Research Article

Isozyme variation in four species of the *Simulium perflavum* species group (Diptera: Simuliidae) from the Brazilian Amazon

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Abstract

Electrophoretic studies of isozymes were done with four closely related species of the *Simulium perflavum* species group (Diptera: Simuliidade) in the Brazilian Amazon, using last-instar larvae collected in the field. Ten enzymes were studied, which yielded 11 loci. Diagnostic loci were not found between *Simulium maroniense* cytotype D and *Simulium rorotaense*. *Simulium maroniense* and *S. rorotaense* differed from *Simulium trombetense* by two diagnostic loci (*Me* and *Xdh*), and *Simulium perflavum* differed from the other three species by four diagnostic loci (*Me, Xdh, Mdh*, and *Got)*. The mean number of alleles per locus ranged from 1.30 to 2.30, the percentage of polymorphic loci ranged from 18.2 to 63.6% and the mean heterozygosity values observed ranged from 0.062 to 0.108. Genetic distances among the species ranged from 0.010 to 0.581. The lowest value was obtained between *S. maroniense* and *S. rorotaense,* and the highest between *S. perflavum* and *S. trombetense*. The genetic relationships among the four *S. perflavum* group species indicate that they are closely related. The high similarity at the isozyme level, allied to previous studies of morphology and polytene chromosomes, may suggest that the divergence time since the separation of *S. maroniense* and *S. rorotaense* is still too recent for diagnostic loci to have evolved.

Key words: Simulium perflavum species group, isozymes, genetic divergence, biochemical systematic, Brazilian Amazon.

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Introduction

Enzymatic markers are helpful tools for estimating genetic variability and gene flow among populations and for distinguishing closely related and/or sibling species in different animal groups, especially insects (Steiner *et al*., 1982; Kreutzer *et al*., 1990; Farid *et al*., 1991; Tabachnick, 1992; Krafsur *et al*., 1993; Lopes and Moreno, 1995; Almeida and Sodré, 1998). In the genus *Simulium*, several species have been studied using these markers (May *et al*. 1977; Petersen, 1982; Snyder, 1982; Snyder and Linton, 1983; Mebrahtu *et al*., 1987; Charalambous *et al*., 1993; Davies *et al*., 2000). However, relatively few loci were interpreted in most of the studies (Mebrahtu *et al*., 1987; Charalambous *et al*., 1993; Davies *et al*., 2000).

The *Simulium perflavum* species group is distributed in South America from the non-Andean northern to northern Argentina. Systematic studies on this species group are controversial, with the number of species believed to exist varying from four to seven due to high similarity at the mor-

phological level for some species (Coscarón, 1990; Py-Daniel and Sampaio, 1995; Crosskey and Howard, 1997; Shelley *et al*., 1997; Hamada and Adler, 1999). Currently, this species group is composed of seven species: *Simulium perflavum* Roubaud 1906, *Simulium maroniense* Floch and Abonnenc 1946, *Simulium rorotaense* Floch and Abonnenc 1946, *Simulium kabanayense* Ramírez Pérez and Vulcano 1976, *Simulium ignacioi* Ramírez Pérez and Vulcano 1977, *Simulium suarezi* Ramírez Pérez, Rassi and Ramírez 1977 and *Simulium trombetense* Hamada, Py-Daniel and Adler 1999, based on the chromosomal, morphological and ecological evidence (Hamada, 1993, 1998; Hamada and Adler, 1998, 1999; Hamada and McCreadie, 1999; Alencar *et al*., 2001a, b).

Shelley *et al.* (1984, 1997) placed *S. maroniense*, formerly considered a good species by Coscarón (1990), and *S. ignacioi* in synonymy with *S. rorotaense*. Later, Hamada and Adler (1998, 1999) showed that *S. rorotaense* and *S. maroniense* can be distinguished at the morphological, cytotaxonomic and ecological levels and that *S. ignacioi* also has a distinct chromosomal configuration (N. Hamada, P.H. Adler and M.E. Grillet, unpublished data).

Based on polytene chromosome patterns, two groups of sister species were established for four species analyzed

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from this group: 1) *S. perflavum* and *S. trombetense*, which share one fixed inversion (IIIL-1) located in the terminal region of chromosome IIIL, and 2) *S. maroniense* and *S. rorotaense,* which share one fixed inversion (IIIL-5) also located in the terminal region of chromosome IIIL (Hamada and Adler, 1999). In addition, within the *S. maroniense* taxon four cytotypes were recognized, denominated A, B, C, and D (Hamada and Adler, 1999). Each cytotype has a different sex chromosome and different polymorphisms on the autosomal chromosomes, and each is associated with different environmental conditions such as landscape type, temperature, altitude, and geographical distribution. Cytotype A occurs in the rainforest on Mount Pacaraima, Roraima state (Brazil), cytotype B is represented by populations in savanna and forested areas in Roraima state (Brazil), cytotype C is found in Amapá and Pará states (Brazil), and cytotype D occurs in Amazonas state (Brazil). All cytotypes of *S. maroniense* can be distinguished from *S. rorotaense* by a fixed terminal inversion on the long arm of chromosome III (IIIL-5) and gill-histoblast morphology of last-instar larvae and pupae. In the present study, only *S. maroniense* cytotype D was included in the analyses.

In this study, we investigated the genetic variability and genetic divergence of populations of *S. maroniense* cytotype D, *S. rorotaense*, *S. trombetense*, and *S. perflavum*, based on isozyme electrophoresis, as an attempt to better understand the evolutionary relationships among these closely related species. This is the first study applying enzymatic markers to Simuliidae from Brazil.

Material and Methods

Black-fly collection

Black-fly specimens were collected in Presidente Figueredo and Manaus Counties, Amazonas state, Brazil. *Simulium rorotaense* was collected in Acará stream, Reserva Florestal Adolfo Ducke (02°57' S; 59°57' W), Manaus County. The other three species were collected in Presidente Figueiredo County: *S. maroniense* cytotype D in the Canoas stream (01°49' S; 60°04' W), BR-174 Highway km 134; *S. trombetense* in the Pantera stream (02°02' S; 59°50' W), AM-240 Highway km 20; and *S. perflavum* in the Escada stream (02°02' S; 59°52' W), AM-240 Highway km 16. *Simulium maroniense*, *S. rorotaense* and *S. trombetense* are found in larger, more shaded streams with substrates of boulders or bedrock, while *S. perflavum* occurs in smaller, slower, more open streams with sandy or pebble bottoms. The *S. maroniense* habitat has significantly higher pH than the habitats of the other three species.

Larvae were collected on all of the possible substrates in the streams, placed in Petri dishes with wet filter paper and maintained on ice until arrival in the laboratory at the Instituto Nacional de Pesquisas da Amazonia (INPA), Manaus, Amazonas. The last-instar larvae were identified according to Hamada and Adler (1998). Subsequently, they were placed in Eppendorf tubes, labeled and stored at -70 °C until electrophoretic analyses.

Isozyme electrophoresis

Individual larvae of the four species were homogenized in 20 µL of 0.5% β-mercaptoethanol solution (v:v) on ice. The resulting suspensions were absorbed in Whatman number 3 papers, applied to the gels, and electrophoresed at 8 °C. The isozymes were analyzed using two types of electrophoresis horizontal supports: starch (12%) and starch-agarose (2 and 0.8%, respectively) gels (Scarpassa *et al.*, 1999). Thirteen enzymes were tested (MDH, ME, PGM, ACON, IDH, GOT, PGI, XDH, EST, LAP, 6-PGD, HK, and GDH). However, the three latter enzymes were not considered in the analyses due to their poor resolutions in the electrophoretic profiles, which did not allow accurate genotype counts. The enzyme systems, including symbol, Enzyme Commission number, and number of studied loci were: esterase (EST, E.C.3.1.1.1, 1 locus), leucine aminopeptidase (LAP, E.C.3.4.11.1, 2 loci), phosphoglucomutase (PGM, E.C.5.4.2.2, 1 locus), isocitrate dehydrogenase (IDH, E.C.1.1.1.42, 1 locus), aconitase (ACON, E.C.4.2.1.3, 1 locus), malate dehydrogenase (MDH, E.C.1.1.1.37, 1 locus), malic enzyme (ME, E.C.1.1.1.40, 1 locus), xanthine dehydrogenase (XDH, E.C.1.2.1.37, 1 locus), phosphoglucose isomerase (PGI, E.C.5.3.1.9, 1 locus), and glutamate-oxaloacetate transaminase (GOT, E.C.2.6.1.1, 1 locus). The buffer solutions and reaction mixtures were prepared according to Steiner and Joslyn (1979) and Scarpassa *et al*. (1999) with minor modifications.

Statistical analysis

Statistical analyses were performed using the Biosys-1 program (Swofford and Selander, 1989). Based on the allele frequencies, the genetic variability of each species was estimated using the mean number of alleles per locus (A), proportion of polymorphic loci (P), mean observed (H_o) and expected (H_e) heterozygosity, and test for conformance to Hardy-Weinberg equilibrium by chi-square analysis. The genetic relationship among species was measured by the Nei genetic distance values and dendrogram. Genetic distance (Nei, 1978) values were used to produce the dendrogram (Figure 1) based on the unweighted pair-group method using the arithmetic average (UPGMA). The no-criteria option from the Biosys-1 program was used to estimate the percentage of polymorphic loci. Thus, a locus was considered polymorphic regardless of the frequency of alleles detected. For each locus, the most frequent allele was designated as 100 and the other alleles numbered according to their mobility in relation to the most common allele.
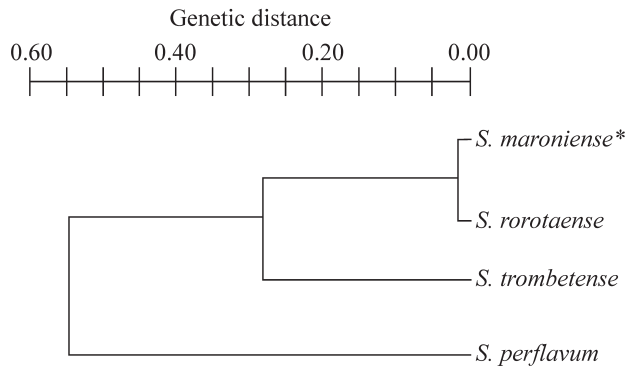
Genetic distance

Figure 1 - Dendrogram of the Nei genetic distance among four species of the *Simulium perflavum* group, based on the UPGMA method. Cophenetic correlation = 0.996. * *Simulium maroniense* cytotype D.

Results

The ten enzymes examined yielded 11 loci in the four species studied. All enzymes showed electronegative migration (anodic). Of the 11 loci, two (*Lap-1* and *Lap-2*) were monomorphic for the same allele in the four species (Table 1). The remaining loci were either polymorphic for at least one of the species or were monomorphic within each species but had diagnostic alleles among the species, *e.g.*, the *Xdh* and *Me* loci. Table 1 shows sample size, allele frequency and Hardy-Weinberg equilibrium for each locus

in the four species. Homogenous allelic frequencies were observed for 11 loci between *S. maroniense* and *S. rorotaense.* Consequently, none of the 11 loci was diagnostic between the two species; only the *Pgi* locus showed some evidence for differentiation in the most common allele Pgi^{100} between *S. maroniense* (0.825) and *S. rorotaense* (0.604). *Simulium maroniense* and *S. rorotaense* differed from *S. trombetense* by two diagnostic loci (*Me* and *Xdh*), which were fixed for different alleles. Me^{100} and Xdh^{100} alleles were fixed in *S. maroniense* and *S. rorotaense*, whereas the Me^{103} and Xdh^{98} alleles were fixed in *S. trombetense.* *Simulium perflavum* differed from *S. maroniense*, *S. rorotaense* and *S. trombetense* by four diagnostic loci (*Me*, *Xdh, Mdh,* and *Got*). The chi-square test applied to the Hardy-Weinberg model showed that most loci were in equilibrium (Table 1). However, two loci out of 19 comparisons (10.5%) showed significant deviations from the expected Hardy-Weinberg equilibrium, due to deficiency of heterozygote individuals for the *Pgm* and *Got* loci in *S. maroniense* and *S. trombetense*, respectively. Three-banded heterozygotes were stained in four enzymes (MDH, IDH, GOT, and PGI), indicating a dimeric structure for these enzymes, and three enzymes (ACON, PGM, and EST) showed two bands in the heterozygotes, a pattern typical of monomeric enzymes. The enzymes ME, XDH, and LAP were monomorphic within each species (*i.e.* no heterozygote individual was detected). Therefore, it was not possible to know the structures of these proteins in the studied species.

Table 1 - Allele frequencies in four species of the *Simulium perflavum* species group.

Locus	Allele	Species			
		S. maroniense[*]	*S. rorotaense*	*S. trombetense*	*S. perflavum*
Mdh	n	67	51	36	31
	113	0.022^b	0.000	0.000	0.000
	109	0.000	0.000	0.000	1.000^b
	105	0.097^b	0.000	0.000	0.000
	100	0.873^b	0.990^b	0.972^b	0.000
	96	0.000	0.010^b	0.000	0.000
	91	0.007^b	0.000	0.028^b	0.000
χ^2_{H-W}[a]		4.45	0.00	0.01	
Acon	n	20	20	24	24
	105	0.000	0.000	0.021	0.000
	103	0.000	0.000	0.104	0.000
	102	0.100	0.000	0.000	0.000
	100	0.900	1.000	0.875	1.000
χ^2_{H-W}		0.18		0.40	
Me	n	21	19	18	25
	118	0.000	0.000	0.000	1.000^b
	103	0.000	0.000	1.000^b	0.000
	100	1.000^b	1.000^b	0.000	0.000

Table 1 (cont.)

Locus	Allele	Species			
		S. maroniense[*]	*S. rorotaense*	*S. trombetense*	*S. perflavum*
Pgm	n	57	28	41	32
	102	0.035	0.143	0.049	0.422
	100	0.860	0.786	0.915	0.578
	97	0.061	0.054	0.037	0.000
	94	0.044	0.018	0.000	0.000
$\chi^2_{\text{H-W}}$		17.11***(df = 6)	1.88	0.30	1.07
Idh	n	53	37	27	23
	108	0.019	0.000	0.000	0.000
	100	0.981	1.000	1.000	1.000
$\chi^2_{\text{H-W}}$		0.01			
Got	n	47	36	47	22
	117	0.011[b]	0.000	0.000	0.000
	106	0.000	0.181[b]	0.032[b]	0.000
	100	0.968[b]	0.819[b]	0.936[b]	0.000
	94	0.021[b]	0.000	0.032[b]	0.000
	89	0.000	0.000	0.000	1.000[b]
$\chi^2_{\text{H-W}}$		0.03	0.01	30.38***(df = 3)	
Pgi	n	60	48	63	23
	106	0.025	0.021	0.016	0.152
	100	0.825	0.604	0.968	0.261
	93	0.133	0.375	0.016	0.587
	88	0.017	0.000	0.000	0.000
$\chi^2_{\text{H-W}}$		2.55	0.96	0.05	6.08
Xdh	n	19	20	27	44
	102	0.000	0.000	0.000	1.000[b]
	100	1.000[b]	1.000[b]	0.000	0.000
	98	0.000	0.000	1.000[b]	0.000
Est	n	53	33	36	20
	100	0.962	1.000	0.972	1.000
	98	0.038	0.000	0.028	0.000
$\chi^2_{\text{H-W}}$		0.06		0.01	
Lap-1	n	26	20	27	31
	100	1.000	1.000	1.000	1.000
Lap-2	n	31	25	33	31
	100	1.000	1.000	1.000	1.000

[*] *Simulium maroniense* cytotype D. df = degree freedom.
[a] Chi-square for Hardy-Weinberg Equilibrium; *** p < 0.0001.
[b] Diagnostic alleles.

The measures of genetic variability (Table 2) showed that the mean ± SE number of alleles per locus ranged from 1.30 ± 0.20 to 2.30 ± 0.40; the lowest value was for *S. perflavum* and the highest value for *S. maroniense*. The percentage of polymorphic loci ranged from 18.2 to 63.6%, with the lowest and the highest values for *S. perflavum* and *S. maroniense*, respectively. The mean ± SE heterozygosity observed ranged from 0.062 ± 0.025 to 0.108 ± 0.056, with the lowest value for *S. trombetense* and the highest for *S. rorotaense* and *S. maroniense*. The latter two species had similar values for this measure.

Table 3 shows the values for similarity and genetic distance (above and below the diagonal, respectively) among the species. The genetic distance between *S.*

Table 2 - Measures of genetic variability in four species of the *Simulium perflavum* species group.

Species	Mean sample size per locus	Mean number of alleles per locus (A)	Percentage of polymorphic[a] loci (P)	Mean heterozygosity	
				Observed (H_o)	Expected (H_e)
S. maroniense*	41.3	2.3 (0.4)[b]	63.6	0.101 (0.035)	0.104 (0.035)
S. rorotaense	30.6	1.6 (0.3)	36.4	0.108 (0.056)	0.108 (0.056)
S. trombetense	34.5	1.9 (0.3)	54.5	0.062 (0.025)	0.062 (0.023)
S. perflavum	27.8	1.3 (0.2)	18.2	0.088 (0.060)	0.097 (0.066)

* *Simulium maroniense* cytotype D.
[a] A locus was considered polymorphic regardless of the frequency of the detected alleles.
[b] The values within parenthesis are patterns errors.

Table 3 - Matrix of similarity (above) and distance (below) in four species of the *Simulium perflavum* species group.

Species	*maroniense**	*rorotaense*	*trombetense*	*perflavum*
S. *maroniense**	-	0.990	0.799	0.572
S. *rorotaense*	0.010	-	0.785	0.597
S. *trombetense*	0.225	0.242	-	0.559
S. *perflavum*	0.559	0.515	0.581	-

* *Simulium maroniense* cytotype D.

maroniense and *S. rorotaense* was 0.010, 0.559 between *S. perflavum* and *S. maroniense*, 0.515 between *S. perflavum* and *S. rorotaense*, and 0.581 between *S. perflavum* and *S. trombetense*. *Simulium trombetense* is more closely related to *S. maroniense* and *S. rorotaense* than to *S. perflavum* (D = 0.225 and 0.242, respectively). The genetic distance dendrogram (Figure 1) based on UPGMA methods showed that *S. maroniense* and *S. rorotaense* are highly similar. *Simulium perflavum* is the most divergent species among the four species studied in the *S. perflavum* group.

Discussion

In the *S. perflavum* species group, the mean heterozygosity ranged from 0.062 to 0.108, which was similar to average values found in invertebrates (0.112 ± 0.072) (Nevo, 1978) and in other Diptera (0.115 ± 0.009) (Graur, 1985), as well as being very similar to values found in mosquitoes, such as *Toxorhynchites rutilus* (Hilburn *et al*., 1984), *Anopheles darlingi* (Manguin *et al*., 1999), *Anopheles nuneztovari* (Scarpassa *et al*., 1999), and *Aedes aegypti* (Souza *et al*., 2000). *Simulium maroniense* had the highest values for the two indices of variability (A and P). *Simulium maroniense* and *S. rorotaense* showed the highest values for mean heterozygosity (H_o and H_e). Thus, the highest values for the three indices detected for *S. maroniense* may indicate a large population size with low probability of inbreeding, perhaps because populations of this species occupy a wide niche and/or because these populations are ex-

posed to great environmental heterogeneity in the central Brazilian Amazon (McDonald and Ayala, 1974; Narang, 1980). *Simulium perflavum* showed the lowest values for the first two indices (A and P); however, this species did not have the lowest heterozygosity value. These results agree, partially, with those obtained by polytene chromosome analysis, where *S. perflavum* was almost monomorphic throughout the study area in Brazil and Venezuela (Hamada and Adler, 1999). *Simulium perflavum* is of specialist habitat and occupies a narrow niche (Hamada, 1993; Hamada and McCreadie, 1999), which may be reflected in the low levels of polymorphism indicated by izozymes and polytene chromosomes.

The isozyme markers used to estimate genetic relationships among the four species in the *S. perflavum* group suggest that these species are closely related (Distance = 0.010-0.581). Similar genetic distance values were found among sibling species in the *Simulium jenningsi* complex: D = 0.10-0.58 (May *et al*., 1977). However, our values were higher than those observed among sibling species of the *S. venustum* and *S. verecundum* complexes: D = 0.06-0.29 (Snyder, 1982). In anophelines mosquitoes, the average genetic distance between pairs of sibling species is often between 0.10 and 0.30 (Bullini and Coluzzi, 1982). Values above this range have usually been found between species that already show some degree of morphological differentiation (Cianchi *et al*., 1981; Manguin *et al*., 1995; Dias-Rodrigues, 1998; Schaffner *et al*., 2000). In the *Drosophila willistoni* group, mean genetic distances for various stages of evolutionary divergence are as follows: between local populations 0.03; between subspecies 0.23; between sibling species 0.58; between non-sibling species 1.06 (Ayala *et al*., 1975). In the present study, the distance values found are closer to those obtained among sibling species in *Simulium* and *Drosophila*.

In the present study, the four species analyzed can be morphologically distinguished at the pupal stage. They therefore cannot be considered to be sibling species. Four diagnostic loci allowed separation of *S. perflavum* from the

other three species, while two diagnostic loci distinguished *S. trombetense* from *S. maroniense* and *S. rorotaense*. Thus, *S. perflavum* is genetically well diverged from the other species. This conclusion agrees with the polytene chromosome data, where *S. perflavum* is the most divergent from the other species, with six fixed inversions. Although two diagnostic loci were found between *S. trombetense* and *S. maroniense*/*S.rorotaense*, the genetic distance values were low (D = 0.225 - 0.242, similar to values found between sibling species of *Simulium* and *Anopheles*, and between subspecies of *Drosophila*). This result suggests that the morphological differences by which these species are well defined arose with little differentiation at the isozyme level. Interestingly, the polytene chromosome data indicated that *S. trombetense* is more closely related to *S. perflavum* than to *S. maroniense* and *S. rorotaense*. The discrepancy between chromosome and isozyme data could be explained by the IIIL-1 inversion that is fixed and shared in *S. trombetense* and *S. perflavum*, which is really part of the ground plan of the *S. perflavum* group, rather than a synapomorphy for these two species. If this is the case, *S. trombetense*, *S. perflavum*, and *S. maroniense* / *S. rorotaense* would be in an unresolved chromosomal trichotomy. On the other hand, analysis of additional isozyme loci may provide a better explanation for this distinctive result, but it has been difficult to identify many loci with good resolution in these *Simulium* species.

Diagnostic loci were not found between *S. maroniense* cytotype D and *S. rorotaense*, and the genetic distance was very low (D = 0.010). The level of heterozygosity was also similar between the two species. Of the 11 loci, only the *Pgi* locus appears to be in the process of differentiation. Similar distance values have frequently been observed among populations of same species (Ayala *et al.*, 1975; Manguin *et al.*, 1995; Scarpassa *et al.*, 1999; Sukowati *et al.*, 1999). Results somewhat similar to these of the present study have been obtained between sister taxa *Prosimulium fuscum* and *P. mixtum*. Of the 11 loci studied, only one (*6-Pgd*) allowed identification between the two *Prosimulium* species; the remaining loci showed similar allele frequencies and the monomorphic loci had identical alleles (Snyder and Linton, 1983). Low distance values have also been found between *Drosophila heteroneura* and *D. silvestre* (Sene and Carson, 1977) and among fossorial mole rats in the *Spalax ehrenbergi* complex (Nevo and Cleve, 1978), which were attributed to recent divergence. In the present study, the low genetic distance value and the absence of diagnostic loci should be interpreted cautiously because the three other *S. maroniense* cytotypes were not used for comparison with *S. rorotaense*, so that diagnostic loci might be found in these cytotypes, especially in the most divergent ones. At the morphological level, although these two species are isomorphic in the adult stage, the four *S. maroniense* cytotypes can be distinguished from *S. rorotaense* using gill-histoblast morphology of last-instar larvae and pupae and also by a fixed inversion on the long arm of chromosome III (IIIL-5), with a confidence level of 99.4%. Alternatively, the examination of additional isozyme loci or other genetic markers that evolve faster, such as mtDNA (mitochondrial DNA) and highly variable loci (microsatellites), may permit better separation between the *S. maroniense* cytotype D and *S. rorotaense*. However, although the three other cytotypes were not used for comparison in this study, the high similarity found at the isozyme level, allied with previous data on morphology and cytotaxonomy, may indicate that the evolutionary divergence time since the separation of these two species is still very recent.

In summary, the genetic relationships suggested by the comparison of 11 isozyme loci in four species of the *S. perflavum* group indicate that these species are closely related. This result supports those from the external morphology data and agrees, at least in part, with the relationships established from polytene chromosome rearrangements. This is the first study applying enzymatic markers to Simuliidae in Brazil, and the results suggest that these markers can provide useful insights into the evolutionary history of this family.

Acknowledgments

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