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USE OF ISOZYME ELECTROPHORESIS IN BLACK FLY SYSTEMATICS

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ABSTRACT

Isozymes were used to characterize six species of Simulium collected in Chiriqui Province, Panama: S. chiriquense, S. metallicum, S. ochraceum, S. panamense, S. quandrivittatum, S. rubicundulum. Comparative studies were made on 22 enzymes. Tests for Hardy-Weinberg equilibrium of the phosphoglucomutase (PGM) alleles were made of four samples of S. metallicum and one sample of each of the other five species. For these tests it was first assumed that the PGM bands observed in each species were allozymes (allelic isozymes), then expected genotypic frequencies were calculated from the electrophoresis data. Chi-square tests were done to determine if the observed numbers fit the expected Hardy-Weinberg proportions. S. metallicum was the only species that showed a highly significant deviation from the expected frequencies.

Morphological variation has been observed in S. metallicum. Trichomes on the pupal thorax may be bifid, trifid or multiple. We developed a method to examine morphological and enzyme variation from the same pupa. Crushing without grinding produced tissue homogenate for electrophoresis while preserving the pupal morphological characters. When zymogram data were regrouped according to the branching pattern of pupal trichomes two of the PGM alleles were found only among the specimens having bifid trichomes. When zymogram data of the pupae with bifid trichomes were retested for Hardy-Weinberg equilibrium the results did not differ significantly from expected. 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.

INTRODUCTION

In comparison with many of the other families of the order Diptera, the family Simuliidae has received quite a lot of attention from entomologists. Black flies are studied bacause they may be intolerable pests and because they may be vectors of diseases such as human and animal onchocerciasis and Leukocytozoon of birds. Black flies receive attention because they are impossible to ignore. However, black flies are of intrinsic interest also, for example, serving as models of speciation. As Dr. Rothfels has shown earlier in this symposium chromosome banding patterns are a rich store of information about genetic differentiation. (See also: Rothfels 1979). The family Simuliidae offers excellent opportunities for the study of the processes of evolutionary change. The raw material for these processes of change is inheritable variation. I would like to discuss genetic variation within single species of black flies; and the evolution I will be referring to is microevolution, that is, small evolutionary changes such as amino acid substitutions in a given enzyme. In so doing I will try to bridge the gap between alpha-taxonomy and population genetics.

Enzymes that are slightly different structurally may show the same enzymatic activity. These molecular variations are called isozymes. Isozymes have a genetic basis, their structure being determined by the sequence of bases in the DNA of one or more genes. Many isozymes can be detected by electrophoresis which is the migration of charged particles in an electric field. The number of charges on a molecule and whether those charges are positive or negative will determine if a molecule moves in an electric field and its rate and direction of migration.

In the first paper to report electrophoretic patterns in black flies, William Coker, (1973) examined 6 enzymes in Simulium damnosum collected in Ghana, West Africa. Coker reported the relative mobilities of the electrophoretic bands he observed. A few years later, Harold Townson (1976) collaborating with Coker examined 5 members of the Simulium damnosum complex: S. yahense, S. sanctipauli, S. damnosum (sensu str.) S.squamosum and S. sirbanum. Of the 15 enzymes studied, one, phosphoglucomutase (PGM) gave clear-cut differences among members of the S. damnosum complex. Townson reported that PGM bands in S. yahense were different from those found in the other cytotypes. Flies emerging from pupae at one locality and flies collected biting man at that same site were of the same PGM type. Larvae from this site were identified cytologically as S. yahense. Isozyme electrophoresis together with the study of polytene chromosome banding patterns was used to identify the flies biting man as S. yahense. Townson and Coker are thus credited with the first confirmation of the man biting activity of the S. yahense cytotype.

In 1977 May and his co-workers described inter-and intraspecific protein variation in 3 closely related species of the S. jenningsi complex from Maine. Three species within the S. jenningsi complex are morphologically similar in the adult stage although they are identifiable in the larval and pupal stages. The morphological similarity of the adults prevented the determination of the species responsible for biting humans in the Penobscot River watershed of Maine. A study of the isozyme frequencies of 4 key enzymes showed that S. penobscotensis was the principal anthropophilic black fly.

At the Gorgas Memorial Laboratory in Panama, we are investigating genetic

variation in S. metallicum. We have obtained preliminary evidence from enzyme studies that S. metallicum is made up of at least two genetically distinguishable groups.

METHODS

The black flies used in this study were collected in Chiriqui Province, Western Panama (Figure 1). Larvae and pupae were collected from streams and immediately placed on ice for transport to Panama City. At the laboratory the larvae and pupae were stored in a deep freeze at -55 degree C until needed for electrophoresis. Six species of Simulium were screened for 22 different enyzme systems to determine which enzymes were polymorphic. Figure 2 is a diagram showing the relative mobilities of five alleles of phosphoglucomutase (PGM), in Simulium metallicum. The origin is the application point of the tissue homogenate from one individual black fly. Either a larva, a pupa, or an adult fly may be used. The numbers on the left represent the relative mobilities of the various alleles. 100 is the reference band. The reference band, by convention, is the most common allele. The distance from the origin to bands that migrate to "115" divided by the distance from the origin to point "100" times 100 equals 115. Likewise, the distance from the origin to point "67" divided by the distance from the origin to point "100" times 100 equals 67. The patterns shown in Figure 2 represent all the possible combinations of five alleles taken two at a time, that is, 15 different combinations. Stated another way, five alleles are expected to show 15 different genotypes.

To study intraspecific variation relatively large numbers of individuals of the same species were run using just one enzyme system and the number of different bands observed was counted. If these bands are the product of a single genetic locus and certain other assumptions are met their frequency distribution should fit a pattern known as the Hardy-Weinberg equilibrium. Tests for equilibrium of the alleles of phosphoglucomutase (PGM) were made of four samples of S. metallicum and one sample of each of five other species. For these tests it was assumed that all the PGM bands observed in one species were allelic isozymes, that is, alleles at a single gene locus then expected genotyped frequencies were calculated based on the allele frequencies from the electrophoresis data. Chi-square tests were done to determine if the observed numbers fit the expected values assuming Hardy-Weinberg equilibrium.

RESULTS AND DISCUSSION

S. metallicum is the only species that shows highly significant deviation from Hardy-Weinberg equilibrium (Table 1). There are a number of possible explanations for this. Small sample size can lead to sampling error that results in deviations from Hardy-Weinberg equilibrium. That is not likely in this case. Sample size for S. metallicum was comparable to that of the other species tested and all four replicates deviated in the same way: i.e. deficiency of PGM^{100}/PGM^{86} heterozygotes. Another possibility is that there is a balanced polymorphism of alleles in S. metallicum with some of the classes missing or reduced in numbers. A third possibility is that S. metallicum is really a mixture of genetically distinguishable groups. Hardy-Weinberg equilibrium assumes random mating of the population being tested. Mating would not be

random among sampled black flies if, in fact, S. metallicum were a mixture of two or more genetically distinguishable groups. The data in Table 1 tell us only that the four samples of S. metallicum do not meet the assumptions of Hardy-Weinberg equilibrium, but based on this information alone we do not know why the samples tested deviated from expected.

To test the hypothesis that S. metallicum is a mixture of genetically distinguishable groups we decided to look for morphological variation. In order to interpret morphological variation in terms of enzyme frequencies we needed to do the electrophoretic analysis without grinding up the entire specimen and destroying the morphological characters. The procedure we adopted was to firmly crush the black fly pupae in a small amount of electrophoretic buffer. Crushing without grinding produced tissue homogenate while preserving the pupal morphological characters. The homogenate was then used for the enzyme electrophoresis and the rest of the pupa was preserved in 70% ethanol. Later, each pupa was cleared, and mounted on a microscope slide and kept as a voucher specimen.

Table 1. Tests for equilibrium of phosphoglucomutase alleles of various Simulium species.

Species	Sample size	Number of alleles	χ^2	(d.f.)	p
S. chiriquense	187	3	1.9	(3)	p>.5 n.s.
S. metallicum(1)	111	5	43.5	(10)	p<.001 ***
S. metallicum(2)	209	5	41.96	(10)	p<.001 ***
S. metallicum(3)	162	6	96.0	(15)	p<.001 ***
S. metallicum(4)	216	5	72.23	(10)	p<.001 ***
S. ochraceum	161	5	18.63	(10)	p<.05 *
S. panamense	232	5	8.4	(10)	p>.5 n.s.
S. quadrivittatum	70	3	2.87	(3)	p>.3 n.s.
S. rubicundulum	104	2	0.06	(1)	p>.7 n.s.

p = Probability
d.f. = Degrees of freedom
n.s. = not significant
* = significant
*** = highly significant

All of the pupae were dissected to show the cephalic capsule, the thorax, respiratory filaments and abdomen. The morphological characters of greatest interest were the thoracic and cephalic trichomes (Figure 3). We found that the trichomes of S. metallicum could be bifid, trifid or multiple. 214 specimens

were prepared that had a readable zymogram plus a mounted pupa. When the zymogram data were split into two groups - one group of 70 pupae with bifid trichomes and another group of 144 with multiple-branched trichomes, two of the PGM alleles, PGM^{75} and PGM^{67} were found only among the specimens having bifid trichomes. When the zymogram data of the 70 specimens with bifid trichomes were retested for Hardy-Weinberg equilibrium the results did not differ significantly from expected (Table 2).

Table 2. Phosphoglucomutase (PGM) allele frequencies of Simulium metallicum by stage and breeding site.

Allele	Pupae			Larvae	
	Mixed (n=214)	Multiple (n=144)	Bifid (n=70)	Sample 1 (n=94)	Sample 2 (n=81)
PGM^{115}	.049	.063	.021	.053	.086
PGM^{100}	.650	.774	.414	.431	.395
PGM^{86}	.238	.163	.386	.340	.352
PGM^{75}	.014	0	.050	.059	.080
PGM^{67}	.049	0	.129	.117	.087
H-W	72.2(10) ***	25.2(3) ***	15.7(10) n.s.	10.6(10) n.s.	11.3(10) n.s.

H-W = Results of Chi-square tests for Hardy-Weinberg equilibrium (degrees of freedom).

The above results are consistent with the hypothesis that a subgroup of S. metallicum can be characterized by the presence of bifid trichomes. Another subgroup is (or other subgroups are) characterized by multiple-branched trichomes.

This work has been extended to a study of larval stages. Larvae of the ultimate instar (those with dark, well-developed respiratory histoblasts) are cut into 3 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 (Figure 4). Anterior and posterior sections are preserved in 70% ethanol until they can be cleared in hydroxide, dissected on a microscope slide to show morphologically important characters and mounted in euparal as permanent voucher specimens. The middle part is triturated and used for electrophoresis. Larvae collected from small streams that flow through pasture grasses such as "estrella africana" or "star grass" (Cynodon plectostachyus) have been characterized electrophoretically. The results are presented in Table 2. Note that the allele frequencies from the two larval collections do not differ significantly from Hardy-Weinberg equilibrium and show the alleles PGM^{75} and PGM^{67}. Pupae collected from these same sites were characterized by the presence of

bifid trichomes. This suggests that one subgrouping of Simulium metallicum may be characterized as follows: (1) morphologically by presence of bifid trichomes on the pupal thorax; (2) genetically by presence of the alleles PGM^{75} and PGM^{67} of the enzyme phosphoglucomutase; and ecologically by breeding sites in pastures. Another subgrouping of S. metallicum may be characterized by multiple-branched trichomes in the pupa; absence of the alleles PGM^{75} and PGM^{67}; and preliminary evidence indicates this subgrouping breeds in rocky streams.

CONCLUSION

Isozyme analysis is just beginning to make important contributions to the study of the biology and taxonomy of black flies. This technique has great potential for helping to elucidate problems associated with species complexes such as S. damnosum (sensu latu) in Africa and S. metallicum in the Americas. Among the advantages of isozme electrophoresis are the following: (1) Isozymes can be studied in all stages of the life cycle - larvae, pupae and adults. (2) Some isozymes can be useful as species diagnostic characters. (3) Isozymes can be used as biochemical characters in the recognition of sibling species and other species groups.

This is by no means a definitive study. Much work remains to be done, for example, associating adult morphological markers with enzyme data. But the study so far has generated testable hypotheses and we have the means to attain a better understanding of black fly systematics.

SUMMARY: SIMULIUM METALLICUM

Character	Group A	Group B
Pupal trichomes	Bifid	Multiple
Pgm^{75} and Pgm^{67}	Present	Absent
Breeding sites	Pastures	Rocky streams (?)

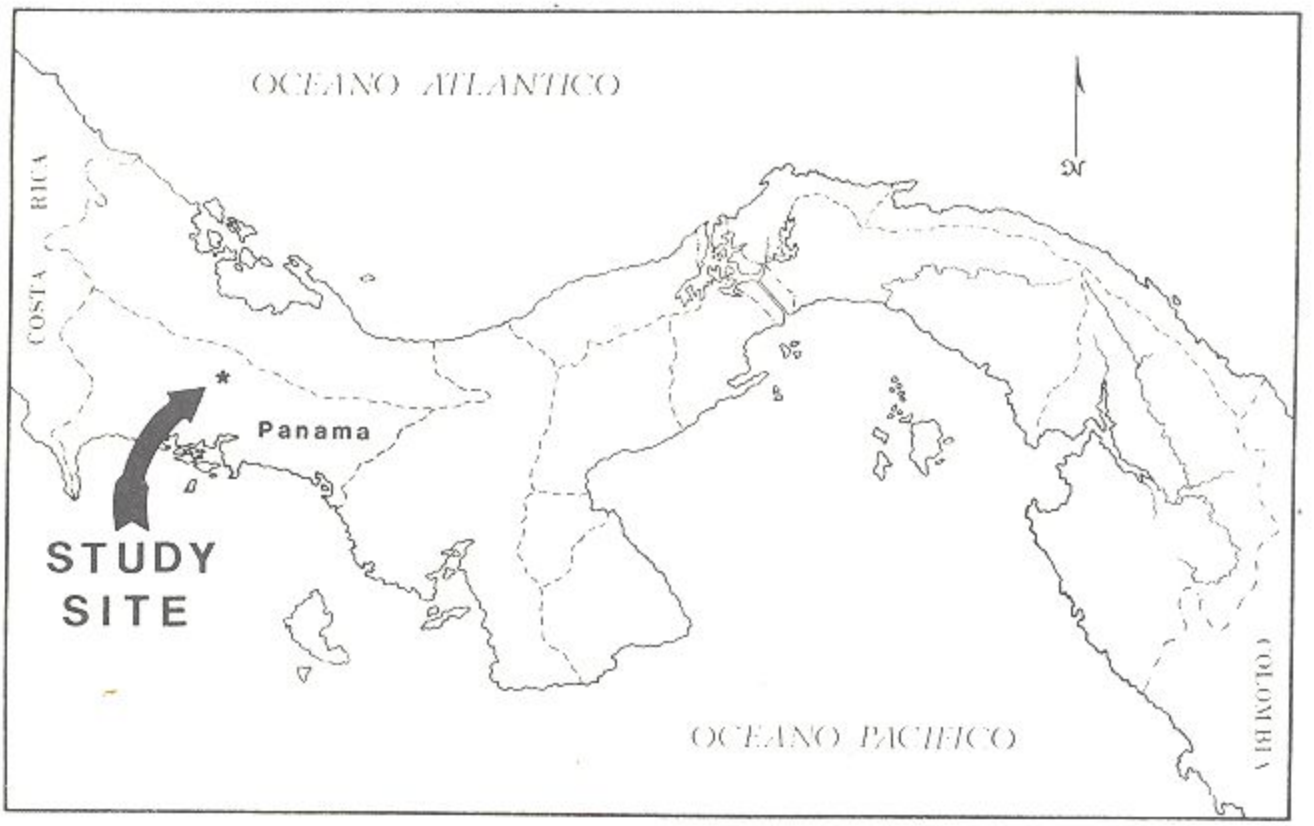

Figure 1. Location of study site.

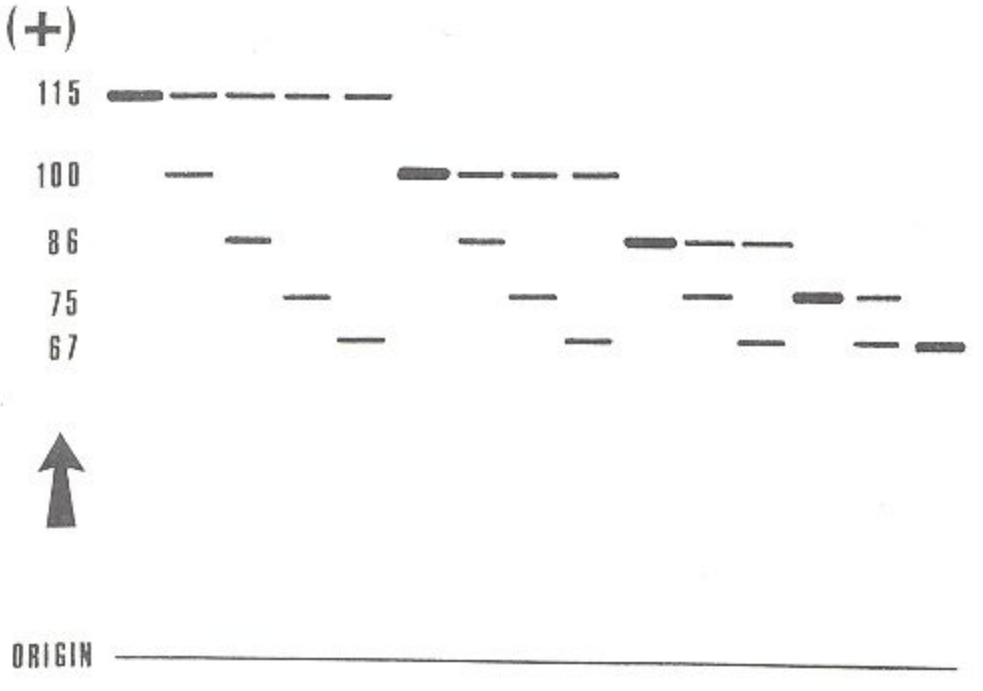

Figure 2. Relative mobility of phosphoglucomutase (PGM) genotypes in Simulium metallicum.

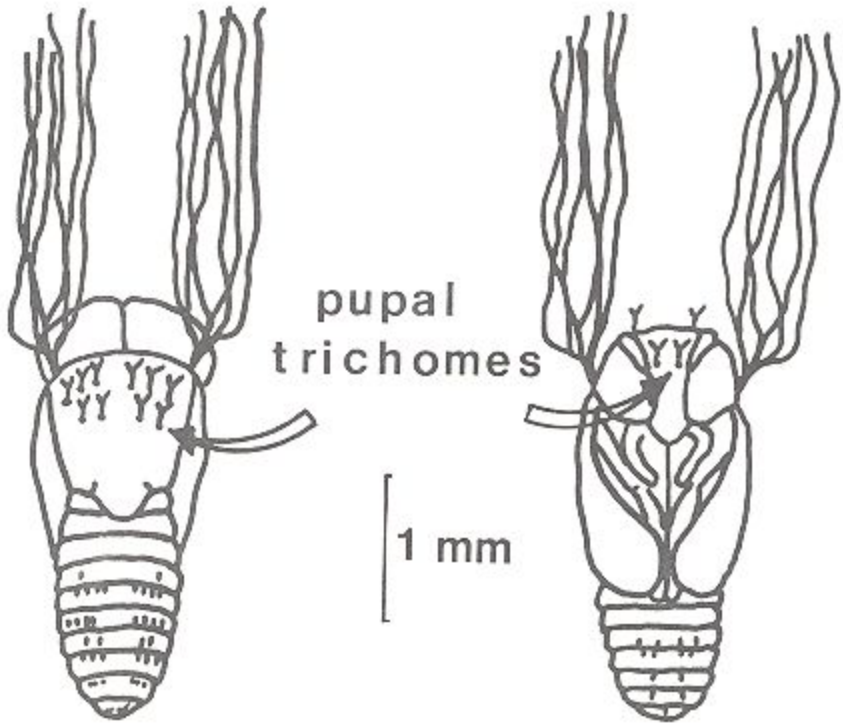

Figure 3. Simulium metallicum pupa: Dorsal and ventral views.

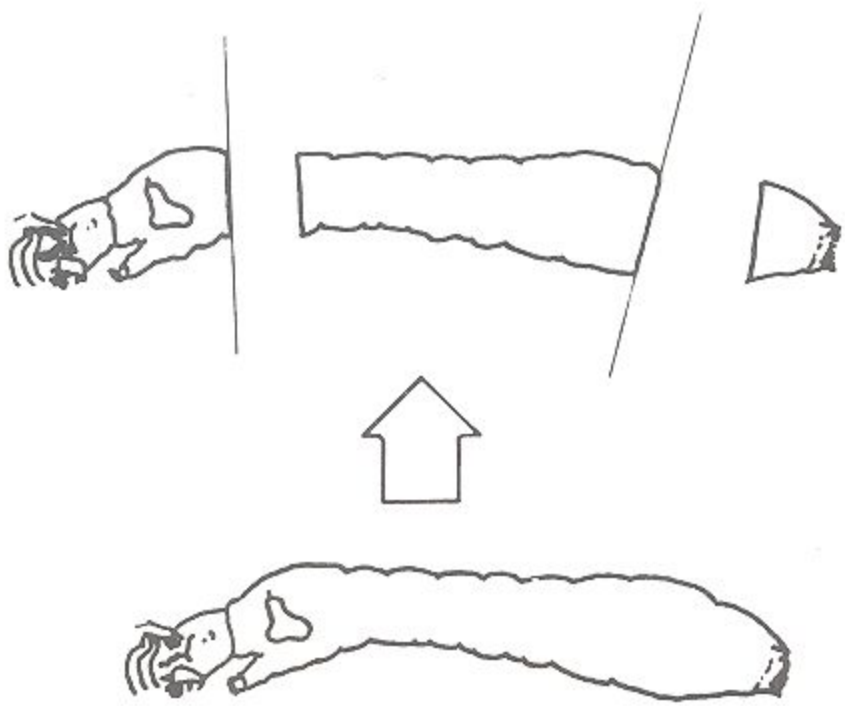

Figure 4. Simulium metallicum larva: Showing cuts prior to electrophoresis.

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