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Genetic Evidence for the Existence of Cryptic Species in the *Anopheles albitarsis* Complex in Brazil: Allozymes and Mitochondrial DNA Restriction Fragment Length Polymorphisms[1]

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Allozyme and mitochondrial DNA (mtDNA) restriction studies were undertaken to determine the extent of genetic divergence among field populations of Anopheles albitarsis *in Brazil. Two sympatric species,* An. deaneorum *and* An. marajoara, *were identified in collections from Costa Marques (CM), Rondonia. Genetic evidence includes (1) the presence of two types of individuals, each with diagnostic allelic clusters (for* Had-1, Pgi-1, Pep-1, Mpi-1, *and* Idh-1*), (2) a deficiency of heterozygotes, and (3) characteristic mtDNA haplotypes. In addition, two allopatric cryptic species of* An. marajoara *were identified, one from Iguape (*An. marajoara *form IG), Sao Paulo state, and the other from the Island of Marajo (*An. marajoara *form MA). Though form IG and form-MA resemble form CM in wing spot morphology, they differ from it in diagnostic allozymes and mtDNA haplotypes.* An. marajoara *form CM had a higher*

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variability (mean heterozygosity, H = *0.22, and percentage of polymorphic loci,* P = *66.7) than did form IG and form MA (*H = *0.08 in both, and* P = *25.0 and 33.3, respectively). Form MA and form IG are genetically more similar to each other than both are to form CM. Based on wing morphology, estimates of* F *statistics, and genetic similarities, we propose that* An. albitarsis *in Brazil is a species complex. It comprises at least two morphologically distinguishable species: (1)* An. deaneorum *(currently one taxon) and (2) the* An. marajoara *species complex, which further consists of at least three cryptic forms,* marajoara *form MA,* marajoara *form IG, and* marajoara *form CM.*

KEY WORDS: *Anopheles albitarsis;* allozymes; mitochondrial DNA; restriction fragment length polymorphism; cryptic species.

INTRODUCTION

Anopheles albitarsis (Lynch Arribalzaga), a neotropical species in the subgenus *Nyssorhynchus,* has been incriminated as one of the three principal vectors of human malaria in Brazil and the neighboring countries. It is widely distributed in Central and South America, ranging from Northern Argentina to Southern Panama. *Anopheles albitarsis* was originally described from Baradero, Buenos Aires state, Argentina, in 1878. In 1943, Galvao and Grieco recognized two distinct subspecies on the basis of behavioral differences, (1) *An. albitarsis domesticus* (Galvao and Grieco, 1943; Rios *et al.,* 1984), an indoor biting mosquito and thus the principal vector of human malaria, and (2) *An. albitarsis albitarsis,* an outdoor biting mosquito with a secondary role in malaria transmission. In addition, other investigators observed regional differences in morphology, behavior and ecology (see review by Linthicum, 1988) and described them as races, varieties, subspecies, or forms. Consequently, different scientific names (*An. albitarsis limai, An. albitarsis imperfectus,* and *An. alopha*) were given to the variant forms without adequate descriptions to support their taxonomic status. Therefore, it is uncertain whether some of these subspecific and specific descriptions refer to sibling species or merely polymorphic forms of a single species.

During the last two decades, three investigators attempted to address this question using polytene chromosomes and allozymes (Kitzmiller *et al.,* 1974; Kitzmiller, 1976, 1977; Steiner *et al.,* 1982, Rosa-Freitas, 1989; Rosa-Freitas *et al.,* 1990). These studies indicated that *An. albitarsis* is a species complex, which consists of three or more sympatric and allopatric forms. These authors observed a high degree of variation in the morphological taxonomic characters in field samples (Kitzmiller and Steiner, personal communications). Unfortunately they neither published descriptions of these variants nor made correlations between chromosomal or allozyme types and the previously described morphological or behavioral variants.

Consequently, the taxonomic relationships between the morphological variants and the chromosomal and allozyme forms remained unknown.

Linthicum (1988), in a review of the subgenus *Nyssorhynchus,* used morphological characters and grouped all previously described forms of *albitarsis* into two species, *An. albitarsis* and *An. marajoara.* According to him, "true albitarsis" is restricted to Argentina, Uruguay, southern Brazil, and Paraguay. The other *albitarsis* forms (which occur in Bolivia, middle and northern Brazil, Colombia, Costa Rica, Guatemala, the Guianas, Panama, northern Paraguay, Trinidad, and Venezuela) are conspecific with *An. marajoara* (which does not occur in Argentina and Uruguay).

There are two major problems with the revision of the *albitarsis* by Linthicum (1988). First, he lumped all "typical Brazilian" forms of *albitarsis* into one taxon, *An. marajoara,* disregarding published genetic evidences to the contrary. For example, Kitzmiller *et al.* (1974) and Kreutzer *et al.* (1976) documented the presence of two cytologically distinguishable sympatric sibling species (lack of inversion heterozygotes) in southern and eastern Brazil. They also showed that a third allopatric chromosomal form occurs in Colombia and Venezuela. Furthermore, Steiner *et al.* (1982) demonstrated the occurrence of at least three sibling forms in Brazil by enzyme electrophoresis. Two of these occurred sympatrically (based on lack of heterozygotes at numerous allozyme loci) in central Brazil. Since these authors did not describe morphological variations associated with the cryptic taxa, it is not possible to determine whether any of those taxa are conspecific with one or more of the previously described varieties and forms of *An. albitarsis* or with *An. marajoara* (described in detail by Linthicum, 1988) or with *A. deaneorum,* a new species in the *Albitarsis* complex (Rosa-Freitas, 1989).

The purpose of this paper is to present evidence (morphological, electrophoretic, and mtDNA restriction patterns) which invalidates the taxonomic revision of *albitarsis* by Linthicum (1988). We prove that the "typical Brazilian" forms of albitarsis are not conspecific with *An. marajoara.* On the contrary, the *An. albitarsis* complex in Brazil consists of at least two morphological distinguishable species, *An. deaneorum* and *An. marajoara.* The latter is further comprised of at least three allopatric forms, CM, IG, and MA.

MATERIALS AND METHODS

Sample Collection. Samples were collected from three geographical sites (Fig. 1). These included (1) Costa Marques (CM), Rondonia—collections made in both 1988 and 1989 contained *Anopheles marajoara* and *An. deaneorum;* (2) *An. marajoara* from Iguape (IG), Sao Paulo state, in 1989; and (3) *An. marajoara* from the Island of Marajo (MA), Para State, in 1989.

Fig. 1. Collection sites of the *Anopheles albitarsis* species complex in Brazil. IG, Iguape, state of Sao Paulo; CM, Costa Marques, state of Rondonia; MA, Island of Marajo.

During 1988, adult mosquitoes collected from CM were separated into *marajoara* and *deaneorum* based on wing spot differences, frozen in liquid nitrogen, hand carried to the U.S. Department of Agriculture Laboratory in Gainesville, and stored at −70°C until used for allozyme and DNA restriction fragment length polymorphism (RFLP) studies. During 1989, eggs were obtained from individual gravid females collected from CM, IG, and MA. Individual progeny were reared separately for morphological taxonomy. Voucher specimens were deposited at the Smithsonian Institution, Washington, D.C. The remainder of each progeny was frozen in liquid N_2 and transported to Gainesville for genetic analysis.

Allozyme Analysis. Starch gel electrophoresis was conducted according to Steiner and Joslyn (1979) with a few modifications (Narang *et al.*, 1989a,d). Two buffer systems were used to analyze 18 gene–enzyme systems (Table I) comprising 24 allozyme loci. A laboratory stock, Q_2 of *An. quadrimaculatus* species A (Lanzaro, 1986), homozygous for 24 loci, was used as a standard reference for identification of alleles in natural populations. The most common allele at each locus was given a mobility value (R_f)

Table I. List of Enzymes Analyzed and Buffer Systems Used for Starch Gel Electrophoresis of the *An. albitarsis* Species Complex[a]

Enzyme (E.C. No.)	Abbreviation	Buffer
Aconitase hydratase (EC 4.2.1.3)	ACON	TC-5.5
Adenylate kinase (EC 2.7.4.3)	ADK	TC-5.5
Aldehyde oxidase (EC 1.2.3.1)	AO	TC-5.5
Esterase (EC 3.1.1.1)	EST	TC-5.5, CA-8
Hexokinase (EC 2.7.1.1)	HK	TC-5.5
Phosphoglucose isomerase (EC 5.3.1.9)	PGI	TC-5.5
Phosphoglucomutase (EC 2.7.5.1)	PGM	TC-5.5
6-Phosphogluconate dehydrogenase (EC 1.1.1.44)	6PGD	TC-5.5
a-Glycerophosphate dehydrogenase (EC 1.1.1.8)	aGPDH	CA-8
Glucose oxidase	GO	CA-8
Hydroxy acid dehydrogenase (EC 1.1.99.6)	HADH	CA-8
Malate dehydrogenase (EC 1.1.1.37)	MDH	CA-8
Malic enzyme (EC 1.1.1.40)	ME	CA-8
Peptidase (EC 3.4.1.1)	PEP	CA-8
Glutamate oxaloacetate transaminase (EC 2.6.1.1)	GOT	CA-8
Isocitrate dehydrogenase (EC 1.1.1.42)	IDH	CA-8
Mannose-6-phosphate isomerase (EC 5.3.1.8)	MPI	CA-8
Xanthine dehydrogenase (EC 1.2.1.37)	XDH	CA-8

[a]TC-5.5: gel buffer—0.02 *M* Tris, 8 m*M* citric acid, pH 5.5; electrode buffer—0.16 *M* Tris, 0.07 *M* citric acid, pH 5.2. CA-8 (Steiner and Joslyn, 1979): gel buffer—0.074 *M* Tris, 9 m*M* citric acid, pH 8.45; electrode buffer—cathode tray, 0.34 *M* Tris, 0.08 *M* citric acid; anode tray, 0.28 *M* Tris, 0.063 *M* citric acid, pH 8.1.

of 100. The R_f values of other alleles of the corresponding locus were calculated relative to the reference band of R_f 100. In addition, the R_f of each allele was determined relative to the reference allele in the Q_2 stock.

Identification of sympatric sibling species (in collections from CM) was first accomplished by the "allelic cluster method" of Narang and Seawright (1988) as used by Narang *et al.* (1989b). Statistical analysis of genotypic data was performed using a Fortran IV computer program, BIOSYS-1 (Swofford and Selander, 1981). Various estimates of genetic variability and differentiations were calculated according to Narang *et al.* (1989c,d). These included (1) Hardy–Weinberg test, (2) genotypic fixation index "*F*," (3) linkage disequilibrium between loci by contingency chi-square analysis, (4) Wright's *F* statistics [three levels of genetic differentiation (F_{is}, F_{it}, F_{st}) to determine degree of genetic subdivision], (5) chi-square analysis to determine the significance of gene frequency differences among cryptic taxa, (6) chi-square to determine the significance of the F_{is} values, (7) diagnostic values of allozyme loci for identification of cryptic species, and (8) genetic similarity and distance among population pairs.

Mitochondrial DNA RFLP. The methods for isolation of total DNA from individual mosquitoes, digestion of DNA with restriction endonucle-

ases, gel electrophoresis, Southern blotting, hybridization, and autoradiography were the same as described earlier (Kim and Narang, 1990; Mitchell, 1990). The total DNA from individual mosquitoes was partitioned into eight equal aliquots. Each aliquot was digested with a different restriction enzyme. The mtDNA plasmid clones of *An. quadrimaculatus* species A (supplied by Dr. S. Mitchell, USDA-ARS, Gainesville, Florida) were used as probes for Southern blot hybridization. The mitochondrial similarity between each pair of taxa, based on the proportion of identical fragments (F), was calculated by the method of Nei and Li (1979).

RESULTS

Allozyme Variability, Diagnostic Loci, and Phenetic Relationships

Statistical analysis of genotypic frequency data revealed that whereas samples from IG and MA were in Hardy–Weinberg equilibrium, the CM samples (collected in 1988 and 1989) were not. There were significant heterozygote deficiencies at seven loci, *Idh-1, Had-1, Me-1, Pgi-1, Pep-1, Mpi-1,* and *Pgm-1* (Table II). This suggested the occurrence of at least two sympatric cryptic taxa. Therefore, the genotypic data from CM were partitioned into two data sets by the allelic cluster method of Narang and Seawright (1988). Statistical analysis showed that each data set was in Hardy–Weinberg equilibrium. Based on examination of wing spots from samples of corresponding progeny (stored at −70°C), all samples assigned to one set (by allelic cluster method, see underlined alleles in Table III) were

Table II. Deviations in Number of Observed Heterozygote Electromorphs from Those Expected Under Hardy–Weinberg Equilibrium at Some Loci in a Sample of "*albitarsis*" from Costa Marques[a]

Locus	Sample size[b]	No. heterozygotes		F	χ^2
		Obs.	Exp.		
Idh-1	86	2	50	0.96	+
Had-1	43	0	21	1.00	+
Me-1	108	3	53	0.94	+
Pgi-1	172	10	85	0.88	+
Pep-1	98	9	38	0.76	+
Mpi-1	81	8	42	0.80	+
Pgm-1	130	16	65	0.75	+

[a] +, significant deviation by chi-square test; F, fixation index.
[b] According to Linthicum (1988), the sample in this region should contain only one species, *An. marajoara.*

Table III. Allele Frequencies in Members of the *Anopheles albitarsis* Species Complex[a]

	Population			
		marajoara		
Locus	deaneorum (CM)	Form MA	Form CM	Form IG
Idh-1				
(*N*)	(73)	(32)	(47)	(76)
75	0.01	0.00	0.00	0.00
91	0.91	1.00	0.02	1.00
100	0.01	0.00	0.96*	0.00
105	0.02	0.00	0.00	0.00
113	0.05	0.00	0.02	0.00
Had-1				
(*N*)	(47)	(22)	(23)	(63)
71	0.03	0.93	0.00	0.85
100	0.04	0.02	1.00*	0.13
123	0.85	0.05	0.00	0.02
146	0.08	0.00	0.00	0.00
Mdh-1				
(*N*)	(50)	(32)	(41)	(54)
78	0.00	0.00	0.05	0.00
81	0.00	0.00	0.19	0.00
100	1.00	0.98	0.72	1.00
124	0.00	0.02	0.04	0.00
Me-1				
(*N*)	(79)	(32)	(66)	(58)
94	0.00	0.00	0.01	0.02
96	0.01	0.00	0.02	0.00
100	0.02	1.00	0.93	0.98
104	0.96	0.00	0.04	0.00
113	0.01	0.00	0.00	0.00
Pgi-1				
(*N*)	(109)	(32)	(104)	(76)
95	0.02	0.00	0.00	0.00
98	0.01	0.00	0.00	0.00
100	0.96	0.34	0.03	0.00
105	0.01	0.66	0.97	1.00
Pep-1				
(*N*)	(55)	(20)	(40)	(67)
82	0.92	0.00	0.00	0.00
90	0.02	0.00	0.03	0.02
96	0.02	1.00	0.12	0.98
110	0.02	0.00	0.85*	0.00
115	0.02	0.00	0.00	0.00
Pep-2				
(*N*)	(94)	(32)	(84)	(76)
57	0.00	0.00	0.01	0.00
80	0.00	0.00	0.01	0.00
100	1.00	0.94	0.98	1.00
128	0.00	0.06	0.00	0.00

Table III. (continued)

Locus	Population			
	deaneorum (CM)	marajoara		
		Form MA	Form CM	Form IG
Got-1				
(*N*)	(56)	(18)	(49)	(16)
100	0.99	1.00	0.94	1.00
107	0.01	0.00	0.06	0.00
Mpi-1				
(*N*)	(63)	(30)	(43)	(49)
80	0.05	0.00	0.01	0.00
90	0.94	0.07	0.09	0.00
94	0.00	0.00	0.05	0.72*
100	0.01	0.93	0.85	0.28
Mpi-3				
(*N*)	(47)	(25)	(25)	(76)
86	0.00	0.04	0.14	0.00
100	1.00	0.96	0.86	1.00
Xdh-1				
(*N*)	(33)	(16)	(22)	(41)
82	0.08	1.00	0.48	0.06
100	0.77	0.00	0.41	0.90
108	0.15	0.00	0.11	0.01
132	0.00	0.00	0.00	0.03
Pgm-1				
(*N*)	(90)	(30)	(72)	(57)
93	0.01	0.00	0.00	0.00
100	0.74	0.05	0.14	0.29
130	0.23	0.95	0.86	0.69
144	0.02	0.00	0.00	0.00
176	0.00	0.00	0.00	0.02
Acon-1				
(*N*)	(36)	(28)	(20)	(51)
94	0.03	0.00	0.00	0.00
100	0.97	1.00	0.90	0.99
105	0.00	0.00	0.10	0.01
6-Pgd				
(*N*)	(54)	(22)	(36)	(76)
86	0.00	0.07	0.23	0.00
100	0.96	0.93	0.68	1.00
106	0.00	0.00	0.06	0.00
110	0.04	0.00	0.03	0.00
Ao-1				
(*N*)	(76)	(19)	(71)	(50)
94	0.02	0.02	0.06	0.00
100	0.93	0.82	0.09	0.81
109	0.05	0.16	0.85	0.19
Est-1				
(*N*)	(28)	(36)	(13)	(22)
100	0.86	0.00	0.54*	0.00
106	0.14	1.00	0.38	1.00
109	0.00	0.00	0.08	0.00

Table III. (continued)

Locus	Population			
	deaneorum (CM)	marajoara		
		Form MA	Form CM	Form IG
Est-2				
(*N*)	(32)	(27)	(25)	(29)
91	0.14	0.28	0.26	0.02
100	0.84	0.66	0.66	0.93
108	0.02	0.06	0.06	0.05
115	0.00	0.00	0.02	0.00
Go-1				
(*N*)	(27)	(18)	(13)	(56)
100	1.00	1.00	0.92	1.00
117	0.00	0.00	0.08	0.00

[a]Alleles characteristic of *An. deaneorum* are underlined; those characteristic of a member within the *marajoara* group are marked with a superscript asterisk.

identified as *An. deaneorum* (described by Rosa-Freitas, 1989). The samples assigned to second set were morphologically similar to *An. marajoara* (Linthicum, 1988).

Of the 24 loci examined, 18 were polymorphic (Table III). The remaining six loci (*Adk-1, Got-2,* two *Hk* loci, *a-Gpdh-1, Mpi-2*) were monomorphic. *Anopheles deaneorum* differs from *An. marajoara* form CM by alleles at six loci and from each of form IG and form MA by alleles at five loci (alleles underlined in Table III). *An. deaneorum* can be distinguished from three forms of *An. marajoara* by diagnostic loci, *Had-1, Pgi-1, Pep-1, Mpi-1, Idh-1, Me-1,* and *Xdh-1* (Table IV). The most common allele of *Had-1* in *An. deaneorum* migrates about twice the distance compared to that of Q_2, whereas that of *An. marajoara* form CM moves about 1.5 times faster than that of Q_2. In form-IG, the *Had*-1 allele moves about halfway between the distances moved by alleles in form CM and Q_2 (Fig. 2). The most common allele of *Pgi-1* of *An. deaneorum* comigrates with that of Q_2, whereas alleles of three forms of *An. marajoara* move faster (Fig. 3, Table III). *Anopheles marajoara* form CM can be distinguished from form IG and form MA by diagnostic alleles, *Idh*-1 (100) and *Had*-1 (100). Although only one locus, *Xdh-1,* is diagnostic between form MA and form IG, the two showed significant differences in allele frequencies at four loci, *Est-2, Mpi-1, Pgi-1,* and *Pgm-1* (Table III). The *Pgi-1* locus is polymorphic in form MA but monomorphic (at the 0.95 level) in form IG and form CM (Table III). As expected, we observed a large number of heterozygotes for *Pgi-1* in MA samples (Fig. 3).

Table IV. A List of Diagnostic Allozyme Loci (Based on Data in Table III) in Members of the *An. albitarsis* Complex[a]

Diagnostic loci for					
dean (CM) . . mar (CM)	dean (CM) . . mar (MA)	dean (CM) . . mar (IG)	mar (MA) . . mar (CM)	mar (MA) . . mar (IG)	mar (CM) . . mar (IG)
Had-1	*Me-1*	*Me-1*	*Had-1*	*Xdh-1*	*Had-1*
Pgi-1	*Pep-1*	*Pgi-1*	*Pep-1*		*Pep-1*
Pep-1	*Mpi-1*	*Pep-1*	*Idh-1*		*Idh-1*
Mpi-1	*Xdh-1*	*Mpi-1*			
Idh-1					

[a]dean, *An. deaneorum*; mar, *An. marajoara*; CM, Costa Marques; IG, Iguape; MA, Island of Marajo.

Estimation of genetic variability (Table V) showed that *An. marajoara* form CM ranked highest in percentage of polymorphic loci (66.7%) and mean heterozygosity (0.22), followed by *An. deaneorum* form CM (37.5% and 0.11, respectively). *An. marajoara* form IG ranked lowest in genetic variability (less than half of the estimates for the form CM). Analysis of the standardized variance of allele frequencies (F_{st}) at certain loci (data not shown) indicated significant genetic substructuring in samples from Costa Marques. For example, between 83 and 85% of the allele frequency variance (F_{st} = 0.83 to 0.85) at *Idh-1, Me-1,* and *Pep-1* was due to genetic differences between *An. deaneorum* and *An. marajoara* form CM, leaving about 15–17%

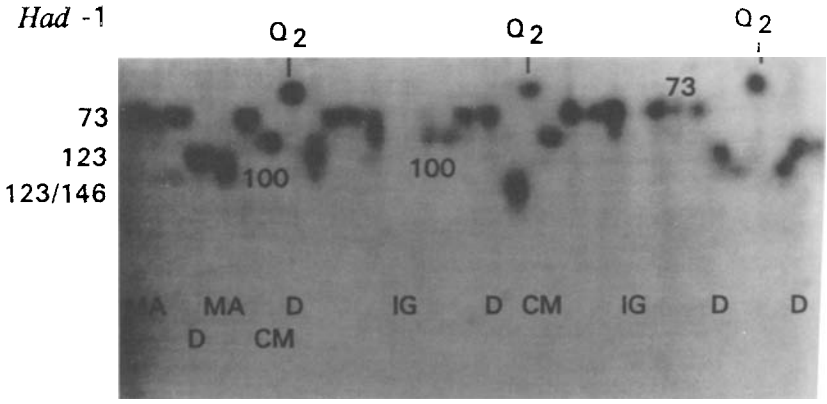

Fig. 2. Electromorph patterns of *Had-1* of the *An. albitarsis* species complex. CM, *An. marajoara* form CM; IG, *An. marajoara* form IG; MA, *An. marajoara* form MA; D, *An. deaneorum.* Q_2: a standard reference strain of *An. quadrimaculatus* species A.

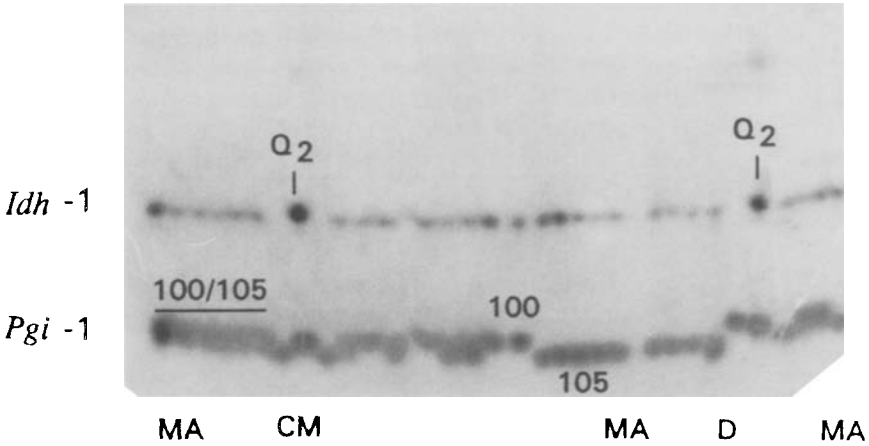

Fig. 3. Electromorph patterns of *Pgi-1* in the *An. albitarsis* species complex. CM, *An. marajoara* form CM; MA, *An. marajoara* form MA; D, *An. deaneorum*. Q_2: a standard reference strain of *An. quadrimaculatus* species A.

of the respective gene diversity within individual taxa. The F_{st} value was 0.75 for *Had-1,* ranged from 0.58 to 0.68 for *Est-1, Mpi-2, Mpi-1,* and *Pgi-1,* and was 0.34 for *Pgm-1. F* statistics on data from samples from Iguape and the island of Marajo showed a lack of substructuring.

As shown in Table VI, *An. deaneorum* and *An. marajoara* form CM are genetically most differentiated ($D = 0.41$, $I = 0.67$). Of the three cryptic forms of the *An. marajoara* species complex, form MA and form IG are the most closely related ($I = 0.93, D = 0.07$). *An. marajoara* form CM showed a similar level of genetic similarity with each of the other two forms (MA and IG).

Table V. Estimates of Genetic Variability Measures in Members of the *An. albitarsis* Complex in Brazil[a]

Species	Mean sample size per locus	Mean No. of alleles per locus	Percentage of loci polymorphic[b]	Mean heterozygosity
deaneorum (CM)	54.8 (4.8)	2.5 (0.3)	37.5	0.11 (0.02)
marajoara				
Form MA	23.6 (2.0)	1.5 (0.1)	33.3	0.08 (0.03)
Form CM	43.9 (4.8)	2.6 (0.2)	66.7	0.22 (0.04)
Form IG	53.1 (4.5)	1.6 (0.2)	25.0	0.08 (0.03)

[a]Standard error in parentheses. Collection sites: CM, Costa Marques; MA, Island of Marajo; IG, Iguape.

[b]A locus is considered polymorphic if the frequency of the most common allele does not exceed 0.95.

Table VI. Estimates of Unbiased Genetic Identity (Above Diagonal) and Genetic Distance (Below Diagonal) for Members of the *An. albitarsis* Complex in Brazil[a]

Species	*deaneorum* (CM)	*marajoara*		
		Form MA	Form CM	Form IG
deaneorum (CM)	—	0.734	0.666	0.762
marajoara				
Form MA	0.309	—	0.792	0.929
Form CM	0.406	0.233	—	0.783
Form IG	0.271	0.074	0.244	—

[a]Collection sites: CM, Costa Marques; MA, Island of Marajo; IG, Iguape.

Mitochondrial Restriction Patterns

Of the 16 patterns obtained by use of eight restriction endonucleases (*Ava*I, *Cla*I, *Eco*RI, *Hin*dIII, *Nsi*I, *Pst*I, *Pvu*II, and *Sca*I), none was diagnostic between *An. deaneorum* and the three cryptic forms of the *An. marajoara* species complex. Some patterns were characteristic of each taxon. Among *An. marajoara,* form MA was fixed for *Hin*dIII haplotype B (fragments in kb: 5.6, 3.2, 2.7, 1.6, 1.3, 0.6), and form IG and form CM (and also *An. deaneorum*) were fixed for haplotype A (8.8, 2.7, 1.9, 1.6). *Pst*I produced a two-band pattern (12.2 and 2.8) in form MA, one band (15 kb) in form IG, and a polymorphic pattern in *deaneorum* (no data on form CM). *Pvu*II can be used to distinguish three forms of *An. marajoara* (Fig. 4; fragments—8.7 and 6.3 in form IG, 6.5, 5.9, and 2.6 in form MA, and 6.7, 6.0, and 2.5 in form CM). *An. deaneorum* was polymorphic for the *Pvu*II pattern. Only one fragment of about 15 kb was observed in restriction digests of DNA by all eight restriction enzymes, in samples of form IG and form CM of *An. marajoara.* Overall, *An. deaneorum* and *An. marajoara* form MA were polymorphic (two or more fragments) for 63 and 12.5% of the restriction enzymes respectively. The mitochondrial similarity (F) values between *deaneorum* and the three forms of *marajoara* (MA, CM, and IG) were 0.35, 0.37, and 0.39, respectively. The F values between form MA and forms CM and IG were 0.65 and 0.57, respectively. The F value between form IG and form CM was 0.89.

DISCUSSION

During the last two decades, a number of morphologically almost indistinguishable but genetically distinct and reproductively isolated taxa (sibling species) have been identified in the genus *Anopheles* (Narang and Seawright, 1990). Most cryptic species in sympatry can be easily identified by one or

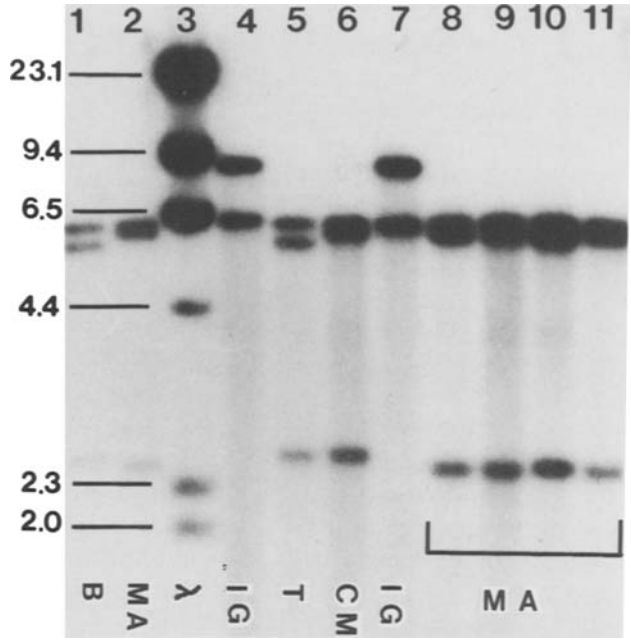

Fig. 4. *Pvu*II restriction patterns of mtDNA of the *An. albitarsis* species complex. CM, *An. marajoara* form CM; IG, *An. marajoara* form IG; MA, *An. marajoara* form MA. B, *An. benarrochi,* and T, *An. triannulatus,* common in collections from Costa Marques are also included for comparison. λ*Hin*dIII was used as a standard molecular size marker.

more techniques, including allozyme electrophoresis, DNA RFLP, and polytene chromosome banding patterns. Our data (diagnostic loci and wing spot morphology) showed that the samples from Costa Marques contained two sympatric cryptic species, *An. deaneorum* (recently identified by Rosa-Freitas, 1989) and *An. marajoara.* Furthermore, our *F* statistics (together with genetic similarity data) show genetic substructuring in *An. marajoara,* in Brazil. It consists of at least three allopatric cryptic forms: (a) form MA (b) form IG, and (c) form CM. Our conclusions are contrary to the claim made by Linthicum's (1988) conclusion that all "Brazilian forms" of *albitarsis* are conspecific with *An. marajoara.*

Though our isozyme data combined with morphological data (wing types) are highly suggestive of the above conclusion, overall, two alternative interpretations are possible. For example, three of the diagnostic loci used to distinguish *deaneorum* and *marajoara* form CM can also be used to distinguish the latter from form IG and form MA (albeit with different alleles). Therefore, the first alternative interpretation is that, instead of

grouping three forms of *marajoara* into a distinct group, separate from *deaneorum,* one could simply propose four electrophoretic phenotypes with varying levels of differences. The second alternative is to group the taxa on the basis of genetic similarities. From the data on genetic similarity in Table VI and on diagnostic loci in Table IV, it is clear that form MA is closely related to form IG (one-locus difference) compared to form CM (three-locus difference) and *deaneorum* (four-locus difference). Therefore, one could argue for the existence of three equivalent forms: two highland forms, *deaneorum* (CM) and *marajoara* form CM, and a third coastal form (IG and MA) (refer to Fig. 1 for locations of the collection sites). To verify the second alternative, one would need to analyze samples from additional coastal and highland locations. However, differences in wing morphology between *deaneorum* and the three forms of *marajora* (forms IG, CM, and MA have the same wing type) would suggest that our conclusion of grouping three forms of *marajoara* in a distinct group is the most logical one.

A review of genetic differentiation among sibling species of anophelines (Narang and Seawright, 1990) showed a wide range in degree of genetic differentiation (based on genetic distance, D, and genetic identity, I). The lowest level occurs in the *gambiae* complex ($D = 0.07$ and $I = 0.93$ between *arabiensis* and *melas;* $D = 0.09$ and $I = 0.92$ between *gambiae* and *arabiensis*). The genetic distances among closely related members in the *maculipennis* complex vary from 0.12 (*messeae:subalpinus*) to 0.25 (*atroparvus: labranchiae*). The genetic distances among sibling species of *An. quadrimaculatus* complex vary from 0.16–0.20 in species A:B, to 0.44–0.61 in species A:C, to 0.61 in species B:C (Narang *et al.,* 1989d). For the sake of comparison, the genetic differentiation among sibling species of *Drosophila* varies considerably but is far higher in most cases than in most anophelines examined so far [for example, $D = 0.49$–0.62 in the *D. willistoni* complex and $D = 0.08$–0.20 in the *D. bipectinata* complex; refer to the review by Narang and Seawright (1990)]. In view of the above, differentiation between *An. deaneorum* and *An. marajoara* form CM is substantial ($D = 0.41$). It is low between form MA and form IG ($D = 0.07$; see Table VI). Unless additional data on cryptic anopheline taxa are available, one cannot reliably infer the level of genetic differentiation among populations from D and I statistics alone.

A comprehensive elucidation of taxonomic status of previously described variants (chromosomal, electrophoretic, behavioral, ecological) would require comparative studies on (i) morphology, (ii) cross-mating relationships [*An. deaneorum* was colonized by Klein *et al.* (1990)], and (iii) polytene chromosomal banding patterns (of parent taxa and interspecies hybrids) and allozyme patterns. The two morphologically distinguishable species, *An. deaneorum* and *An. marajoara* form MA (from Island of Marajo), should be

used as reference standards. Similarly, allopatric forms of *marajoara* should be further characterized by the above techniques.

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