Preliminary Survey of Isozyme Variation in Anthropophilic Panamanian *Lutzomyia species*

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INTRODUCTION

Isozyme electrophoresis is a useful tool in both systematics and taxonomy as well as an aid to the study of population biology (May et al., 1977; Miles, 1979; Munstermann, 1980; Powell et al., 1980; Saul et al., 1977; Stock and Amman, 1980). This technique has been applied to the study of phlebotomine sandflies. Miles and Ward (1978) reported a preliminary isozyme study, employing thin-layer starch gel electrophoresis, of ten enzymes on larvae and adults from two laboratory colonies of Lutzomyia flaviscutellata. This study showed that enzymes with high activity such as MDH, PGM, and PGI were detectable in individual flies and thus could be used in future population genetic studies. Recently, Ward et al. (1981) examined genetic polymorphism in Phlebotomus perniciosus and eight other sandfly species collected in southern France and Tunisia. They concluded that MDH, PGM, PGI, GOT, HK and α-GPD were the most useful diagnostic enzymes enabling biochemical identification of the two most common species collected in southern France, P. ariasi and P. perniciosus.

This paper reports the results of genetic studies based on cellulose acetate electrophoresis of five putative sandfly vectors of leishmaniasis in Panama.

MATERIAL AND METHODS

Sandflies. Of the sixty-eight sandfly species reported from Panama (Christensen, 1972) five are known or suspected to be vectors of Leishmania braziliensis causing human cutaneous leishmaniasis (Christensen and Herrer, 1973): Lutzomyia panamensis, L. ylephiletor, L. gomezi, L. trapidoi and L. sanguinaria. Each of these five species was examinded by cellulose acetate electrophoresis in the present study.

Collecting and storage methods. Sandflies were collected from sunset to 2200 hours using human bait. Specimens were stored on ice in a cooler for transport to the laboratory next day.

Collecting sites

1) Mojinga swamp - located within the boundaries of Fort Sherman on the east bank of the Chagres River, Colon Province (Atlantic watershed).

2) Changuinola - sandflies were collected along the Risco Valley 30 km south of Changuinola, Bocas del Toro Province (Atlantic watershed).

3) Juan Mina - settlement on the Chagres River 25 km northwest of Panama City, Panama Province. Juan Mina is situated approximately in the middle of the Isthmus of Panama.

4) Isla Majé - an island in the man-made lake formed by the Bayano reservoir 100 km east northeast of Panama City, Panama Province (Pacific watershed).

5) El Aguacate - a rural village 25 km southeast of La Chorrera, Panama Province (Pacific watershed).

Sandflies were examined under a stereomicroscope mounted on a cold table, identified to species by external morphological characters and stored at - 55°C in a deep freeze until needed for electrophoresis.

Electrophoresis. Names, abbreviations and enzyme commission (E.C.) numbers for the enzymes assayed are as follows:

acid phosphatase (ACP-E.C. 3.1.3.2);
aldolase (ALD-E.C. 4.1.2.13);
α-amylase (AMY-E.C. 3. 2. 1. 1);
carboxyesterase (EST-E.C. 3.1.1.11);
phosphofructokinase (FK-E.C. 2.7.1.11);
fumarate hydratase (FUM E.C. 4.2.1.2);
α-glycerol-3-phosphate dehydrogenase (NAD^+)
(α-GDH-E.C. 1.1.1.8);
glucose-6-phosphate dehydrogenase (G6PDH-E.C. 1.1.1.49);
glyceraldehyde-phosphate dehydrogenase (GAPDH-E.C.1.2.1.12);

glutamic-oxaloacetic transaminase (GOT-E.C. 2.6.1.1.);
hexokinase (HK-E.C.2.7.1.1.);
isocitrate dehydrogenase ($NADP^+$) (IDH-E.C. 1.1.1.42);
leucine aminopeptidase (LAP-E.C. 3.4.11.1);
malate dehydrogenase (MDH-E.C. 1.1.1.37);
malate dehydrogenase (oxaloacetate decarboxylating) ($NADP^+$) (ME-E.C.1.1. 1.40);
6-phospho-D-gluconate: $NADP^+$ 2-oxidoreductase (decarboxylating) 6PGDH-E.C.1.1.1.44);
glucosephosphate isomerase (PGI-E.C. 5.3.1.9);
phosphoglucomutase (PGM-E.C.2.7.5.1); xanthine oxidase (XOX-E.C.1.2.3.2).

With the exception of α-amylase (AMY) the staining technique used were those previously described from this laboratory for the study of mosquitoes (Kreutzer et al., 1977; Kreutzer, 1979; Kreutzer and Galindo, 1980) and Leishmania spp. (Kreutzer and Christensen, 1980). The staining technique for α-amylase was that given by Harris and Hopkinson (1976).

Numerical superscripts of the alleles shown in Table 1 to 4 were based on relative electromorph migration. For each enzyme locus, the most common electromorph of L. panamensis was designated the standard and assigned a value of "100". All other designations were derived from measurements relative to the standard.

Data analysis. Observed genotypic frequencies were compared to values expected if random mating were occuring (Hardy-Weinberg equilibrium). Contingency chi-square tests, based on the observed numbers of each allele at a single locus, were used to compare allele frequencies among the different collecting sites.

RESULTS

The enzymes HK, ME, PGI and PGM were very active and stained rapidly with good to excellent resolution. All presented both inter- and intra-specific variation (Tables 1 to 4).

Two enzymes, MDH and EST, stained well, produced bands with good resolution and were polymorphic, but could not be interpreted because it was impossible to assign the electromorphs to particular loci. It was obvious from the multiple bands produced that more than a single locus was coding for the active enzyme.

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* Original count data for each allele can be obtained from Tables 1 to 4 by doubling the number of flies examined (each fly carries 2 alleles at each genetic locus) and multiplying by the appropriate allele frequencies.

AMY and GOT stained well and were polymorphic but not enough samples were analyzed to warrant statistical analysis. Both anodal and cathodal migration were noted in GOT.

The following enzymes showed either no activity or poor resolution of the enzyme bands preventing rational interpretation of the zymograms: ACP, ALD, FK, FUM, GDH, G6PDH, GAPDH, IDH, LAP, 6PGDH, and XOX.

Geographic variation of allele frequencies in L. panamensis is presented in Table 1. When observed numbers* of each allele at the Pgi locus were compared by contingency chi-square tests over all five collecting sites the results were highly significant (X^2 = 90.5, d.f. = 12, $p < 0.001$) indicating statistically significant differences among sites with respect to allele frequencies. The only sites which do not differ significantly from each other when tested by pair-wise comparisons were Mojinga and El Aguacate (X^2 = 3.3, d.f. = 2, $p > 0.1$). Contingency chi-square tests indicated lack of significant difference between collections from Mojinga and El Aguacate for both the Me locus (X^2 = 0.2, d.f. = 1, $p > 0.5$) and the Pgm locus (X^2 = 2.1, d.f. = 2, $p > 0.1$).

Table 1

GEOGRAPHIC VARIATION OF ALLELE FREQUENCIES IN LUTZOMYIA PANAMENSIS

Enzyme Phenotypes	Mojinga	Aguacate	Majé	Juan Mina	Changuinola
Pgi^{131}	.12	.07	.05	.03	.03
Pgi^{100}	.44	.45	.59	.51	.74
Pgi^{75}	.44	.48	.32	.45	.23
Pgi^{56}	0	0	.04	.01	0
(n)*	(110)	(135)	(127)**	(107)	(76)**
	A***	A	B	C	D
Me^{111}	.07	.05	-	-	-
Me^{100}	.93	.95	-	-	-
(n)	(14)	(21)	(0)	(0)	(0)
	E	E			
Pgm^{108}	.14	.08	-	-	-
Pgm^{100}	.56	.65	-	-	-
Pgm^{85}	.30	.27	-	-	-
(n)	(33)	(62)	(0)	(0)	(0)
	F	F			

* (n) = Number of flies examined.

** Significant chi-square ($p < 0.05$) deviation from Hardy-Weinberg equilibrium.

*** Columns ending with the same symbol are not significantly different from each other by contingency chi-square tests.

Table 2

GEOGRAPHIC VARIATION OF PGI ALLELE FREQUENCIES IN FOUR LUTZOMYIA SPECIES

	L. gomezi			L. sanguinaria		L. ylephiletor			L. trapidoi	
Enzyme phenotype	Mojinga	Aguacate	Juan Mina	Majé	Aguacate	Mojinga	Aguacate	Juan Mina	Mojinga	Aguacate
Pgi^{150}	.26	.21	.29							
Pgi^{155}				.01	0					
Pgi^{131}						.06	0	0		
Pgi^{125}	.21	.23	.16	.76	.82				.05	.03
Pgi^{113}						.18	.17	.24		
Pgi^{104}									.67	.64
Pgi^{100}	.49	.56	.55	.23	.17					
Pgi^{88}						.76	.82	.76	.28	.33
Pgi^{75}	.04	0	0	0	.01	0	.01	0		
Pgi^{63}									0	.01
(n)*	(121)** A' ***	(102) B'	(63) B'	(41) C'	(110)** C'	(80) D'	(148) E'	(105)** E'	(12) F'	(156)** F'

* (n) = Number of flies examined.

** Significant chi-square ($p < 0.05$) deviation from Hardy-Weinberg equilibrium.

*** Columns ending with the same symbol are not significantly different from each other (contingency chi-square test).

Table 3

VARIATION OF ME ALLELE FREQUENCIES IN FOUR LUTZOMYIA SPECIES COLLECTED IN TWO SITES

	L. gomezi		L. panamensis		L. sanguinaria		L. ylephiletor	
Enzyme Phenotype	Mojinga	Aguacate	Mojinga	Aguacate	Mojinga	Aguacate	Mojinga	Aguacate
Me^{130}					0	.12		
Me^{115}					1.00	.88	.14	.21
Me^{111}			.07	.05				
Me^{107}							.86	.79
Me^{100}			.93	.95				
Me^{96}	1.00	1.00						
(n)*	(11)	(21)	(14) A"**	(21) A"	(13) B"	(17) B"	(11) C"	(17) C"

* (n) = Number of flies examined.

** Columns ending with the same symbol are not significantly different from each other by contingency chi-square tests.

Table 4

SPECIES VARIATION IN HEXOKINASE PHENOTYPE OF FLIES COLLECTED IN EL AGUACATE

Enzyme Phenotype	L. gomezi	L. panamensis	L. sanguinaria	L. ylephiletor
Hk^{104}				1.00
Hk^{100}	1.00	1.00	.83	
Hk^{91}			.17	
(n)*	(20)	(20)	(20)**	(20)

* (n) = Number of flies examined.

** Significant chi-square ($p<0.05$) deviation from Hardy-Weinberg equilibrium.

Geographic variation of Pgi allele frequencies in four other Lutzomyia species (L. gomezi, L. sanguinaria, L. ylephiletor, and L. trapidoi) are presented in Table 2. When the allele frequencies of L. gomezi were compared over the three sites the results were significant ($X^2 = 19.9$, d.f. = 6, $p < 0.05$), but were not significant when just El Aguacate and Juan Mina were compared ($X^2 = 4.2$, d.f. = 2, $p > 0.1$). The two field collections of L. sanguinaria were not significally different from each other ($X^2 = 4.8$, d.f. = 3, $p > 0.1$). Likewise, the two field collections of L. trapidoi were not significantly different from each other ($X^2 = 1.9$, d.f. = 3, $p > 0.5$). However, the three field collections of L. ylephiletor were significantly different from each other when tested over all three collecting sites ($X^2 = 38.3$, d.f. = 6, $p < 0.001$).

Table 3 presents the geographic variation of Me allele frequencies in four Lutzomyia species collected at Mojinga swamp and El Aguacate. No significant geographic variation of Me was observed between samples from these sites. The allele Me^{96} is "fixed" in L. gomezi, i.e., there is no variation at this genetic locus. L. panamensis is the only species of the four tested that showed the alleles Me^{100} and Me^{111}. L. sanguinaria and L. ylephiletor showed the allele Me^{115} while only L. sanguinaria showed Me^{110} and only L. ylephiletor showed Me^{102}.

Table 4 presents species variation in hexokinase (HK) in sandflies collected from El Aguacate. L. ylephiletor is fixed for

the allele Hk^{104} and both L. gomezi and L. panamensis are fixed for Hk^{100}. L. sanguinaria is the only species polymorphic for HK with the alleles Hk^{100} and Hk^{91} occuring at a frequency of 0.83 and 0.17, respectively.

Significant deviations from Hardy-Weinberg equilibrium were found in certain groups of sandflies (Tables 1, 2 and 4).

DISCUSSION

Interspecific comparisons. These data show that the Lutzomyia species examined are clearly differentiated genetically. With the single exception of L. trapidoi (for which insufficient data are presently available), each of the Lutzomyia species examined could be distinguished electrophoretically from every other species tested. L. gomezi was uniquely characterized by the allele Me^{96} (Table 3), L. panamensis by the presence of the alleles Me^{100} and Me^{111} (Table 3), and L. ylephiletor by the allele Hk^{104} (Table 4). L. sanguinaria was characterized by a combination of traits: presence of Me^{130} or Me^{115}, but absence of Hk^{104} (Tables 3 and 4). In addition, L. trapidoi was characterized by Pqi^{106} which occured with a frequency greater than 60 per cent and was found in no other Lutzomyia species (Table 2). These results are evidence that biochemical methods of identifying sandflies may be as reliable and as specific as morphological characters and may have an important role to play when morphological characters fail, as in the case of sibling species.

Intraspecific comparisons. The patterns of genetic variation observed within species did not suggest that any species tested was composed of sibling or cryptic species. Significant deviations from Hardy-Weinberg equilibrium may indicate that factors such as the Wahlund effect (pooling of genetically different groups) were disrupting the theoretical equilibrium so that expected phenotypic frequencies were not observed. Significant heterozygote deficiencies of two major alleles (frequencies > 0.10) is an indication of the Wahlund effect, but was never observed during this study. Other factors that contribute to significant Hardy-Weinberg deviations are small sample size, inbreeding, linkage disequilibrium, selection and the presence of a null (silent) allele. Sample sizes were small, as evidenced by the frequency of "expected" values less than five during the chi-square tests of significance. While we can not rule out any of the alternative hypotheses mentioned above, small sample size seems to be the most likely explanation for the significant deviations from Hardy-Weinberg equilibrium.

Cellulose acetate electrophoresis has revealed isozyme markers that uniquely characterize some important vectors of human cutaneous leishmaniasis and significant differences in geographic variation are beginning to be mapped. Hopefully, these genetic

markers will have wider application, serving as reference points in future studies on genetic variation in host preference, microhabitat, and most importantly, vectorial capacity with respect to transmission of human leishmaniasis. Such genetic markers may be useful in detecting variation in transmission of Leishmania to man and may be used to identify epidemiologically dangerous strains.

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