An alloenzymic comparison of *Psychodopygus wellcomei* — an incriminated vector of *Leishmania braziliensis* in Pará State, Brazil — and the sympatric morphospecies *Ps. complexus* (Diptera, Psychodidae) [1]

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Summary

The series Psychodopygus squamiventris *contains a number of morphospecies which are separated according to male morphological characters alone. Females of this series, presumed to be* Ps. wellcomei, *had been incriminated as vectors to man of* Leishmania braziliensis *in southeast Amazonia, Brazil, but the recent discovery of* Ps. complexus *males in the same forests has called into doubt the vectorial and biological status of* Ps. wellcomei. *Enzyme polymorphism was investigated in males and in laboratory-reared F1 females of both morphospecies in a search for diagnostic female characters and for evidence of biological separateness. 16 enzymes were studied using electrophoresis (all 16 with cellulose acetate and 3 with starch gel as well). Only Glucose-phosphate isomerase (E.C. 5.3.1.9.), Mannosephosphate isomerase (E.C. 5.3.1.8) and Phosphoglucomutase (E.C. 2.7.5.1) displayed substantial polymorphism at any one locus. No diagnostic electromorphs were found, but GPI allele frequencies and genotype frequencies were significantly different for populations of* Ps. wellcomei *and* Ps. complexus. *This result supports the conclusion of rearing experiments, namely that these two taxa are distinct biological species.*

Key words : Phlebotomines — Vectors — Alloenzymes — Electrophoresis — Leishmaniasis — Brazil.

Résumé

Une comparaison alloenzymique entre Psychodopygus wellcomei — un vecteur incriminé de Leishmania braziliensis — et l'espèce morphologique sympatrique Ps. complexus (Diptera, Psychodidae). *La localité-type de* Psychodopygus wellcomei *est la Serra dos Carajás dans l'État brésilien du Pará, où les femelles de ce phlébotome ont été incriminées comme vectrices de* Leishmania braziliensis, *l'agent étiologique de la leishmaniose cutanée régionale. Récemment, le statut vectoriel et biologique de* Ps. wellcomei *a été mis en doute par les études écologiques qui ont montré la présence de* Ps. complexus *dans la Serra dos Cara-*

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jás. Les deux espèces appartiennent au même groupe d'espèces (ou série), celui de Psychodopygus squamiventris, *qui se compose de plusieurs espèces morphologiques qu'on ne peut classer que selon les caractères mâles.*

La variation enzymatique a été recherchée chez les mâles et chez les femelles (F1) des deux espèces, afin de découvrir des différences biologiques et des caractères diagnostiques chez les femelles. L'électrophorèse a été appliquée aux 16 enzymes (toutes les 16 sur acétate de cellulose, et 3 seulement sur amidon). Seules les enzymes GPI (E.C. 5.3.1.9), MPI (E.C. 5.3.1.8) et PGM (E.C. 2.7.5.1) étaient fortement polymorphes à n'importe quel locus.

L'électrophorèse n'a pas mis en évidence de caractères diagnostiques mais les fréquences alléliques et génotypiques de l'enzyme GPI étaient nettement différentes chez Ps. wellcomei *et* Ps. complexus. *Ce résultat renforce les conclusions des expériences d'élevage, à savoir que les deux taxons sont des espèces biologiques : les mâles F1 et F2 issus d'une même femelle ont toujours été du même type morphologique.*

Mots-clés : Phlébotomes — Vecteurs — Alloenzymes — Electrophorèse — Leishmaniose — Brésil.

The series *Psychodopygus squamiventris* contains a number of morphospecies whose males are morphologically distinct, but whose females cannot presently be distinguished (Ready *et al.*, 1982). Females of this series, presumed to be *Psychodopygus wellcomei* Fraiha, Shaw and Lainson, have been incriminated as vectors to man of *Leishmania braziliensis* s.l. in southeast Amazônia, Brazil (Lainson *et al.*, 1973). The original epidemiological investigations were carried out in a small forested area of the Serra dos Carajás hills in Pará State, where *Ps. wellcomei* was thought to be the only representative of the *squamiventris* series *sensu stricto* and to predominate in catches of phlebotomines from human bait (Ward *et al.*, 1973). However, the recent discovery of the males of another member of the series, *Ps. complexus* Mangabeira, in the same forests called into doubt the identity of the infected female sandflies found by those workers (Ready *et al.*, 1984).

The present publication reports an investigation of enzyme polymorphism in *Ps. wellcomei* and *Ps. complexus*, which was undertaken to search for characters diagnostic for females and to help elucidate the biological status of these two morphospecies — taxonomic preliminaries to any studies on their roles as vectors of cutaneous leishmaniasis.

1. Materials and methods

Male and female phlebotomines were aspirated from Shannon traps (Shannon, 1939) set in evergreen seasonal forests covering the summit and the base of the Serra Norte, the northern range of the Serra dos Carajás hills situated in southeast Pará State, Brazil (6° S-50°18′ W). Two forest sites were sampled : " N2 " at 600-700 m above sea-level, the site where *Ps. wellcomei* females infected with *Le. braziliensis* were found by Lainson *et al.* (1973) ; and, " Paranapanema " at 150-200 m above sea-level, at the eastern base of Serra Norte near the river Parauapebas. *Psychodopygus wellcomei* predominated at the former site and *Ps. complexus* at the latter (Ready *et al.*, 1984).

Phlebotomines were collected from 18.30 (dusk) to 21.00 hr (= GMT — 3 hours) on several dates between November 1982 and March 1983. At the end of an evening's capture, the total catch was left in a holding cage to feed overnight (in the forest) on a restrained hamster. The following morning all living males and unfed females were stored in liquid nitrogen ; blood engorged females were individually captured in tubes and their progeny reared, as isofemale lines, in the insectary of The Wellcome Parasitology Unit in Belém (25 ± 3°C ; 90 % RH ; ca. 12 hour daily photoperiod) following the methods of Ward (1977) and Ready and Croset (1980). One day after emergence F1 adults were stored in liquid nitrogen.

Enzyme polymorphism in individual flies (or parts of them) was studied using cellulose acetate electrophoresis (Helena system) and starch gel electrophoresis (Lanham *et al.*, 1981 ; Miles *et al.*, 1980). For cellulose acetate electrophoresis (CAE), ultra-violet fluorescent methods were used to visualize the enzymes Aspartate aminotransferase (E.C. 2.6.1.1.), Alanine aminotransferase (E.C. 2.6.1.2.) and Esterase (E.C. 3.1.1.1.) (Lanham *et al.*, 1981) ; for all other enzymes, tetrazolium staining was employed, with the " developer " (of substrate, coenzyme and linking enzymes) incorporated into a 10 % agar gel (Kreutzer and Christensen, 1980 ; Lanham, 1982). Phlebotomines were sorted and prepared for electrophoresis on ice-packs. Each

fly was ground with a glass rod in 15 µ of " enzyme stabilizer " (Miles and Ward, 1978).

2. Results

2.1. Cellulose acetate electrophoresis

Each homogenate of an individual fly provided material sufficient for the revelation of four to six enzymes.

Glucosephosphate isomerase (E.C. 5.3.1.9. GPI)

The revelation of GPI gave strong, discrete anodic electrophoretic bands (= electromorphs) which, based on the results of isofemale broods, represented four allelomorphs at one locus (Fig. 1).

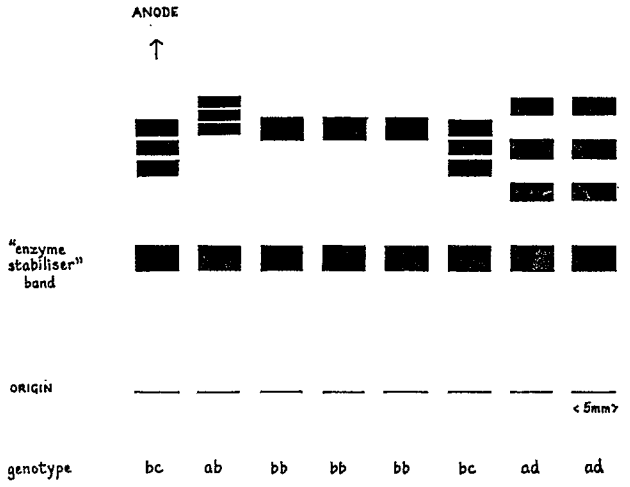

Figure 1. — Electromorphs revealed after separation of the alloenzymes of Glucosephosphate isomerase (GPI) on cellulose acetate.

All allelomorphs of this dimeric enzyme were common to *Ps. wellcomei* and *Ps. complexus* (i.e. non-discriminating) but, quantitatively, enzyme polymorphism was significantly less for *Ps. wellcomei* (Table I). For each population, the genotypes were united into the two groups (Table II) that best reflected the differences between *Ps. wellcomei* (alleles " a " and " d " infrequent ; allele " c " frequent) and *Ps. complexus* (alleles " a " and " d " frequent ; allele " c " infrequent). So arranged, pairs of populations were compared (in 2 × 2 contingency tables) by using Fisher's Exact test which, unlike the χ^2 test, is not invalidated by theoretical (= " expected ") numbers of less than 5 (Bailey, 1969 : 61-65). Genotype frequencies were found to be significantly different for the two morphospecies regardless of population origin ($p < 0.05$ for two-tailed Exact test, for all interspecific comparisons in Table II). Similarly, allele frequencies were found to be significantly different for the two morphospecies.

By forming five genotype groups (b/b, b/c, b/d, b/a and " others ") χ^2 tests were performed to compare observed frequencies with those expected in the case of Hardy-Weinberg equilibrium. No significant deviation from Hardy-Weinberg equilibrium could be demonstrated for the combined *wellcomei* and *complexus* populations from Paranapanema or for the combined populations from both sites ($0.95 > p\ (\chi^2) > 0.05$). This result indicates the desirability of increasing the sample size.

All four electromorphs of GPI could be revealed routinely using only the thorax of individual flies.

Mannosephosphate isomerase (E.C. 5.3.1.8., MPI)

The revelation of MPI gave intense, discrete anodic electromorphs which, based on the results of isofemale broods, were the products of five alleles at one locus. All allelomorphs of this monomeric enzyme were common to *Ps. wellcomei* (N = 46) and *Ps. complexus* (N = 26) and the allele and genotype frequencies were not significantly different for their populations. All five electromorphs could be revealed routinely using an extract of the thorax of individual flies.

Phosphoglucomutase (E.C. 2.7.5.1., PGM)

Two (presumed) anodic loci were revealed, but electromorphs at the faster were seen on only one occasion. The slower locus had four allelomorphs common to *Ps. wellcomei* (N = 22) and *Ps. complexus* (N = 25). PGM is monomeric in these species, but the electrophoretic bands were rarely intense and often difficult to visualize, even in extracts of whole flies.

Isocitrate dehydrogenase (E.C. 1.1.1.42., ICD)

Two intense, discrete anodic electromorphs, apparently representing two loci (from the distance between them), were common to *Ps. wellcomei* (N = 22) and *Ps. complexus* (N = 24). Another *Ps. wellcomei* had a slightly faster band at the faster locus.

TABLE I

Allele and genotype frequencies for the enzyme GPI (* Alleles designated a, b, c, d in descending order of anodic mobility).

Collection site		N2		PARANAPANEMA		PARANAPANEMA	
Morphospecies (♂♂ and F1 ♀♀)		*wellcomei*		*wellcomei*		*complexus*	
N°. of specimens		40		20		39	
N°. of alleles		4		4		4	
Allele frequency a* =		0.012		0.025		0.141	
b =		0.713		0.700		0.654	
c =		0.263		0.250		0.051	
d =		0.012		0.025		0.154	
Genotypes :		frequency	number	frequency	number	frequency	number
Observed and (theoretic)	b/b =	0.500 (0.508)	20 (20.3)	0.450 (0.490)	9 (9.8)	0.461 (0.428)	18 (16.7)
	b/c	0.375 (0.375)	15 (15.0)	0.400 (0.350)	8 (7.0)	0.103 (0.067)	4 (2.6)
	c/c =	0.075 (0.069)	3 (2.8)	0.050 (0.063)	1 (1.3)	0.000 (0.003)	0 (0.1)
	b/d =	0.025 (0.017)	1 (0.7)	0.050 (0.035)	1 (0.7)	0.103 (0.201)	4 (7.8)
	d/d =	0.000 (0.001	0 (0.0)	0.000 (0.001)	0 (0.0)	0.051 (0.024)	2 (0.9)
	d/a =	0.000 (0.001)	0 (0.0)	0.000 (0.001)	0 (0.0)	0.103 (0.043)	4 (1.7)
	b/a =	0.025 (0.017)	1 (0.7)	0.050 (0.035)	1 (0.7)	0.179 (0.184)	7 (7.2)
	Others = — a/a, a/c, c/d	0.000 (0.012)	0 (0.5)	0.000 (0.025)	0 (0.5)	0.000 (0.050)	0 (2.0)

TABLE II

Grouped genotype frequencies for the enzyme GPI (* All males).

Morphospecies	Collection site	N°. (%) in genotype groups b/b + b/c + c/c	Others
complexus	Paranapanema	22 (56.4 %)	17 (43.6 %)
wellcomei	Paranapanema	18 (90.0 %)	2* (10.0 %)
wellcomei	N2	38 (95.0 %)	2* (5.0 %)
wellcomei	Combined	56 (93.3 %)	4* (6.7 %)

Hexokinase (E.C. 2.7.1.1., HK) and Aminopeptidase (cytosol) (E.C. 3.4.11.1., PEP)

The revelation of each enzyme gave two well-separated anodic electromorphs (apparently representing two loci) common to *Ps. wellcomei* (N = 8, 11) and *Ps. complexus* (N = 7, 3). The slower band was always the more intense.

Malic enzyme (E.C. 1.1.1.40., ME), Glucose 6-phosphate dehydrogenase (E.C. 1.1.1.49., G6PD) and 6-Phosphoglucose dehydrogenase (E.C. 1.1.1.44., 6PGD)

For each enzyme, one intense, discrete anodic electromorph was found to be common to *Ps. wellcomei* (N = 9, 9, 9) and *Ps. complexus* (N = 23, 3, 3).

Malate dehydrogenase (E.C. 1.1.1.37., MDH)

Two intense, discrete electromorphs, one anodic and the other cathodic, were common to *Ps. wellcomei* (N = 24) and *Ps. complexus* (N = 7).

Aspartate aminotransferase (E.C. 2.6.1.1., ASAT), Alanine aminotransferase (E.C. 2.6.1.2., ALAT), Aconitate hydratase (E.C. 4.2.1.3., ACON), Lactate dehydrogenase (E.C. 1.1.1.27., LDH), Glutamate dehydrogenase (E.C. 1.4.1.2., GD) and Esterase (E.C. 3.1.1.1., EST; 4-methylumbelliferyl acetate esterolytic)

ALAT, ACON, LDH and GD gave no electrophoretic bands, while the results with ASAT or EST were poor or impossible to interpret (N = 10 *wellcomei* and 4 *complexus* for each enzyme).

2.2. Starch gel electrophoresis

ASAT

One intense, discrete anodic electromorph was common to all *Ps. wellcomei* (N = 13) and *Ps. complexus* (N = 8). Two additional anodic bands were revealed for one *Ps. wellcomei* (making a three-band pattern typical of an heterozygote of a dimeric enzyme). Strong extracts of females of both morphospecies produced an additional, cathodic electromorph.

ALAT and EST

No discrete electrophoretic bands were revealed (N = 21 *wellcomei* and 8 *complexus*).

Other electrophoretic bands

The enzyme stabilizer was often responsible for an intense anodic band on CAE plates. This band was revealed when extracts of phlebotomines were made in enzyme stabilizer or when enzyme stabilizer alone was run as a control, but never appeared in extracts of flies in double-distilled water (which was rarely used because of poor revelation).

3. Discussion and Conclusions

Enzyme electrophoresis is one, often powerful, tool for the study of species groups and sibling species, see Avise (1975). However, speciation is not always accompanied by extensive enzymic divergence and there are some morphologically and biologically distinct species of *Drosophila* which show little or no enzyme differentiation (Sene and Carson, 1977).

In the field of medical entomology, too, the results of enzyme studies have been mixed : enzyme variants are diagnostic between members of the *Anopheles gambiae* complex, but not at some localities (Miles, 1978) ; and there is limited enzyme variation in the *Simulium damnosum* complex (Townson and Meredith, 1979). Investigations of enzyme variation in phlebotomines have been few and have not produced results of epidemiological significance (Miles and Ward, 1978 ; Tibayrenc *et al.*, 1980 ; Ward *et al.*, 1981a, 1981b ; Petersen, 1982).

In the present investigation, enzyme characters diagnostic for *Ps. wellcomei* and *Ps. complexus* were not demonstrated ; reproductive isolation was not proven by finding unique alloenzymes. Nevertheless, the available evidence does strongly suggest that the two taxa represent good biological species : significant differences in GPI allele and genotype frequencies did indicate that genetic exchange does not occur freely between their populations ; and, more importantly, F1 and F2 isofemale (= single family) broods reared in the laboratory have never produced a mixture of the two male morphs (unpublished observations). The major morphological differences between the external genitalia of the males of *Ps. wellcomei* and *Ps. complexus*, alone, would indicate reproductive isolation to most taxonomists. In addition, there is substantial physical isolation of the two morphospecies : they are sympatric in most of their known ranges in Amazônia, but *Ps. wellcomei* is abundant only in submontane forests (Ready *et al.*, 1984).

It should be remembered that this report refers to attempts to characterize but 16 enzymes, of which only 11 were successfully revealed.

Methods are available for the detection (in different media) of more than 80 enzymes and, therefore, it is quite likely that more extensive investigations could uncover enzyme variants diagnostic for the females of *Ps. wellcomei* and *Ps. complexus*.

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BIBLIOGRAPHY

AVISE (J.), 1975. — Systematic value of electrophoretic data. *Syst. Zool.* 23 : 465-481.

BAILEY (N. T. J.), 1969. — Statistical methods in Biology. English Universities Press Ltd, London.

KREUTZER (R. D.) and CHRISTENSEN (H. A.), 1980. — Characterization of *Leishmania* spp. by isoenzyme electrophoresis. *Am. J. Trop. Med. Hyg.*, 29 : 199-208.

LAINSON (R.), SHAW (J. J.), WARD (R. D.) and FRAIHA (H.), 1973. — Leishmaniasis in Brazil : IX. Considerations on the *Leishmania braziliensis* complex : Importance of sandflies of the genus *Psychodopygus* (Mangabeira) in the transmission of *L. braziliensis braziliensis* in north Brazil. *Trans. R. Soc. trop. Med. Hyg.*, 67 : 184-196.

LANHAM (S. M.), 1982. — Kits for isoenzyme characterization of *Leishmania* isolates in the field. PAHO workshop, Washington, D.C., 9-11 December 1980. UNDP/World Bank/WHO, Geneva, 105-115.

LANHAM (S. M.), GRENDON (J. M.), MILES (M. A.). PÓVOA (M.) and DE SOUZA (A. A.), 1981. — A comparison of electrophoretic methods for isoenzyme characterization of trypanosomatids. 1. : Standard stocks of *Trypanosoma cruzi* zymodemes from northeast Brasil. *Trans. R. Soc. trop. Med. Hyg.*, 75 : 742-750.

MILES (S. J.), 1978. — Enzyme variation in the *Anopheles gambiae* Giles group of species (Diptera : Culicidae). *Bull. ent. Res.*, 68 : 85-96.

MILES (M. A.), LANHAM (S. M.), DE SOUZA (A. A.) and PÓVOA (M.), 1980. — Further enzymic characters of *Trypanosoma cruzi* and their evaluation for strain identification. *Trans. R. Soc. trop. Med. Hyg.*, 74 : 221-237.

MILES (M. A). and WARD (R. D.), 1978. — Preliminary isoenzyme studies on phlebotomine sandflies (Diptera : Psychodidae). *Ann. trop. Med. Parasit.*, 72 : 398-400.

PETERSEN (J. L.), 1982. — Preliminary survey of isozyme variation in anthropophilic Panamanian *Lutzomyia* species. PAHO workshop, Washington DC.., 9-11 December 1980. UNDP/World Bank/WHO. Geneva, 105-115.

READY (P. D.) and CROSET (H.), 1980. — Diapause and laboratory breeding of *Phlebotomus perniciosus* Newstead and *Phlebotomus ariasi* Tonnoir (Diptera : Psychodidae) from southern France. *Bull. ent. Res.*, 70 : 511-523.

READY (P. D.), FRAIHA (H.),LANE (R. P.), ARIAS (J. R.) and PAJOT (F. X.), 1982. — On distinguishing the female of *Psychodopygus wellcomei*, a vector of mucocutaneous leishmaniasis, from other *squamiventris* series females. I. Characterization of *Ps. squamiventris squamiventris* and *Ps. s. maripaensis* stat. nov. (Diptera : Psychodidae). *Ann. trop. Med. Parasit.*, 76 : 201-214.

READY (P. D.), LAINSON (R.) and SHAW (J. J.), 1984. — Habitat and seasonality of *Psychodopygus wellcomei* help incriminate it as a vector of *Leishmania braziliensis* in Amazônia and Northeast Brazil. *Trans. R. Soc. trop. Med. Hyg.*, 78 : 543-544.

SENE (F. M.) and CARSON (H. L.), 1977. — Genetic variation in Hawaiian *Drosophila*, IV. Allozymic similarity between *Drosophila sylvestris* and *Drosophila heteroneura* from the island of Hawaii. *Genetics*, 86 : 187-198.

SHANNON (R.), 1939. — Methods for collecting and feeding mosquitos in jungle yellow fever studies. *Am. J. trop. Med.*, 19 : 131-140.

TIBAYRENC (M.), CARRIOU (M. L.), CORNEAU (B.) and PAJOT (F. X.), 1980. — Étude allozymique chez *Lutzomyia umbratilis* (Diptera, Psychodidae), vecteur de la leishmaniose en Guyane française. *Cah. O.R.S.T.O.M., série Ent. méd. et Parasitol.*, 18 : 67-70.

TOWNSON (H.) and MEREDITH (S. E. O.), 1979. — Identification of Simuliidae in relation to onchocerciasis. *In* : Symposia of the British Society for Parasitology. Vol. 17. Blackwell Scientific Publications, Oxford, 145-174.

WARD (R. D.), 1977. — The colonization of *Lutzomyia flaviscutellata* (Diptera : Psychodidae), a vector of *Leishmania mexicana amazonensis* in Brazil. *J. Med. Entomol.*, 14 : 469-476.

WARD (R. D.), BETTINI (S.), MAROLI (M.), MCGARRY (J. W.) and DRAPER (A.), 1981a. — Phosphoglucomutase polymorphism in *Phlebotomus perfiliewi perfiliewi* Parrot (Diptera : Psychodidae) from central and northern Italy. *Ann. trop. Med. Parasit.*, 75 : 653-661.

WARD (R. D.), PASTEUR (N.) and RIOUX (J. A.), 1981b. — Electrophoretic studies on genetic polymorphism and differentiation of phlebotomine sandflies (Diptera : Psychodidae) from France and Tunisia. *Ann. trop. Med. Parasit.*, 75 : 235-245.

WARD (R. D.), SHAW (J. J.), LAINSON (R.) and FRAIHA (H.), 1973. — Leishmaniasis in Brazil : VIII. Observations on the phlebotomine fauna of an area highly endemic for cutaneous leishmaniasis, in the Serra dos Carajás, Pará State. *Trans. R. Soc. trop. Med. Hyg.*, 67 : 174-183.