J. Med. Entomol. Vol. 23, no. 5: 489–492 September 1986

DIAGNOSIS BY ISOZYME METHODS OF TWO CRYPTIC SPECIES, *PSYCHODOPYGUS CARRERAI* AND *P. YUCUMENSIS* (DIPTERA: PSYCHODIDAE)[1]

T. Caillard,[2] M. Tibayrenc,[3,6] F. Le Pont,[3] J.P. Dujardin,[4] P. Desjeux,[5] and F.J. Ayala[6]

Abstract. Eleven enzyme systems were used to compare 2 cryptic species previously treated as *Psychodopygus carrerai.* Two enzyme systems were each completely diagnostic. Three other loci gave evidence of reproductive isolation: the combined genotypic frequencies departed from Hardy-Weinberg equilibrium and the allele frequencies were different. The genetic distance between the 2 species is relatively small, suggesting that they are closely related.

Morphological examination of sand flies near Yucumo, Department of Beni, Bolivia, suggested the existence of 2 sympatric morphs within the taxon *Psychodopygus carrerai* (Barretto, 1946). The only external morphological difference detected is in the mesonotum coloration, which is white in the normal morph and light brown in the other morph. A preliminary isozyme study (Le Pont et al. 1985) revealed 1 enzyme system that separates the 2 sympatric morphs and provides evidence that they are 2 different species. The newly defined species was named *Psychodopygus yucumensis* Le Pont, Caillard, Tibayrene & Desjeux, 1986. We present herein the results of a more extensive isozyme comparison of the 2 morphs that confirm that they are, indeed, different species.

MATERIAL AND METHODS

Females of both color forms were captured exclusively at ground level (*Psychodopygus* is absent from the canopy) at only 1 site near Yucumo, always at the bottom of the same tree. Hence, all the specimens used in this study were sympatric. The captures were made during 1983 for 2 days each month from 1900–2200 h. The 2 color forms were present together throughout the year, except in October (dry season) when both forms were absent. Two collectors caught the sand flies on their arms, legs, and clothes with a mouth aspirator and put them singly into glass tubes. The sample tubes were immediately placed into liquid nitrogen in the field. The genitalia of each sand fly were dissected in the laboratory for use in identification. The head and thorax of each specimen were immediately homogenized in 10 μl of hypotonic enzyme stabilizer (Godfrey & Kilgour 1976). The homogenates were then stored at −70 °C until used.

Electrophoresis was carried out on cellulose acetate plates (Helena Laboratories, Beaumont, Texas), with specimens of the two morphs arranged alternately on each plate. Eleven enzyme systems were studied: adenylate kinase (EC 2.7.4.3, ADK); glucose-6-phosphate dehydrogenase (EC 1.1.1.49, G6PD); glucose-6-phosphate isomerase (EC 5.3.1.9, GPI); αglycerophosphate dehydrogenase (EC 1.1.1.8, αGPD); isocitrate dehydrogenase (EC 1.1.1.42, IDH); malate dehydrogenase (EC 1.1.1.37, MDH); malate dehydrogenase (oxaloacetate decarboxylating) ($NADP^+$) or malic enzyme (EC 1.1.1.40, ME); peptidase 2 (bromelain, EC 3.4.22.4, formerly EC 3.4.4.24, substrate: leucyl-L-alanine); phosphoglucomutase (EC 5.4.2.2, formerly EC 2.7.5.1, PGM); 6-phosphogluconate dehydrogenase (EC 1.1.1.44, 6PGD); and xanthine dehydrogenase (EC 1.2.1.37, XDH). The tank buffers and staining recipes were as previously described (Le Pont et al. 1985). Each enzyme system was assayed with and without its specific substrate.

Genetic distance calculations and statistical analyses were performed on a microcomputer (Casio FX702P) using special programs developed by the authors.

1. This study was carried out with the support of the French Technical Cooperation Program, of the Ministère de l'Industrie et de la Recherche (extension of grant number PVD/81/L-1423), and of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

2. Instituto Boliviano de Biología de Altura (IBBA), Embajada de Francia, Casilla 824, La Paz, Bolivia. Send reprint requests to this address.

3. IBBA, and SSC ORSTOM, 70-74 route d'Aulnay, 93140 Bondy, France.

4. IBBA, and Institut de Médecine tropicale "Prince Leopold," 155 Nationalestraat, B-2000 Antwerpen, Belgium.

5. IBBA, and Institut Pasteur, 28 rue du Docteur Roux, 75015 Paris, France.

6. Department of Genetics, University of California, Davis, California 95616, USA.

TABLE 1. Characteristics of the 11 loci surveyed and of their enzyme products when the data of *Psychodopygus carrerai* and *P. yucumensis* are jointly considered.

Enzyme locus	State	No. alleles	Quaternary structure	No. species
Adk 1	polymorphic	2	monomeric	167
Gpi	polymorphic	3	dimeric	220
Idh	polymorphic	2	monomeric	178
Mdh	polymorphic	3	dimeric	253
*Pep 2**	polymorphic	2	?	102
Pgm	polymorphic	5	monomeric	245
*Xdh**	polymorphic	2	?	163
Adk 2	monomorphic	1	?	167
αGpd	monomorphic	1	?	132
G6pd	monomorphic	1	?	120
6Pgd	monomorphic	1	?	149

* *Pep 2* and *Xdh* are polymorphic when both species are combined, but monomorphic for each species separately.

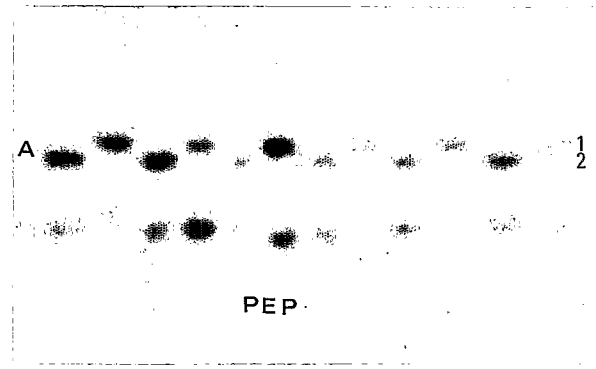

FIG. 1. An electrophoretic plate stained for peptidase. There are 2 zones of activity, but only the fastest one (labeled "A") is considered. Specimens of the 2 species have been placed alternately on the plate: the 1st individual on the left is *P. yucumensis*, the next one is *P. carrerai*, and so on. This enzyme system is fully diagnostic between the 2 species. No heterozygous patterns were found.

RESULTS

Table 1 summarizes the results obtained when the samples of the 2 cryptic species were compared. No color bands appeared on the plates when the specific substrate was not added to the staining solution. The ME plates gave patterns that could not be resolved; this enzyme was not further considered. Nine of the 10 remaining enzymes manifested only the activity of a single gene locus, whereas 2 loci were apparent in the case of adenylate kinase (*Adk 1* and *Adk 2*).

Four loci gave no evidence of genetic variation. Two loci, *Pep 2* and *Xdh*, were polymorphic when the 2 species were compared but monomorphic within each species; hence each of these 2 loci is fully diagnostic. At both loci *P. carrerai* exhibited the fastest migrating allele under our electrophoretic conditions, while *P. yucumensis* had the slowest migrating allele (Fig. 1, 2). The remaining 5 loci were polymorphic in each of the 2 species and had alleles shared by both. From the heterozygous patterns, we could infer the quaternary structure of these enzymes: 2 are dimers (GPI and MDH; 3-banded patterns in the heterozygotes) (Fig. 3, 4) and 3 are monomers (ADK1, IDH, and PGM; 2-banded patterns in the heterozygotes).

Table 2 displays the allele frequencies at the 5 loci that are polymorphic in each species. These frequencies are compared using standard deviate statistics $\epsilon = [(p_A - p_B)/(p \cdot q/N_A + p \cdot q/N_B)^{1/2}]$, where p_A and p_B are the frequencies of a given allele in populations A and B, p is the frequency of that allele in both populations combined, $q = 1 - p$, and N_A and N_B are the numbers of alleles sampled in populations A and B (see Schwartz 1963: 58). Significant differences in allelic frequencies were observed at the 3 highly polymorphic loci *Gpi*, *Mdh*, and *Pgm*; at the other 2 loci, the frequencies of the 2 observed alleles are too close to 1 and to 0 to make this test meaningful.

The numbers of individuals of each genotype observed in each species are given for the 5 polymorphic loci in Table 3. We tested by chi-square whether the genotypic numbers observed in each population were consistent with the expectations of Hardy-Weinberg equilibrium. The only significant deviation occurred at the *Pgm* locus in *P. carrerai* ($\chi^2 = 39.8$, 11 df, $P < 0.001$). When the data for the 2 species were pooled, the observed genotypic numbers departed from the Hardy-Weinberg expectations at the 2 highly polymor-

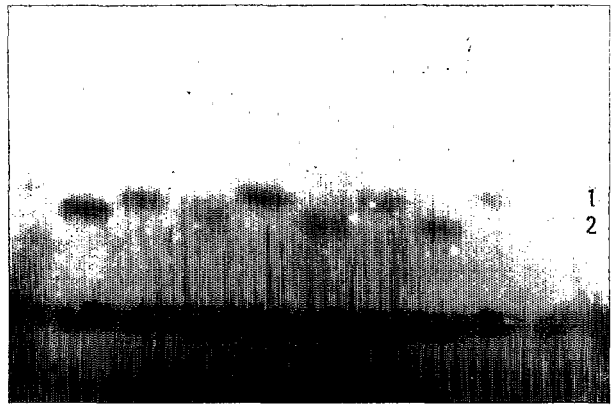

FIG. 2. A plate stained for xanthine dehydrogenase. The 1st specimen on the left is *P. carrerai*, the next is *P. yucumensis*, and so on in alternation. No heterozygous patterns were found. This enzyme system is also fully diagnostic.

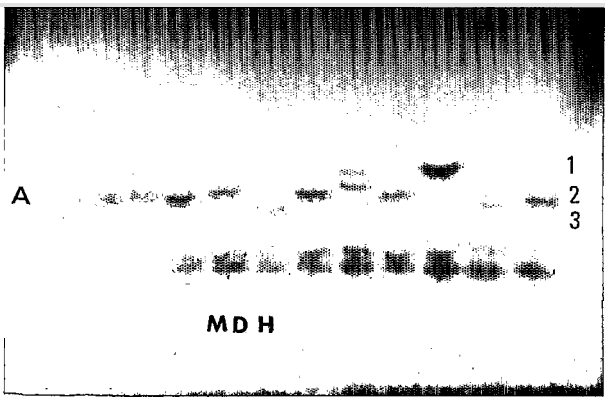

FIG. 3. A plate stained for malate dehydrogenase. Only the fastest migrating zone of activity (A) is considered. Specimens of the 2 species are alternately arranged along the plate. Three alleles were found. On this plate, it would seem that allele *2* is different for the 2 species, but we were unable to ascertain this with other assays. The heterozygotes exhibit a 3-banded phenotype, indicating that this enzyme is a dimer.

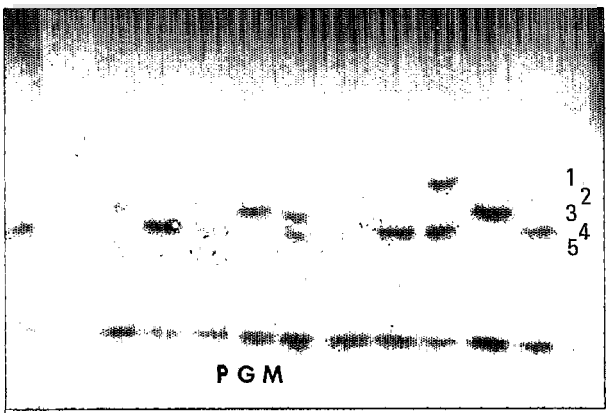

FIG. 4. A plate stained for phosphoglucomutase. Specimens of the 2 species are placed alternately along the plate. Five alleles are apparent. The 2-banded pattern of the heterozygotes indicates that this enzyme is a monomer.

phic loci (*Mdh*: $\chi^2 = 40.8$, 5 df, $P < 0.001$; *Pgm*: $\chi^2 = 47.8$, 12 df, $P < 0.001$), but not at the other 3.

We calculated the average heterozygosity, H (the probability that 1 individual will be heterozygous at a given locus), for the 11 gene loci studied. For *P. carrerai,* the observed $H = 0.099$ and the expected $H = 0.120$; for *P. yucumensis,* observed $H = 0.126$, expected $H = 0.145$. These heterozygosities were within the typical range observed for insects (Ayala 1982), although the number of loci sampled was small.

DISCUSSION

The present results further distinguish the 2 color morphs as 2 separate species, *P. carrerai* and *P. yucumensis.* Two loci, *Pep 2* and *Xdh,* are fully diagnostic between the 2 species; no common alleles were found in more than 80 individuals of each species examined at each locus. We calculated the probability of incorrect diagnosis of the species using the 3 highly polymorphic loci (Ayala & Powell 1972). The probabilities of making the wrong identification were 0.398 for *Gpi,* 0.233 for *Mdh,* and 0.324 for *Pgm.* When these 3 polymorphic loci are used jointly, the probability of correct diagnosis of the species for a single individual is 0.97. Thus, the 3 loci may be used jointly for diagnosis with only a 0.03 probability of error, although the existence of the 2 fully diagnostic loci would normally make superfluous the use of the polymorphic loci.

The differences in allele frequencies are revealed also by use of standard deviate statistics (Table 2) and by the significant departure from Hardy-Weinberg expectations for the pooled genotypic data at 2 of the 3 loci. At the *Pgm* locus, the *P. carrerai* population departs from the Hardy-Weinberg frequencies. This might be due to natural selection or to lack of random mating, which in turn could be the result of recent gene flow or of the presence of 2 or more reproductively isolated populations within *P. carrerai.*

The existence of 2 sympatric species of *Psychodopygus* in this region of Bolivia is of epidemiolog-

TABLE 2. Allele frequencies at 5 polymorphic loci in *Psychodopygus carrerai* and *P. yucumensis.* The similarity of the allele frequencies is examined by the standard deviate statistic ϵ.

Locus and allele	*carrerai*	*yucumensis*	ϵ	P
Adk 1 1	1	0.98	—*	—
Adk 1 2	**0**	**0.02**	—	—
Gpi 1	0.123	0.014	4.54	<0.001
Gpi 2	0.854	0.904	1.61	NS
Gpi 3	0.023	0.082	2.79	<0.02
Idh 1	0.017	0.028	—	—
Idh 2	0.983	0.972	—	—
Mdh 1	0.056	0.295	7.06	<0.001
Mdh 2	0.869	0.472	9.47	<0.001
Mdh 3	0.075	0.232	4.89	<0.001
Pgm 1	0.189	0.012	6.59	<0.001
Pgm 2	0.223	0.159	1.80	NS
Pgm 3	0.235	0.365	3.13	<0.02
Pgm 4	0.273	0.377	2.45	<0.02
Pgm 5	0.080	0.087	0.28	NS

* ϵ was not calculated for *Adk 1* and *Idh* because the allele frequencies in both species are very close to 1 or 0. NS = not significant ($P > 0.05$).

TABLE 3. Genotypic numbers at 5 polymorphic loci in 2 sympatric species of sand flies, *Psychodopygus carrerai* and *P. yucumensis*. The fastest migrating allele is designated "1," the next fastest "2," and so on.

Genotype	*carrerai*	*yucumensis*	Genotype	*carrerai*	*yucumensis*
Adk 1 1/1	83	81	*Pgm 1/1*	9	1
Adk 1 1/2	0	3	*Pgm 1/2*	7	1
Adk 1 2/2	0	0	*Pgm 1/3*	15	0
Idh 1/1	1	0	*Pgm 1/4*	3	0
Idh 1/2	1	5	*Pgm 1/5*	2	0
Idh 2/2	87	84	*Pgm 2/2*	7	5
Gpi 1/1	3	1	*Pgm 2/3*	17	20
Gpi 1/2	21	1	*Pgm 2/4*	15	6
Gpi 1/3	0	0	*Pgm 2/5*	0	3
Gpi 2/2	81	90	*Pgm 3/3*	7	18
Gpi 2/3	5	18	*Pgm 3/4*	8	32
Gpi 3/3	0	0	*Pgm 3/5*	2	4
Mdh 1/1	1	16	*Pgm 4/4*	16	24
Mdh 1/2	7	24	*Pgm 4/5*	7	9
Mdh 1/3	5	19	*Pgm 5/5*	4	3
Mdh 2/2	99	36			
Mdh 2/3	14	24			
Mdh 3/3	0	8			

ical interest, given that the species may be *Leishmania* vectors. The *Leishmania* stocks isolated from the more recently described sand fly species have electrophoretic patterns typical of *Leishmania braziliensis braziliensis* (Desjeux & Le Pont, unpubl. data) and, hence, indicate that *P. yucumensis* is probably a human *Leishmania* vector. The case is different for *P. carrerai*, which yielded an electrophoretically untypical *Leishmania* stock. We have work in progress to ascertain whether these species are able to transmit *Leishmania*.

The genetic distance (D) between the 2 cryptic species is, for all 11 gene loci, 0.256. [D estimates the average number of codon substitutions per gene that have occurred since the phylogenetic divergence of the 2 species (Nei 1972).] This degree of genetic divergence between 2 species is relatively low for insects, even when the species are cryptic (Ayala 1982). This suggests that the evolutionary divergence of the 2 species has occurred in a relatively recent past, which is of course consistent with their great morphological similarity. Nevertheless, we should reiterate that 2 of the 11 loci surveyed are fully diagnostic for these 2 species.

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