. cauchense*, mean = 6.7 branches ($n = 9$, $SD = 1$)] and branching pattern of the dorsal abdominal setae, with branching starting near the base in *S. quadrifidum*, whereas in *S. cauchense* the branching occurs both at the base and at points some distance from the base (Fig. 3). The branching patterns of the gill histoblasts (Figs 6, 7) were often diagnostic, with *S. quadrifidum* exhibiting more proximal branching and *S. cauchense* more distal branching. Sites where both species occurred sometimes had larvae with one petiole branching proximally and the other distally; in these cases the chromosomes permitted as-

signment of the specimen to species. The pigmentation patterns of the body (Figs 4, 5) were highly variable, but color typically was linked to sex in both species, with males being pale brownish or grayish and females dark gray to black, particularly on abdominal segment I.

Polytene chromosomes - The chromosomes of 630 larvae were examined; 162 (25.7%) could be analyzed completely (i.e., all bands were compared with the standard reference map). For *S. quadrifidum* (n = 265), 80 speci-

mens (30 females, 50 males) were analyzed completely, whereas for *S. cauchense* (n = 365), 82 specimens (43 females, 39 males) were analyzed completely (Table II). The low numbers of completely analyzed specimens reflected the poor chromosomal quality, in part because most larvae selected for analysis had mature (dark) gill histoblasts to permit association of gill morphology with chromosomes.

Both species had a chromosomal complement of n = 3,

TABLE I
Sampling sites, dates, and collectors for larvae of *Simulium quadrifidum* and *Simulium cauchense* (Diptera: Simuliidae) in Brazilian Amazonia

Nr	Sampling sites	Coordinates	Date	Collector	Species
Amapá					
1	Highway BR156, Igarapé Água Branca, near Carnot Village	02°40'S/51°21'W	25/07/00	NH	1, 2
2	Highway BR156, 90 km south of Oiapoque	03°11'S/51°32'W	27/07/00	NH	1, 2
Amazonas					
Careiro da Várzea					
3	Road to Purupurú, dirt road Cobra, first bridge	03°23'S/59°38'W	10/06/01	MA; JS; LA	1
4	Road to Purupurú, dirt road Cobra, second bridge	03°23'S/59°38'W	10/06/01	MA; JS; LA	1
Manaus					
5	Road to Riacho Ecológico, entrance of Trigolar Chácara São Sebastião	02°58'S/60°03'W	02/05/96	NH	1
6	Highway AM010 km 30, dirt road Água Branca II, Igarapé Matrinxã, Green Park	02°51'S/59°51'W	05/10/00 12/05/01	MA; JS; SL MA; JS	1, 2 1, 2
7	Highway BR174 km 40, Igarapé Cabeça Branca	02°35'S/60°01'W	16/08/00 17/10/01	MA; JS JS; SC	1 1
Presidente Figueiredo					
8	Highway BR174 km 134, dirt road to comunidade do Castanhal, Igarapé Canoas	01°49'S/60°04'W	16/12/97 03/01/01 17/10/01 05/11/01 12/11/02 08/10/03	NH MA; JS MA; JS AP JS NH	2 2 2 2 2 2
Novo Airão					
9	Highway AM352, Manacapuru, Novo Airão	02°58'S/60°56'W	18/06/01	MA; JS; LA	1
10	Highway AM352, Manacapuru, Novo Airão	02°48'S/60°55'W	19/06/01	MA; JS; LA	1
São Gabriel da Cachoeira					
11	Igarapé Miuá	00°06'S/66°52'W	04/12/00 07/12/00	NH; EP NH; EP	1 1
Apuí					
12	Rio Juma, Balneário Apuí	07°12'S/59°54'W	03/05/99	NH	1
Roraima					
Pacaraima					
13	Highway BR174, dirt road to Maloca Bananal, Igarapé Bananal	04°25'S/61°13'W	10/12/97 13/12/00 20/03/01 25/10/01	NH NH; MA NH; JS NH	1, 2 1, 2 1, 2 1, 2
14	Highway BR174, dirt road to Maloca Bananal, Igarapé Sorocaima	04°25'S/61°13'W	13/12/00 20/03/01 25/10/01	NH; MA NH; JS NH	2 2 2
Rondônia					
Guajará-Mirim					
15	Dirt road to Palheta, after airport	10°47'S/65°12'W	22/09/01	NH	1

Nr: number of collection. Dates are given as day/month/year. Federal highway = BR, Amazonas state highway = AM. Date: day/month/year. Collectors: AP, Ana Maria de Oliveira Pes; EP, Eleny da Silva Pereira; JS, Jeferson Oliveira da Silva; LA, Luis Aquino; MA, Miriam Alvan Aguilar; NH, Neusa Hamada; SC, Sheyla Couceiro; SL, Sérgio Luis Bessa Luz. Species: 1, *Simulium quadrifidum*; 2, *Simulium cauchense*

with standard arm associations (Figs 8-11). The most readily apparent interspecific difference was the position of the nucleolar organizer, which was in the extreme base of IS in *S. cauchense* and in the base of IIIIL in *S. quadrifidum* (Fig. 11). *S. quadrifidum* had a more ex-

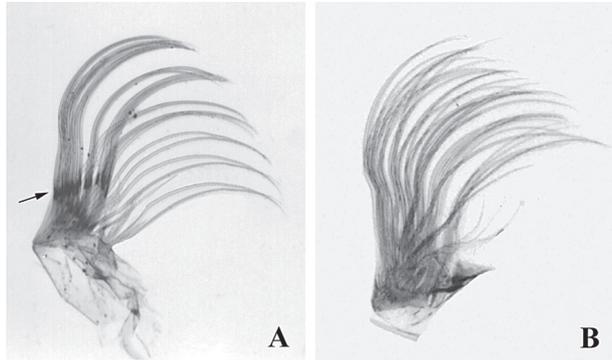


Fig. 2: cephalic fan of larvae of *Simulium quadrifidum* (A) and *Simulium cauchense* (B) (Diptera: Simuliidae); arrow indicates dark band on primary fan.

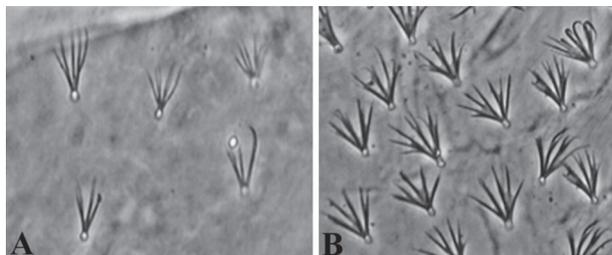


Fig. 3: dorsal abdominal setae of larvae of *Simulium quadrifidum* (A) and *Simulium cauchense* (B) (Diptera: Simuliidae).

panded centromere region in chromosome I than did *S. cauchense* (Fig. 8).

S. cauchense also differed from *S. quadrifidum* by three fixed inversions. Inversion IIIS-1 ran from the middle of section 76 to the end of section 80 (Fig. 10). Inversions IIIIL-1 and IIIIL-2 overlapped broadly (Figs 10, 11). The IIIIL sequence for *S. cauchense* can be derived from that for *S. quadrifidum* by first inverting the IIIIL-1 sequence, followed by the IIIIL-2 sequence (Fig. 10). Polymorphisms were not found in *S. quadrifidum*. Two floating inversions were discovered in *S. cauchense*. Three larvae from Presidente Figueiredo had a subbasal heterozygous inversion in IL (Fig. 11, IL-1). IIIIL-3 (Fig. 10) was a common polymorphism in Hardy-Weinberg equilibrium ($G_{adj.} = 0.1991$, $p > 0.05$) for the one population that was tested (Presidente Figueiredo, 8 October 2003). Both species had undifferentiated sex chromosomes. No evidence of sibling species was found.

DISCUSSION

S. quadrifidum and *S. cauchense* have consistent structural characters that distinguish them in the adult stage (Py-Daniel 1983, Shelley et al. 1997); however, we found that some larval characters previously considered diagnostic (e.g., body pigmentation) overlap, confounding species identification. The polytene chromosomes allow unequivocal assignment of larvae to species.

The polytene chromosomes of *S. quadrifidum* were of higher quality than those of *S. cauchense*. Inferior chromosomal quality has been attributed to factors such as larval age, water temperature, and the quality and quantity of food (e.g., McCreadie & Colbo 1992). In the Amazonian region, the temperature of Simuliidae habitats varies little (Hamada & Adler 2001), suggesting that differ-

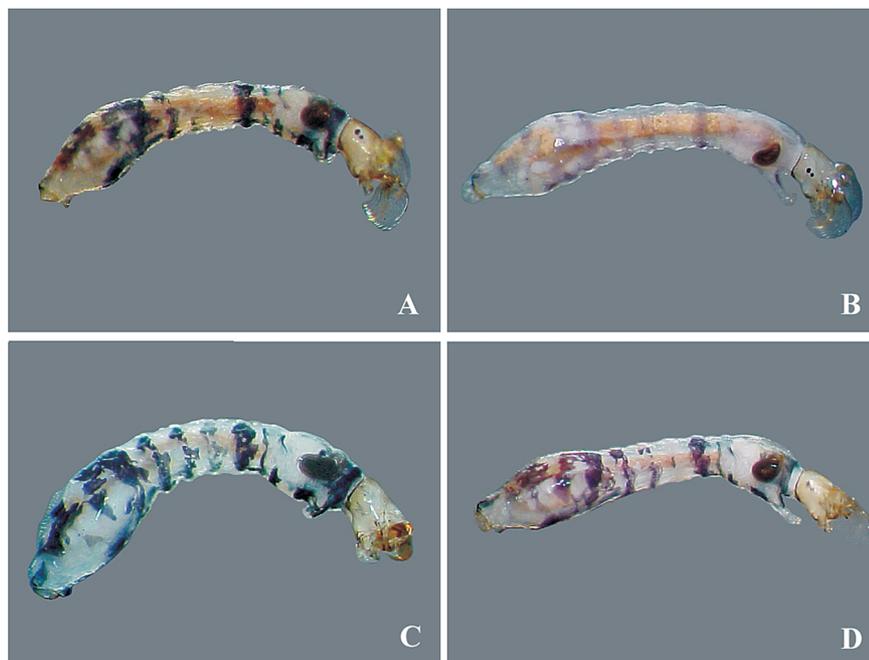


Fig. 4: variation in pigmentation pattern of larvae of *Simulium quadrifidum* (Diptera: Simuliidae). A: female larva, Igarapé Água Branca, AP (site 1); B: male larva, Igarapé Matrinxã, AM (site 6); C: female larva, Igarapé Bananal, RR (site 13); D: female larva, Ramal Palheta, RO (site 15).

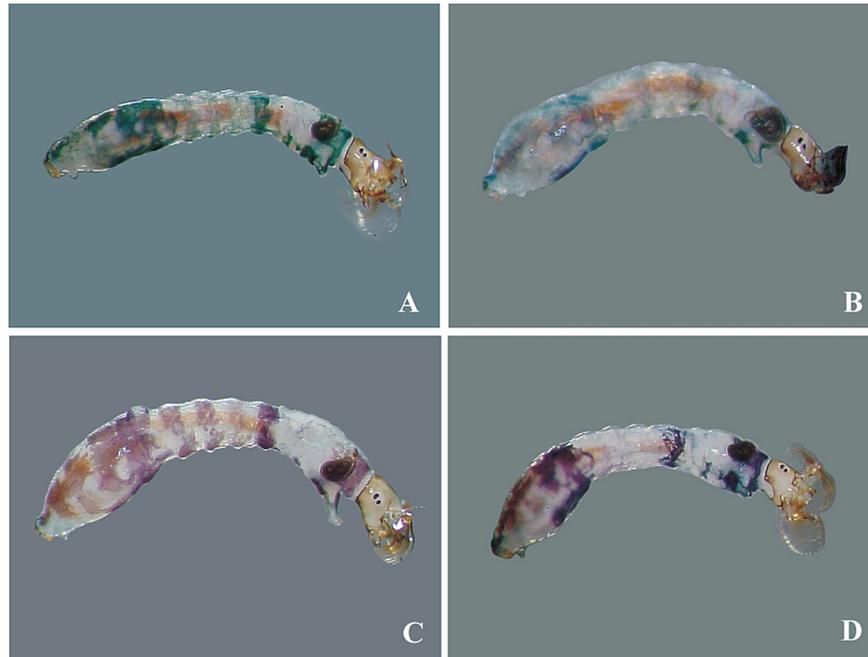


Fig. 5: variation in pigmentation pattern of larva of *Simulium cauchense* (Diptera: Simuliidae). A: female larva, Igarapé Água Branca, AP (site 1); B: male larva, Igarapé Matrinxã, AM (site 6); C: female larva, Igarapé Canoas, AM (site 8); D: female larva, Igarapé Bananal, RR (site 13).

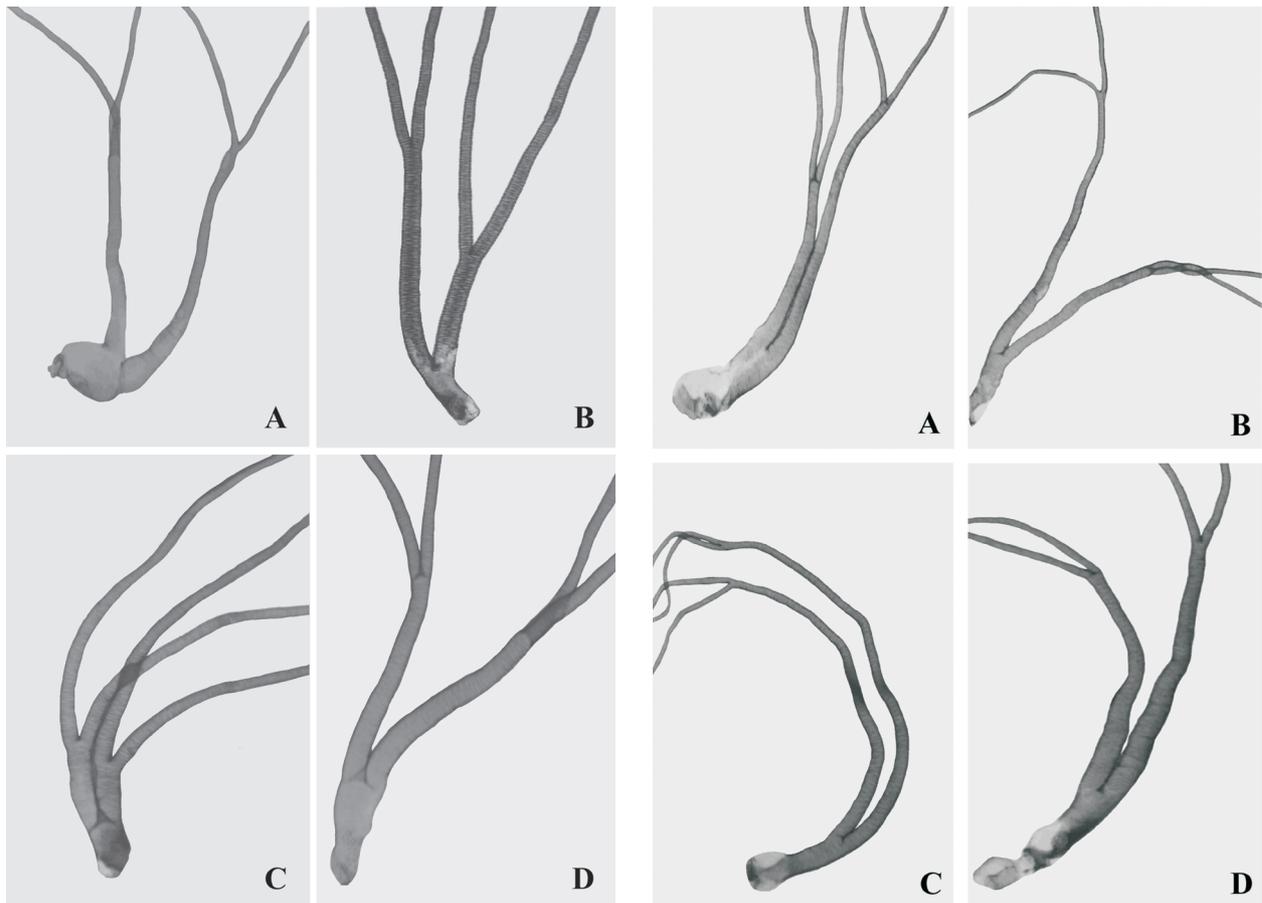


Fig. 6: variation in branching pattern of gill filaments of *Simulium quadrifidum* (Diptera: Simuliidae). A: Igarapé Água Branca, AP (site 1); B: Igarapé Matrinxã, AM (site 6); C: Novo Airão, AM (site 10); D: Ramal Palheta, RO (site 15).

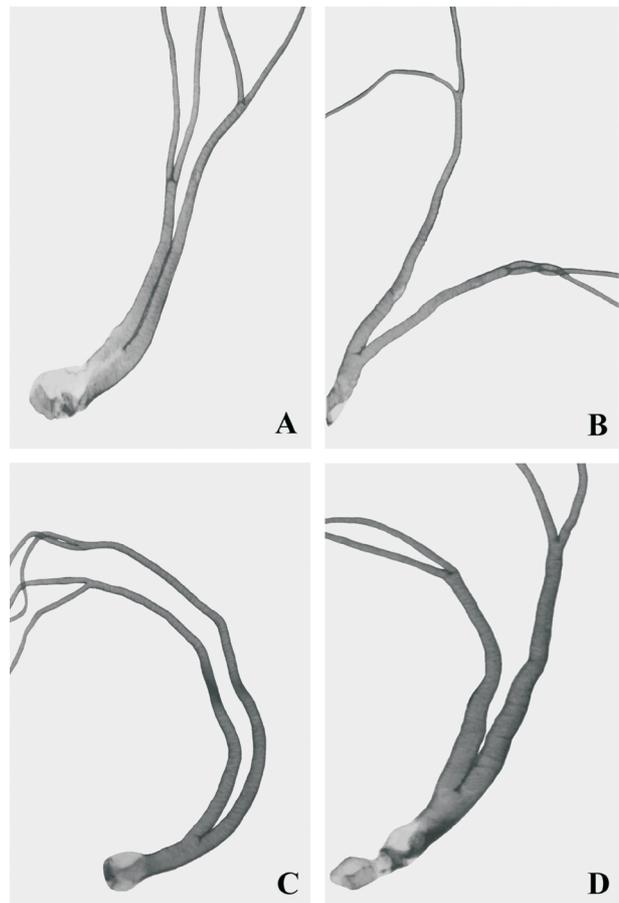


Fig. 7: variation in branching pattern of gill filaments of *Simulium cauchense* (Diptera: Simuliidae). A: Igarapé Água Branca, AP (site 1); B: Igarapé Matrinxã, AM (site 6); C: Igarapé Canoas, AM (site 8); D: Igarapé Bananal, RR (site 13).

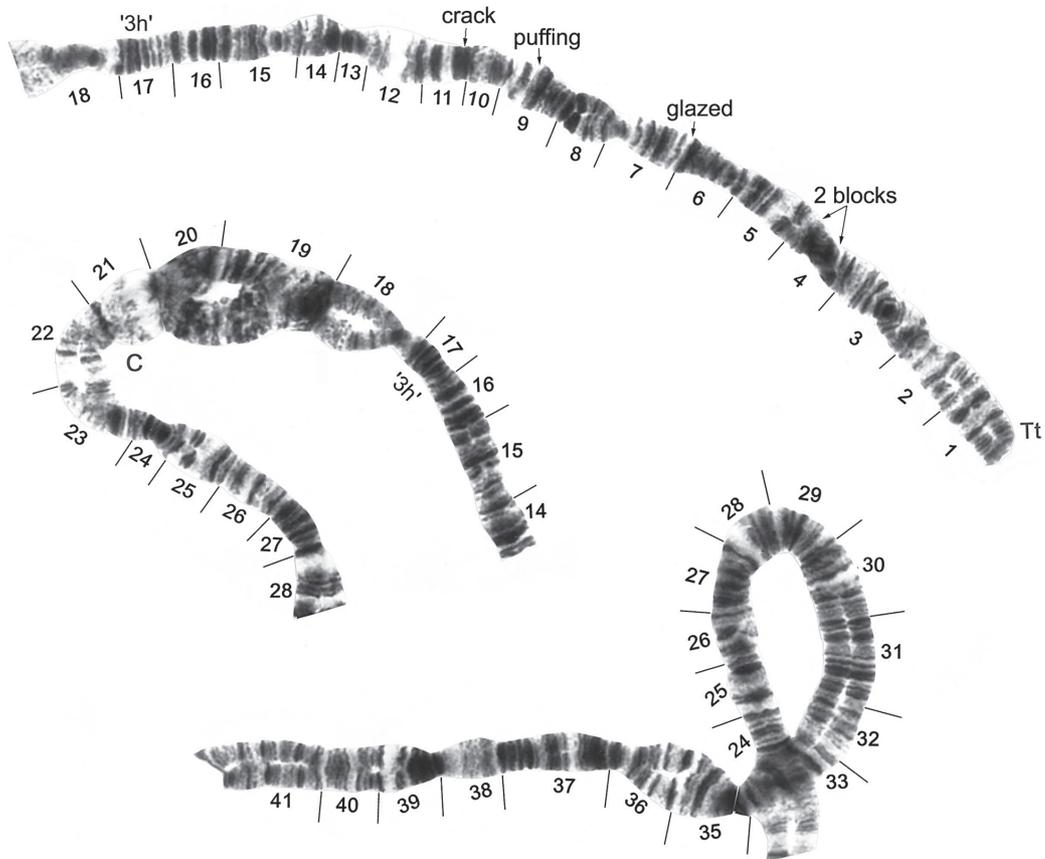


Fig. 8: chromosome I of *Simulium quadrifidum* (Diptera: Simuliidae), showing standard banding sequence of short arm (top), centromere region (middle), and long arm (bottom); C: centromere, Tt: terminal bands, '3h': three heavy bands.

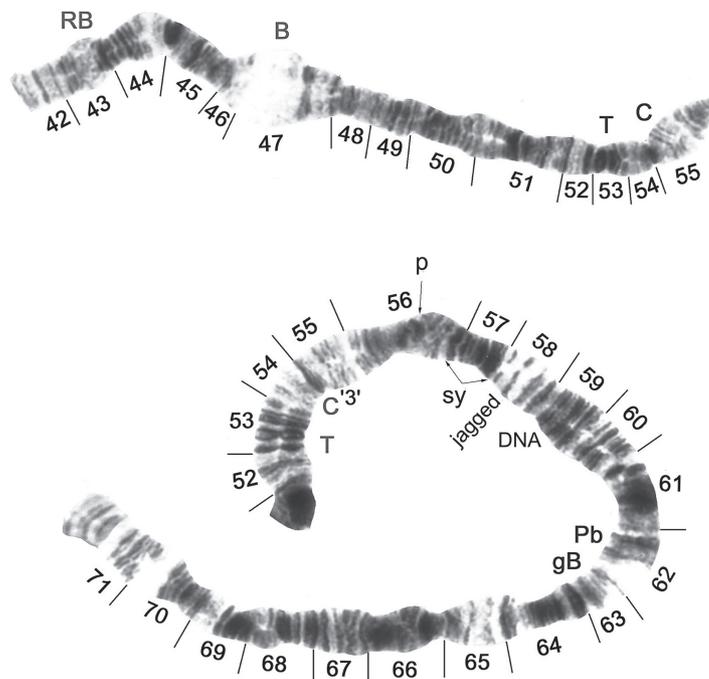


Fig. 9: chromosome II of *Simulium quadrifidum* (Diptera: Simuliidae), showing standard banding sequence of short arm (top) and long arm (bottom); B: bulge, C: centromere, DNA: DNA puff, gB: gray band, p: puffing band, Pb: parabalbiani, Rb: ring of Balbiani, sy: symmetrical, T: trapezoid, '3': three sharp bands.

ences in chromosomal quality between species cannot be attributed solely to water temperature. Ríos-Velásquez et al. (2002) suggested that the chromosomal quality of *S. goeldii* Cerqueira & Nunes de Mello and *S. ulysseesi* (Py-Daniel & Coscarón 2001) is related to stream size and the degree of habitat shading, which are positively correlated with phytoplankton and periphyton production, the main food sources of black flies in Amazonia (Alencar et al. 2001).

Polymorphisms were not found in *S. quadrifidum* and were restricted to two floating inversions in *S. cauchense*. Low levels of inversion polymorphism are characteristic

of other black flies in Brazil (e.g., Campos et al. 1996, 2001, Hamada & Adler 1999, Ríos-Velásquez et al. 2002). The chromosomes of black flies in the central and northern areas of South America, however, are typically polymorphic (e.g., Procnier et al. 1985, Conn et al. 1989, Millett 1992, Hirai et al. 1994). The ecological correlates of chromosomal polymorphism in black flies remain poorly understood.

Although the position of the nucleolar organizer is usually conserved among closely related species (Rothfels 1988, Hamada & Adler 1999), it differs between *S. quadrifidum* and *S. cauchense*. In other members of the

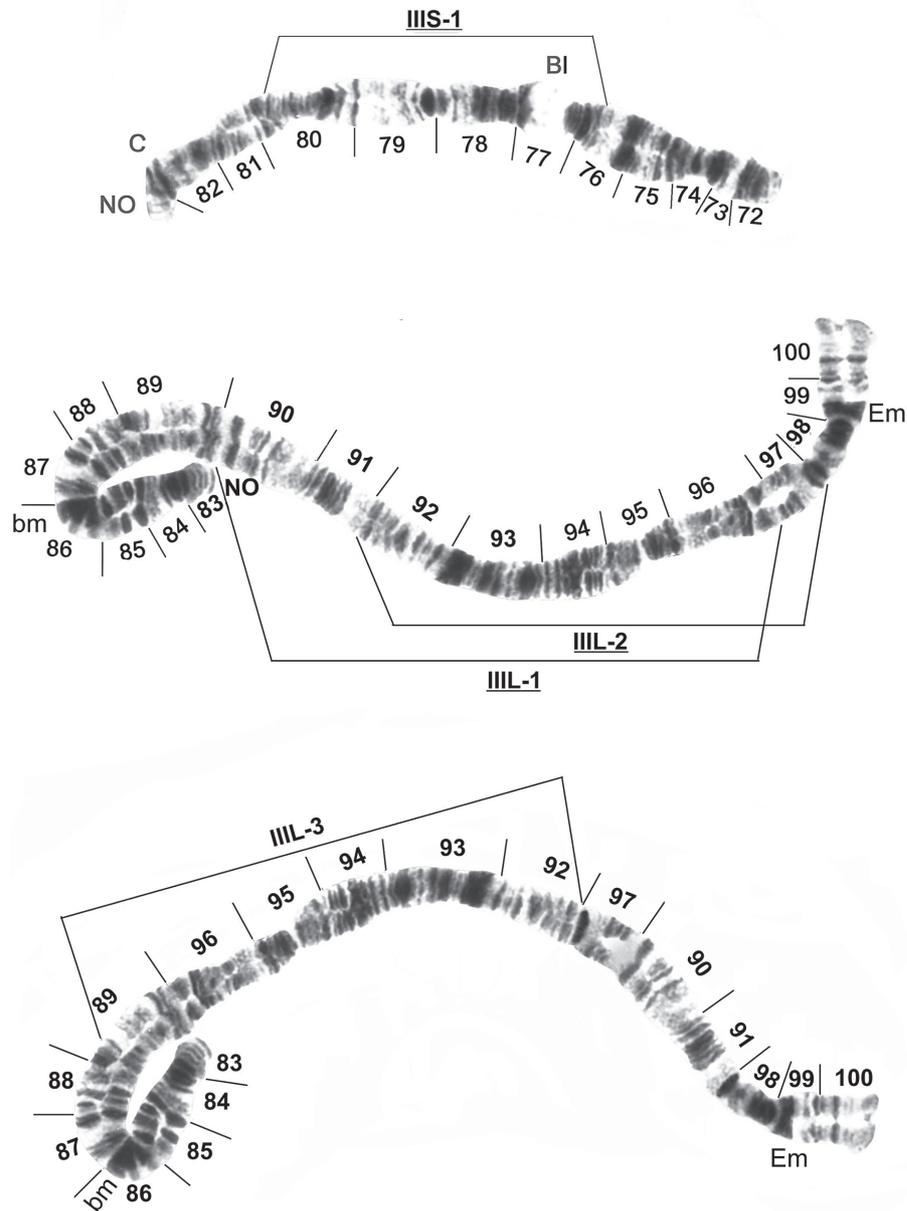


Fig. 10: chromosome III, showing standard banding sequence of short arm (top) and long arm (middle) of *Simulium quadrifidum* and long arm of *Simulium cauchense* (bottom) (Diptera: Simuliidae). Limits of inversions IIS-1, IIL-1, and IIL-2 of *S. cauchense* are indicated by brackets on the standard maps of *S. quadrifidum*. The map of IIL for *S. cauchense* (bottom) was made by cutting and reassembling the standard map of *S. quadrifidum*. The IIL sequence for *S. cauchense* (bottom) can be derived from the *S. quadrifidum* sequence (middle) by first inverting IIL-1 and then inverting IIL-2 on the middle map. The limits of the common floating inversion IIL-3 in *S. cauchense* are bracketed on the map of *S. cauchense* (bottom). BI: blister; bm: basal marker; C: centromere; Em: end marker; NO: nucleolar organizer.

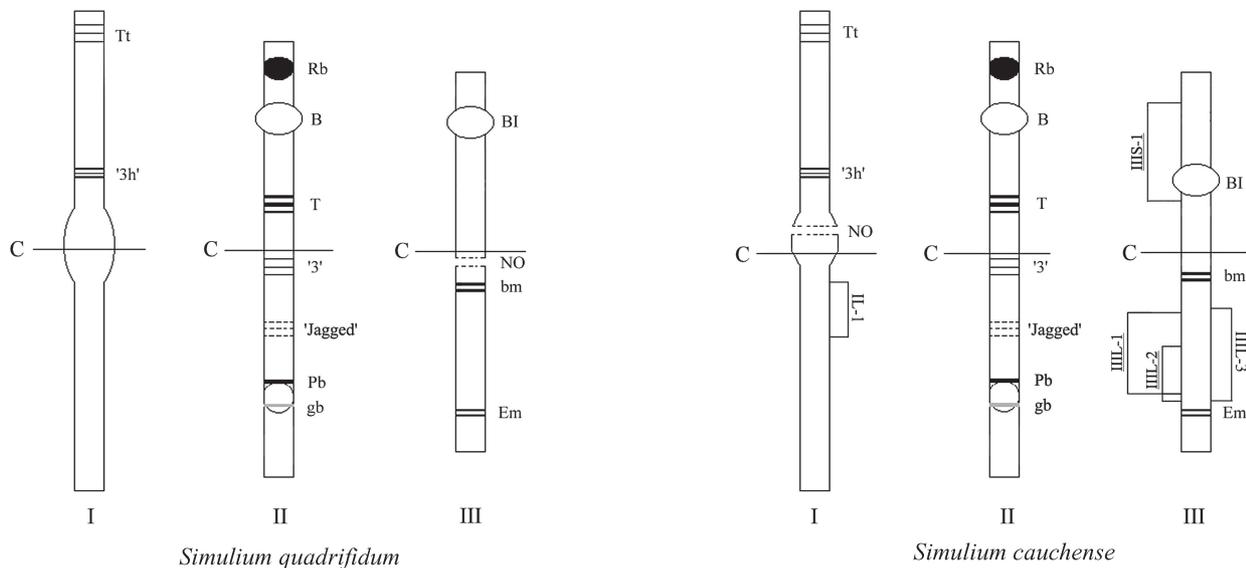


Fig. 11: idiograms of polytene chromosome of *Simulium quadrifidum* and *Simulium cauchense* (Diptera: Simuliidae). I: chromosome I; II: chromosome II; III: chromosome III; C: centromere; B: bulge; BI: blister; bm: basal marker; Em: end marker; gb: gray band; NO: nucleolar organizer; Pb: parabalbiani; Rb: ring of Balbiani; T: trapezoid; Tt: terminal bands; '3h': three heavy bands; '3': three sharp bands. Fixed inversions are underlined and shown on the left side; floating inversions are shown on the right side.

TABLE II

Total number (n) of larvae of *Simulium quadrifidum* and *Simulium cauchense* (Diptera: Simuliidae) examined chromosomally, with the number of female and male larvae analyzed completely, in the Brazilian Amazon region

State	Species					
	<i>Simulium quadrifidum</i>			<i>Simulium cauchense</i>		
Site number	n	Females	Males	n	Females	Males
Amapá						
1	5	1	2	12	0	0
2	5	0	0	12	2	1
Amazonas						
3	8	0	0	-	-	-
4	5	1	0	-	-	-
5	1	0	1	-	-	-
6	53	8	25	10	3	1
7	78	0	6	-	-	-
8	-	-	-	178	24	28
9	3	0	0	-	-	-
10	1	0	0	-	-	-
11	19	3	4	-	-	-
12	13	6	1	-	-	-
Roraima						
13	26	7	7	129	9	6
14	-	-	-	24	5	3
Rondônia						
15	43	2	1	-	-	-
Total	265	30	50	365	43	39

n: total number of larvae identified using at least one diagnostic chromosomal feature (e.g., position of nucleolar organizer), but not necessarily analyzed completely; -: species not collected. Table I provides specific information on sites and dates.

subgenus *Psaroniocompsa* (*sensu* Crosskey & Howard 1997), the nucleolar organizer is located in chromosome I (e.g., *S. daltanhani*, *S. goeldii*, and *S. ulyssesi*) (Ríos-Velásquez et al. 2002, Pereira 2004) or chromosome III (e.g., *S. roraimense* and *S. oyapockense* of the *S. amazonicum* group) (Luz 1999). The position of the nucleolar organizer alone, however, must be used with caution in inferring relationships.

The current study provides a template for comparing the chromosomes of other species in the subgenus *Psaroniocompsa sensu* Crosskey & Howard (1997). The evaluation of additional morphospecies could reveal sibling species and test the validity of the current subgenera and species groups.

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