POPULATION GENETICS OF SOME NEW WORLD SIMULIIDAE

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I. INTRODUCTION

Genetic studies have made several notable contributions to our understanding of black fly problems. The comparison of banding patterns of polytene chromosomes from larval salivary glands has resulted in the description of eight new species of black fly from what used to be called simply Simulium damnosum. (1) Consequently, a major problem has been to associate larval stages (on which the cytotaxonomy is based) with adults which may be vectors of onchocerciasis. Cytotypes cannot usually be determined in the adult stage. Harold Townson has examined the isozyme patterns of five members of the S. damnosum complex: Simulium sanctipauli, S. squamosum, S. sirbanum, S. yahense and S. damnosum (sensu strictu). Townson found that isozymes of phosphoglucomutase (Pgm) in S. yahense were different from those found in the other cytotypes. This information was used to link adults biting man with larvae that could be identified cytologically as S. yahense. This was the first demonstration that S. yahense adults were anthropophilic.(2)

May and his co-workers studied three species of the Simulium jenningsi complex from Maine, USA, which are identifiable in the larval and pupal stages although the adults are morphologically indistinguishable.(3) The lack of diagnostic morphological markers prevented the identification of the species biting man along the Penobscot River watershed. Starch gel electrophoresis was used to identify four key enzymes that enabled May and his co-workers to associate the principal anthropophilic black fly with its larval and pupal stages.

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Subsequently, this undescribed species was named Simulium penobscotensis.(4)

Three Central and South American black fly species-complexes are recognized: (1) S. amazonicum; (2) S. sanguineum; and (3) S. metallicum. In May 1979, an informal workshop on the taxonomy of medically important South American Simuliidae was held in Brazil in part to clear up confusion associated with these species. The following questions were posed:(5)

"(a) Should the question of possible species-complexes existing in South American Simulium pest and vector species be investigated immediately, and if so, how can this be done effectively?

(b) Should cytotaxonomic and enzymotaxonomic techniques be employed as soon as possible as adjuncts to morphological studies, or deferred until conventional taxonomy is further advanced?"

More recently the Guatemala-Japan Joint Conference on Onchocerciasis Research and Control held in Guatemala City, Guatemala, 12-16 January 1981 recognized the problem of the S. metallicum complex and recommended continued study by enzyme electrophoresis and cytotaxonomy, especially in areas endemic for human onchocerciasis.

Our work at the Gorgas Memorial Laboratory in Panama has centered on the investigation of genetic variation in S. metallicum. We have obtained preliminary evidence from isozyme studies that S. metallicum is made up of at least two genetically distinguishable groups.

II. METHODS AND MATERIALS

Six black fly species collected in Chiriqui Province, Western Panama (8° 38' N; 82° 14' W) were studied: Simulium chiriquiense, S. metallicum, S. ochraceum, S. panamense, S. quadrivittatum and S. rubicundulum. Larvae and pupae were collected from streams. Adults were collected by battery-powered, hand-held aspirators as they landed on either humans or cows.

Collected black flies were immediately placed on ice for transport to Panama City where they were stored in a deep freeze at -55°C until needed for electrophoresis. Each species was screened by cellulose acetate electrophoresis (CAE), to determine which species were polymorphic at the genetic locus coding for phosphoglucomutase (Pgm, E.C. 2.7.5.1). The electrophoretic techniques have been previously described. The relative mobilities of the various electromorphs[1] were described as follows:

$$\text{Relative mobility} = \frac{\text{distance of migration of electromorph}}{\text{distance of migration of reference electromorph}}$$

The reference electromorph was defined as the most common band and was designated a value of 100.

To study intraspecific variation, samples of more than 100 individuals of the same species were electrophoresed and scored for Pgm phenotype. The Pgm phenotypes observed in each species were assumed to be allelic isozymes and expected genotype frequencies were calculated directly from the observed phenotypic frequecies and tested for agreement with Hardy-Weinberg equilibrium. If other assumptions are met the observed genotype frequency distribution should fit the values expected according to Hardy-Weinberg equilibrium.(7) Chi-square tests of significance for equilibrium were made on four S. metallicum samples and one sample each of the other five species.

To test the hypothesis that S. metallicum is a mixture of genetically distinguishable groups, morphological variation in pupal, larval and adult stages were examined. To interpret morphological variation as well as enzyme frequencies the electrophoretic analysis was done without grinding up the entire specimen and destroying the morphological characters. Procedures for each stage were as follows:

[1]The term electromorph (=band) is used here because of a lack of data from genetic crosses to demonstrate allelism.

Pupae: Individual black fly pupae were firmly crushed in 5 ul of electrophoretic buffer. Crushing without grinding produced tissue homogenate while preserving the pupal morphological characters. The homogenate was used for enzyme electrophoresis and the rest of the pupa was preserved in 70% ethanol (ETOH). Later, each pupa was cleared, mounted on a microscope slide and kept as a voucher specimen. Pupae were dissected to show the cephalic capsule, thorax, respiratory filaments and abdomen.

Larvae: Samples of larvae were taken from two pasture streams 5 km apart representing different watersheds. Larvae of the last instar (those with dark, well-developed respiratory histoblasts) were cut into three parts: (1) anterior part--head capsule plus thorax; (2) middle part--abdomen; (3) posterior part--tip of abdomen including gills, anal sclerite and circle of hooklets. Anterior and posterior sections were preserved in 70% ETOH until they could be cleared in 10% KOH, rinsed in 10% acetic acid, passed through an alcohol series to 100% ETOH, dissected on a microscope slide to show morphologically important characters and mounted in Euparal (R), GBI Laboratories, Ltd., as permanent voucher specimens. The middle part was triturated and used for electrophoresis.

Adults: Adults were treated in two ways. (1) Head and thorax were separated from the legs and abdomen, glued to paper points, mounted on labeled entomological pins and preserved as voucher specimens. The legs and abdomen were triturated and used for CAE electrophoresis. (2) Th abdomen was separated from the rest of the adult (which was electrophoresed) and placed in 70% ETOH. Later, each abdomen was cleared, dissected and mounted on a microscope slide to show the genitalia and the abdominal sternites.

Representative specimens of the black flies used in this study have been deposited in the U.S. National Museum (Natural History), Washington, D.C., the British Museum (Natural History), London and the Reference Collection of the Gorgas Memorial Laboratory, Panama, Republic of Panama.

Table 1. Results of chi-square (X^2) tests for Hardy-Weinberg equilibrium of phosphoglucomutase alleles of various Simulium species.

Species	Sample size	Number of Electromorphs	X^2	(d.f.)	p
S. chiriquiense	187	3	1.9	(3)	>.5
S. metallicum	111	5	43.5	(10)	<.001
S. metallicum	209	5	41.96	(10)	<.001
S. metallicum	162	6	96.0	(15)	<.001
S. metallicum	216	5	72.23	(10)	<.001
S. ochraceum	161	5	18.63	(10)	<.05
S. panamense	232	5	8.4	(10)	<.5
S. quadrivittatum	136	3	4.7	(3)	>.1
S. rubicundulum	104	2	0.06	(1)	>.7

p = Probability
d.f. = Degrees of freedom

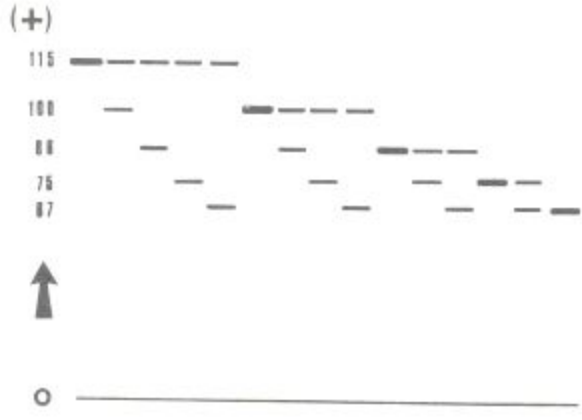
III. RESULTS

Simulium metallicum was the only species to show highly significant deviations from Hardy-Weinberg equilibrium (Tables 1 and 2). Five Pgm electromorphs were detected in S. metallicum (Figure 1). If we assume these electromorphs are alleles at a single genetic locus then the patterns shown in Figure 1 represent all possible combinations of these five alleles taken two at a time. Stated another way, five alleles are expected to show 15 different genotypes. In reality, only 11 genotypes were observed, with the homozygous classes Pgm $^{67/67}$ and Pgm $^{75/75}$, and the heterozygous classes Pgm $^{115/75}$ and Pgm $^{115/67}$ not represented in the samples. Calculation of a Hardy-Weinberg X^2 showed the pooled sample to be lacking equilibirum (X^2 = 72.23, d.f. = 10, $p < 0.001$).

In addition to isozyme variation, morphological variation was observed in S. metallicum: pupal thoracic and cephalic

Table 2. Observed and expected genotype frequencies of phosphoglucomutase in *Simulium metallicum* pupae with bifid and multiple branched trichomes.

Genotype	BIFID		MULTIPLE	
	Observed	Expected	Observed	Expected
Pgm$^{115/115}$	0	0.03	1	0.56
Pgm$^{115/100}$	2	1.26	14	13.94
Pgm$^{115/86}$	1	1.19	2	2.94
Pgm$^{115/75}$	0	0.13	-	-
Pgm$^{115/67}$	0	0.40	-	-
Pgm$^{100/100}$	18	11.70	94	86.33
Pgm$^{100/86}$	14	22.15	21	36.39
Pgm$^{100/75}$	2	2.51	-	-
Pgm$^{100/67}$	4	7.52	-	-
Pgm$^{86/86}$	12	10.48	12	3.84
Pgm$^{86/75}$	2	2.37	-	-
Pgm$^{86/67}$	12	7.12	-	-
Pgm$^{75/75}$	0	0.13	-	-
Pgm$^{75/67}$	2	0.81	-	-
Pgm$^{67/67}$	0	1.20	-	-
	69	69.00	144	144.00
	$X^2 = 14.88$ (d.f. = 10)		$X^2 = 25.18$ (d.f. = 3)	
	$p > 0.1$		$p < 0.001$	



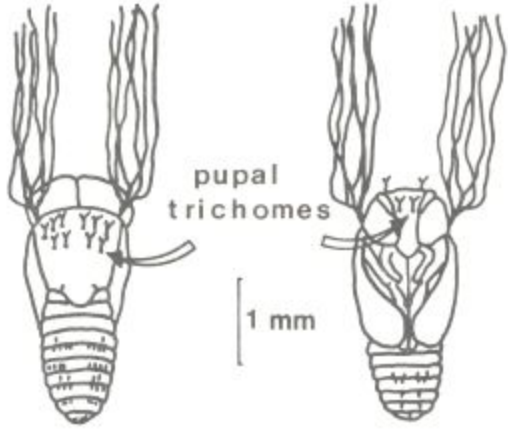


Figure 1. (top) Diagram of the relative mobility of phosphoglucomutase electromorphs in Simulium metallicum showing all combinations theoretically possible. The origin (O) is the application point of the tissue homogenate. The numbers on the left represent the relative mobilities of the electromorphs.

Figure 2. (below) Simulium metallicum pupa: Dorsal and ventral views showing thoracic and cephalic trichomes.

trichomes were bifid, trifid or multiple-branched (Figure 2). Both isozyme and morphological data were recorded for one sample of S. metallicum consisting of 216 pupae (Tables 2 and 3). This sample was partitioned into two groups: one characterized by multiple-branched trichomes (three or more branches) and the other by bifid trichomes (at least some trichomes two branched). These new groups were tested separately for Hardy-Weinberg equilibrium. The group characterized by bifid trichomes did not differ significantly from the expected values ($p > 0.1$) and showed five alleles (Table 2), but the multiple-branched trichome group showed significant deviation from the expected Hardy-Weinberg values ($p < 0.001$, Table 2). The alleles Pgm^{75} and Pgm^{67} were absent from this latter group.

Results from larvae are given in Table 3, columns 4 and 5. Results of chi-square tests of significance for Hardy-Weinberg equilibrium were not significant ($p > 0.1$) for either sample. Allele frequencies of the larval samples from the two watersheds were compared by contingency chi-square tests of significance using the original count data in the test cells.[2] The allele frequencies were not significantly different from each other ($X^2 = 3.09$, d.f. = 4, $p > .5$). Next, allele frequencies of the two larval samples were compared with the allele frequencies of pupae with bifid trichomes in a 3x5 contingency table (Table 3, columns 3, 4 and 5). The samples were not significantly different from each other ($X^2 = 9.6$,, d.f. = 8, $p < 0.1$). No larval diagnostic morphological characters have been found so far which correlate with the isozyme data.

In the samples of adults collected on cow bait and human bait the alleles Pgm^{75} and Pgm^{67} were not observed (Table 3, columns 6 and 7) and the allele frequencies were significantly different from each other when tested by contingency chi-square ($X^2 = 10.78$, d.f. = 2, $p < 0.005$). Adult black flies collected on human bait also showed allele frequencies significantly different from pupae with multiple-branched trichomes (Table 3, column 2) ($X^2 = 39.25$, d.f. = 2, $p < 0.001$).

[2]Original count data can be obtained from Table 3 by doubling the sample size (each individual carries two alleles) and multiplying by the appropriate allele frequency.

Table 3. Phosphoglucomutase allele frequencies of *Simulium metallicum* by stage.

	Pupae[1]			Larvae		Adults	
Allele	All (n=216)[2]	Multiple trichomes (n=144)	Bifid trichomes (n=69)	Sample 1 (n=94)	Sample 2 (n=81)	Cow Bait (n=34)	Human Bait (n=109)
Pgm[115]	.049	.063	.021	.053	.086	.485	.284
Pgm[100]	.650	.774	.412	.431	.395	.471	.592
Pgm[86]	.238	.163	.390	.340	.352	.044	.124
Pgm[75]	.041	0	.044	.059	.080	0	0
Pgm[67]	.049	0	.132	.117	.087	0	0
H-W[3]	72.2(10) $p < 0.001$	25.2(3) $p < 0.001$	14.8(10) $p > 0.1$	10.6(10) $p > 0.1$	11.3(10) $p > 0.1$	4.3(3) $p > 0.1$	1.6(3) $p > 0.5$

[1]Pupae in column 1 were partitioned into two subgroups (columns 2 and 3) based on trichome branching patterns.

[2]Included in this group were three pupae with trichome patterns that could not be read.

[3]H-W = Results of chi-square tests for Hardy-Weinberg equilibrium (degrees of freedom in parenthesis).

IV. DISCUSSION

A number of assumptions underlie the Hardy-Weinberg equation. Since observed genotype frequencies did not agree with the expected frequencies in some samples (i.e. they were not in Hardy-Weinberg equilibrium), this implies that some of these assumptions may not have been met. First, we assumed that the electromorphs detected in each species were alleles at a single locus. This assumption is necessary because none of these species has ever been colonized and inheritance data are lacking. However, this first assumption seems reasonable in view of the phenotypic patterns observed in all six Simulium species examined.

A second major assumption was that we sampled single, randomly mating populations. The results obtained by partitioning and retesting the S. metallicum data suggested that some samples consisted of two or more genetically isolated groups. Looking back at the four original samples of S. metallicum (Table 1) this seems very plausable. In order to test samples of greater than 100 black flies we combined samples from several different breeding sites. The data in Table 3, columns 2 and 3, show that allele frequencies of pupae with multiple-branched trichomes are different from allele frequencies of pupae with bifid trichomes. One way to express this statistically is to ask the question: What is the likelihood of sampling 144 pupae and getting none carrying the allele Pgm^{75} which occurs at a frequency of 0.06? If $p = 0.06$,[3] the likelihood of getting 0 in 144 trials is 1.35×10^{-4}; we reject the null hypothesis that these two groups of pupae could be characterized by the same allele frequency. Likewise, what is the likelihood of sampling 144 pupae and getting none carrying the Pgm^{67} which occurs at a

[3]The allele frequencies $Pgm^{75} = 0.06$ and $Pgm^{67} = 0.11$ were obtained by combining the original count data from pupae with bifid trichomes and both larval samples (Table 3, columns 3, 4 and 5). The best estimate of the parametric allele frequency is the sum of all appropriate observations taken as a whole divided by the total sample size of 244.

frequency of 0.11? If p = 0.11, the likelihood of getting 0 in 144 trials is 5.15×10^{-8}. We come to the same conclusion: these two groups of pupae are genetically isolated. Pooling samples from genetically isolated populations characterized by different allele frequencies and, in the case, two novel alleles ($\underline{Pgm}^{75}$ and $\underline{Pgm}^{67}$), results in a deficiency of heterozygotes when compared to those expected under Hardy-Weinberg equilibrium. This has been classically called a "Wahlund Effect".

A third assumption of Hardy-Weinberg equilibrium is that allele frequencies of the sampled population remain constant from generation to generation, i.e. there is no gain from immigration from flies outside the study area and no loss by emigration. The data from cow-bait and human-bait collections showed allele frequencies statistically different from those of either larvae or pupae. Since adult black flies may fly considerable distances from their breeding sites we may have sampled one population as larvae and another as adults. The data presented show that adult black flies collected on cow and human bait are genetically distinguishable from larvae collected in pasture breeding sites (samples 1 and 2). At present we do not know the location of breeding sites that produce adult flies with allele frequencies like those in Table 3, columns 6 and 7.

The larval collections (Table 3, columns 4 and 5) represent samples from single breeding sites in two different pasture streams 5 km apart. The allele frequencies of the larval samples were not statistically different either from each other or from those of pupae with bifid trichomes (Table 3, column 3). In addition, pupae with bifid trichomes have been collected primarily from pasture streams.

The results presented in Table 3 show that a subgroup of S. metallicum can be characterized by the presence of bifid trichomes in the pupal stage, the presence of the alleles $\underline{Pgm}^{75}$ and $\underline{Pgm}^{67}$, and breeding in pastures. Another subgroup of this species is characterized by multiple-branched trichomes in the pupal stage and absence of the alleles $\underline{Pgm}^{75}$ and $\underline{Pgm}^{67}$. Preliminary evidence indicates this subgroup breeds in rocky streams, but this needs to be confirmed.

These conclusions are most strongly supported by the statistical tests of independence (contingency tables) employing the original count data for each allele. The chi-square tests of significance for Hardy-Weinberg equilibrium are not as meaningful because of the large number of cells with expected values less than 5. Results of Hardy-Weinberg tests of equilibrium are presented here for completeness, but our major conclusions are based on the results of tests of independence of the data presented in Table 3.

Our results show that there is a broad pattern to the variation found in S. metallicum. Genetic, morphological and ecological patterns are associated indicating separate subgroups within the species. Important questions are: Do these subgroups play different roles in the epidemiology of human onchocerciasis? S. metallicum is reported from Mexico through Central America to northern South America (Colombia and Venezuela). Do these subgroups explain the different epidemiological patterns attributed to S. metallicum in Venezuela where it is the primary vector of onchocerciasis and Guatemala where it is a poor vector? These are questions that can only be answered by additional study in the endemic areas.

A recent paper by black fly taxonomists working in Guatemala describes S. horacioi, a new species of Simulium which is morphologically similar to S. metallicum.(8) The two species occur in the same area and human-bait collections may contain both. We have obtained specimens of S. horacioi from Guatemala and are comparing them with our material to determine if the new species exists in Panama. S. horacioi is characterized by bifid trichomes in the pupa. Arrangements are being made to do isozyme electrophoresis on Guatemalan S. metallicum and S. horacioi.

The present state of confusion in the taxonomy of Central and South American black flies exists because of lack of good reference collections. This need emphasizes the importance of depositing voucher specimens from surveys and ecological studies in national museums. It also emphasizes the need for association and synthesis of the various types of data collected: morphological, physiological, genetic, ecological, etc. An

example here is appropriate. When black flies suspected as vectors of onchocerciasis are dissected for examination for developing Onchocerca volvulus larvae the corpse of the dead fly can be used for two additional studies: (1) the genitalia can be preserved in ethanol for microscopic study and can later be permanently preserved on a microscope slide as a voucher specimen; and (2) the remaining homogenate can be triturated for enzyme electrophoresis. Since the CAE technique permits genetic analysis of part of a black fly larva, isozyme studies could also be done in conjunction with cytotaxonomy.(9) Larval salivary glands could be dissected, stained and squashed, while the rest of the larva is electrophoresed. Such associated data might be a great help in resolving taxonomic as well as epidemiological puzzles.

Cellulose acetate electrophoresis was used to characterize six Simulium species collected in Chiriqui Province, Panama (S. chiriquiense, S. metallicum, S. ochraceum, S. panamense, S. quadrivittatum and S. rubicundulum). Chi-square tests for Hardy-Weinberg equilibrium of phosphoglucomutase (Pgm) alleles were made of four S. metallicum samples and one sample each of the other species. Only S. metallicum showed highly significant deviation from equilibrium. S. metallicum also showed morphological variation: pupal thoracic trichomes were bifid, trifid or multiple. In order to examine morphological and enzyme variation from the same pupa, specimens were crushed without grinding to produce tissue homogenate for electrophoresis while preserving the pupal morphological characters. When enzyme data were partitioned based on pupal trichome branching patterns, two Pgm alleles occurred only in specimens with bifid trichomes. Data for pupae with bifid trichomes did not differ significantly from expected Hardy-Weinberg equilibrium. This result is consistent with the hypothesis that a subgroup of S. metallicum can be characterized by the presence of bifid trichomes; another subgroup or other subgroups are characterized by multiple-branched trichomes. Ecological data support this hypothesis.

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