Isoenzimatic Analysis of Four *Anopheles* (*Kerteszia*) *cruzii* (Diptera: Culicidae) Populations of Brazil

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Anopheles cruzii *is a small sylvatic mosquito and primary human* Plasmodium *vector in Southern Brazil. The distribution of this bromeliad-breeding mosquito follows the Atlantic forest coastal distribution, where bromeliads are abundant. Morphological, genetic, and molecular polymorphisms among different populations have been reported and it has recently been suggested that* An. cruzii *is a complex of cryptic species. The aim of this work is to analyze the gene flow between different populations of* An. cruzii *collected in four localities within the geographic distribution range of the species, and to examine if* An. cruzii *is a complex of cryptic species. The genetic distances show that populations of the states of Santa Catarina, São Paulo, and Rio de Janeiro are genetically closer (0.032 to 0.083) than populations of Bahia (0.364 to 0.853) based on profiles from 10 distinct isoenzyme loci. The* F_{st} *was lower (0.077) when the Bahia population was excluded than when it was included (0.300) in the analyses. The inferred number of migrants per generation was 2.99 individuals among populations from the states of Santa Catarina, São Paulo, and Rio de Janeiro and 0.58 migrants per generation among all populations. Results suggest that* An. cruzii *is a complex of species and that the specimes of state of Bahia can be considered as belonging to a species that is distinct from other three closely-related populations studied.*

Key words: isoenzyme - *Anopheles cruzii* - *Kerteszia* - cryptic species

Malaria vectors in Brazil belong to the *Nyssorhynchus* and *Kerteszia* subgenus of genus *Anopheles* (Deane 1986, Consoli & Lourenço de Oliveira 1994). Members of *Kerteszia* are typically small tropical mosquitoes distributed from Southern Mexico to Southern Brazil where immatures develop within bromeliads.

Anopheles (Kerteszia) cruzii Dyar and Knab is a primary vector of human *Plasmodium* which is endemic in Southern and Southeastern Brazil (Rachou 1958, Aragão 1964). Currently *An. cruzii* is involved in the maintenance of several species of human and simian malaria that occurs in the valleys of the Atlantic Coastal Rain Forest in both states Rio de Janeiro and São Paulo (Carvalho et al. 1988, Azevedo 1997, Branquinho et al. 1997). In addition, this species is a vector of the simian malarias in Brazil (Deane 1986).

Morphological differences were observed among populations of *An. cruzii* from Rio de Janeiro and Santa Catarina (Zavortink 1973). Ramirez and Dessen (1994) and Ramirez et al. (1994) described a high genetic polymorphism based on polytene chromosome banding pattern of *An. cruzii* from southern state of São Paulo. In comparison to other species of the same subgenus, the authors relate this phenomenon to the plasticity in selection of breeding places, especially in terms of the size and shade.

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Malafronte et al. (1997) observed a high polymorphism in restriction profiles of the ribosomal DNA (ITS2) among *An. cruzii* populations collected in five distinct areas of state of São Paulo.

Ramirez and Dessen (2000a), studying the polytene chromosomal banding pattern of *An. cruzii* from São Paulo and Santa Catarina, observed the existence of two distinct chromosomic forms and the absence of heterozygotes, which can indicate that *An. cruzii* is a complex of cryptic species. Ramirez and Dessen (2000b) found a third chromosomic form in two areas of São Paulo and concluded that the proposed complex was formed by at least three cryptic species.

The aim of this work is to analyze the gene flow between different populations of *An. cruzii* collected in four localities within the geographic distribution range of the species, and to examine if *An. cruzii* is a complex of cryptic species.

MATERIALS AND METHODS

Mosquito sampling - All mosquitoes included in this study were captured at forest borders of the states of Santa Catarina, São Paulo, Rio de Janeiro, and Bahia (Fig. 1).

All captured females were killed by freezing, morphologically identified using the Consoli and Lourenço de Oliveira's key (1994) and then stored in liquid nitrogen for isoenzymatic analysis.

Isoenzyme electrophoresis was performed on agarose gels as described by Hjèten (1961) using modifications proposed by Salles et al. (1986). Briefly, females were individually processed in 40 µl lysis buffer (0.05 mM Tris-Hcl pH = 8.0, 0.01 mM EDTA, 0.01 mM ε-amino n-caproic acid and 1% Triton-100X) in 96 well plates in an ice bath.

Sixteen enzyme systems were analyzed by agarose gel

Fig. 1: source of specimens of *Anopheles cruzii* used in isoenzyme analyses.

electrophoresis using 2 µl of the lysate for approximately 2 h at 4ºC in appropriate buffers. Detection of each enzyme was carried out by overlaying the electrophoresis gel with a solution of 10% agarose supplemented with specific substrates, co-enzymes and co-factors (for more details see Rosa-Freitas et al. 1992). After development in darkness for 20-60 min at room temperature, the enzymatic reactions were stopped by adding of 5% acetic acid. The gels were dried at room temperature and the analysis genotypic frequencies performed using the BYOSIS software (Swofford & Selander 1981).

RESULTS

Among the of 16 enzymatic systems tested, 10 revealed clear banding patterns [phosfoglucomutase – EC 5.4.2.2 (PGM), hexokinase – E.C. 2.7.1.1 (HK), fumarate hydratase EC 4.2.1.2 (FUM), malic enzyme EC 1.1.1.40 (ME), malate dehydrogenase – E.C. 1.1.1.37 (MDH, glucose-6-phosphate isomerase- EC 5.3.1.9 (GPI), Isocitrate dehydrogenase - EC 1.1.1.42 (IDH), leucyl aminopeptidase - EC 3.4.11.1 (PEP-1), tripeptide aminopeptidase – EC 3.4.11.4 (PEP-2), membrane alanyl aminopeptidase - EC 3.4.11.2 (PEP-3)], with ME showing two distinct loci. Allelic frequencies of all *An. cruzii* loci analyzed are listed in Table I. A monomorphic pattern was observed for HK and FUM enzymes in all samples, whereas GPI and PEP-3 were monomorphic for Bahia and Santa Catarina populations, respectively (Table I).

The mean number of alleles per locus ranged from 2.5 to 3.0, and the percentage of polymorphic loci ranged from 72.7% for the Santa Catarina and Bahia populations to 81.8% for the São Paulo and Rio de Janeiro populations. The mean heterozygosity among the studied samples ranged from 0.211 to 0.386 (Table II).

The genetic distance estimed according to Nei (1978) shows that populations from the states of Santa Catarina, São Paulo, and Rio de Janeiro are genetically closer to each other (0.032 to 0.083) than to the population from Bahia (0.364 to 0.853). Genetic identities varied from 0.921 to 0.968 among populations of the states of Santa Catarina, São Paulo, and Rio de Janeiro and from 0.426 to 0.530 between these populations and the samples from Bahia (Table III, Fig. 2).

Considering that the Bahia population exhibited a higher genetic distance from other three populations analyzed, the F-statistics (Fst), was estimated the population from Bahia both included and excluded. As a result, when the population from Bahia was excluded from analysis, the F_{st} was lower (0.077) than when it was included (0.300) (Table IV). The inferred migrants per generation were 2.99 individuals among populations of the states of Santa Catarina, São Paulo, and Rio de Janeiro and 0.58 migrants per generation among all populations.

DISCUSSION

Zavortink (1973) suggested that *An. cruzii* could represent more than a single species. After a long gap lasting up until only a few years ago, Ramirez and Dessen (2000a), studying the polytene chromosomes banding pattern of distinct populations of *An. cruzii* from Brazil, showed that the population studied have high genetic diversity and thus *An. cruzii* may represent a complex of cryptic species. The results of the present analysis are in agreement with both Zavortink (1973) and Ramirez and Dessen (2000a) hypotheses. Additionally, the present results also show that the population of *An. cruzii* from Bahia has a distinct isoenzymatic profile in comparison to the profiles of the other populations included in this study.

As shown in Table III, *An. cruzii* samples from Southern and Southeastern Brazil (Santa Catarina, São Paulo, and Rio de Janeiro) show genetic distances and identities, which vary from 0.032 to 0.083 and from 0.921 to 0.968, respectively. Nevertheless, the Bahia population is shown to be quite distant from the others, showing values for genetic distance and identity of 0.364 to 0.853 and from 0.426 to 0.530, respectively. In conclusion, isoenzyme analysis revealed that *An. cruzii* populations of Santa Catarina, São Paulo, and Rio de Janeiro are closely related to each other and are genetically distinct from population of the Bahia.

According to Bullini (1982), genetic distance values greater than 0.2 are observed in individuals belonging to cryptic species complexes of mosquitoes. The critical value for genetic distance in order to distinguish between species is 0.35 (Thorpe & Solé-Cava 1994). Considering these values, our results suggests that (i) *An. cruzii* is a complex of species, (ii) specimens of the Bahia may be a distinct species, which is not *An. cruzii*, and (iii) *An. cruzii* populations of Santa Catarina, São Paulo, and Rio de Janeiro are closely related.

Excluding population of Bahia from the analysis, the results of F statistics, i. e. F_{is} and F_{it}, did not show significative alterations. However, the F_{st} value, which indicates

TABLE I

Allelic frequencies of *Anopheles cruzii* populations Santa Catarina (SC), São Paulo (SP), Rio de Janeiro (RJ), Bahia (BA)

	Populations			
Locus	SC	SP	RJ	BA
PGM				
n	30	30	30	30
A	0.033	0.000	0.017	0.000
B	0.033	0.000	0.000	0.000
C	0.833	0.583	0.600	0.000
D	0.100	0.317	0.300	0.017
E	0.000	0.100	0.083	0.000
F	0.000	0.000	0.000	0.917
G	0.000	0.000	0.000	0.067
HK				
n	30	30	30	30
A	1.000	1.000	1.000	1.000
FUM				
n	30	30	30	30
A	1.000	1.000	1.000	1.000
ME-1				
n	30	30	31	30
A	0.017	0.000	0.032	0.667
B	0.017	0.083	0.339	0.333
C	0.967	0.917	0.629	0.000
ME-2				
n	30	30	30	30
A	0.900	0.467	0.550	0.000
B	0.100	0.300	0.450	0.000
C	0.000	0.233	0.000	0.100
D	0.000	0.000	0.000	0.900
MDH				
n	30	30	30	30
A	0.000	0.167	0.083	0.000
B	0.600	0.350	0.450	0.133
C	0.217	0.383	0.333	0.700
D	0.183	0.100	0.133	0.167
GPI				
n	30	24	26	30
A	0.067	0.146	0.058	1.000
B	0.917	0.708	0.846	0.000
C	0.017	0.146	0.096	0.000
IDH				
n	30	30	30	30
A	0.000	0.000	0.167	0.933
B	0.033	0.283	0.283	0.067
C	0.917	0.667	0.550	0.000
D	0.050	0.050	0.000	0.000
PEP-1				
n	29	32	31	29
A	0.0534	0.359	0.597	0.328
B	0.190	0.156	0.000	0.362
C	0.207	0.266	0.161	0.034
D	0.069	0.188	0.210	0.086
E	0.000	0.031	0.032	0.190
PEP-2				
n	30	30	31	32
A	0.517	0.217	0.000	0.250
B	0.350	0.483	0.419	0.047
C	0.117	0.283	0.290	0.188
D	0.017	0.017	0.290	0.469
E	0.000	0.000	0.000	0.047
PEP-3				
n	30	30	30	30
A	0.000	0.033	0.000	0.000
B	0.000	0.050	0.000	0.000
C	1.000	0.717	0.750	0.633
D	0.000	0.200	0.250	0.367

n: individuals

TABLE II

Means and standard errors of samples per locus and alleles per locus, percentage of polymorphic locus, and mean heterozygozity and standard errors for four *Anopheles cruzii* populations from Santa Catarina (SC), São Paulo (SP), Rio de Janeiro (RJ), Bahia (BA)

Population	Mean sample size/locus	Mean number of alleles/locus	Percentage of polymorphic locus	Mean heterozygosity	
				Ho	He
SC	29.9 ± 0.1	2.6 ± 0.4	72.7	0.211 ± 0.062	0.244 ± 0.076
SP	29.6 ± 0.6	3.0 ± 0.4	81.8	0.353 ± 0.067	0.442 ± 0.082
RJ	29.9 ± 0.4	2.7 ± 0.3	81.8	0.386 ± 0.070	0.430 ± 0.73
BA	30.1 ± 0.2	2.5 ± 0.4	72.7	0.251 ± 0.068	0.298 ± 0.083

Ho: observed heterozygosity; He: expected heterozygosity

TABLE III

Matrix of genetic identities (above diagonal) and distances (below diagonal) (Nei 1978) among four *Anopheles cruzii* populations

Population	SC	SP	RJ	BA
SC	-	0.948	0.921	0.426
SP	0.053	-	0.968	0.498
RJ	0.083	0.032	-	0.530
BA	0.853	0.698	0.364	-

SC: Santa Catarina; SP: São Paulo; RJ: Rio de Janeiro; BA: Bahia

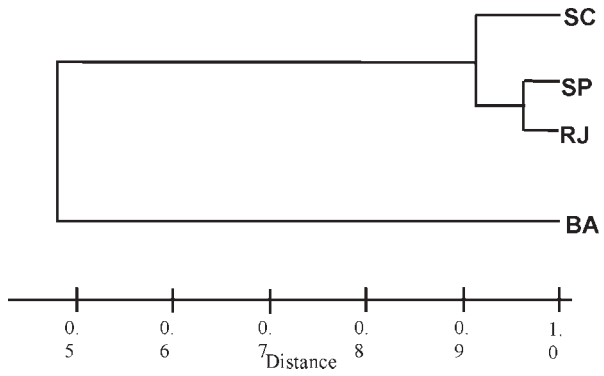

Fig. 2: cladogram of genetic distances between *Anopheles cruzii* populations. SC: Santa Catarina; SP: São Paulo; RJ: Rio de Janeiro; BA: Bahia

TABLE IV

Wright fixation indexes (F_{is}, F_{it}, and F_{st}) for *Anopheles cruzii* populations with and without Bahia population

Locus	F_{is} [a]	F_{it}	F_{st}
PGM	0.199 / 0.230	0.531 / 0.270	0.414 / 0.051
ME-1	0.171 / 0.122	0.555 / 0.253	0.464 / 0.150
ME-2	0.129 / 0.162	0.513 / 0.275	0.441 / 0.134
MDH	0.019 / 0.010	0.118 / 0.022	0.101 / 0.032
GPI	0.153 / 0.153	0.636 / 0.186	0.570 / 0.039
IDH	0.131 / 0.152	0.523 / 0.235	0.451 / 0.098
PEP-1	0.128 / 0.161	0.184 / 0.190	0.065 / 0.034
PEP-2	0.230 / 0.218	0.337 / 0.297	0.140 / 0.101
PEP-3	0.090 / 0.019	0.184 / 0.077	0.103 / 0.095
Média	0.137 / 0.135	0.396 / 0.201	0.300 / 0.077

a: with Bahia population/without Bahia population

the degree of genetic structure, was high (0.300) when the Bahia population was included in the analysis. On the other hand, when this population was excluded, the F_{st} value was relatively low (0.077). When the Bahia population was not taken into consideration, the number of migrating individuals per generation (2.99) shows the occurrence of genetic flow among populations of Santa Catarina, São Paulo, and Rio de Janeiro, despite the current fragmentation of the Brazilian Atlantic Rain Forest. Including *An. cruzii* population from Bahia in the analyze, the F_{st} value was high and almost no migrating individuals were observed, which indicates that there are no genetic exchanges between populations of *An. cruzii* from the Southern and Southeastern regions of Brazil and the Bahia population, located in the Northeastern region of the country. We did not find distinct isoenzymatic patterns in the samples from the states of São Paulo and Santa Catarina, which could be indicative of the presence of distinct populations of *An. cruzii* in the studied localities, as suggested by Ramirez and Dessen (2000a, b).

The results of the analysis are in agreement with and reinforce the hypothesis that *An. cruzii* is a complex of cryptic species, suggesting that the *An. cruzii* population of Bahia constitute an isolated population or distinct species.

We cannot infer if this new species is able to transmit malaria but, interestingly, *An. cruzii* never was considered a vector in Bahia. In the past, when malaria was endemic in the region, other anophelines of *Nyssorynchus* subgenus were considered responsible for transmission.

Studies are continued to verify if there are morphological differences between various isoenzymatic populations of *An. cruzii*.

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