Isoenzymatic Analysis of Four *Anopheles (Kerteszia) bellator* Dyar & Knab (Diptera: Culicidae) Populations

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Anopheles bellator *is a small silvatic bromelia-breeding mosquito and is a primary human malaria vector species in Southern Brazil. The bromelia-breeding habitat of the species should accompany the Atlantic forest coastal distribution, where bromeliads are abundant. Nonetheless, records on* An. bellator *collections show a gap in the species geographical distribution.* An. bellator *has been recorded in Southern Brazil and in the Brazilian states of Bahia and Paraíba. It appers again in the island of Trinidad, in Trinidad and Tobago. The aim of this work was to measure gene flow between different populations of* An. bellator *collected in the northern and southern extremes of the geographic distribution of this species. Mosquitoes were captured in forest borders in Santa Catarina, São Paulo, and Bahia states in Brazil and in the island of Trinidad in Republic of Trinidad and Tobago. Genetic distances varied between 0.076 and 0.680, based on enzymatic profiles from 11 distinct isoenzymes. Results indicate the existence of low-level gene flow between Brazilian populations of* An. bellator, *and a gene flow was even lower between the Brazilian and the Trinidad populations. This finding lead us to hypothesize that* An. bellator *did not spread along the coast, but reached northeastern areas through inland routes.*

Key words: isoenzyme - *Anopheles bellator* - *Kerteszia*

Malaria vectors in Brazil belong to the subgenera *Nyssorhynchus* and *Kerteszia* of the genus *Anopheles*. Subgenus *Kerteszia* has 12 described species which are typically small in size and closely associated with forests, distributed from Southern Mexico to Southern Brazil. With the exception of *An. bambusicolus* Komp, which is found in bamboo internodes, *Kerteszia* immatures develop in bromeliads (Zavortink 1973, Deane 1986, Consoli & Lourenço-de-Oliveira 1994). Four species are known malaria vectors: *An. neivai* Howard, Dyar & Knab, *An. cruzii* Dyar & Knab, *An. homunculus* Komp, and *An. bellator* Dyar & Knab (Zavortink 1973). The geographic distribution of one of these species, *An. bellator*, a primary human malaria vector species in Southern Brazil (Rachou 1958, Forattini 1962, Coutinho & Rachou 1966) is intriguing. Zavortink (1973) described its occurrence on the Atlantic coast of South America, from Eastern Venezuela to Southern Brazil. However, no records of *An. bellator* are reported for the Brazilian Northeastern coastal states of Pará, Piauí, Maranhão, and Ceará. The northernmost report of *An. bellator* on the Brazilian coast is the state of Paraíba (Aragão 1964). The original description of *An. bellator* is based on mosquitoes collected on the island of Trinidad, Republic of Trinidad and Tobago. Thus, the geographic distribution of this species is apparently discontinuous.

Interruption of gene exchange between populations can be attributed to several factors including discontinuity of geographic distribution, which may act as a starting point for genetic differentiation, race production, and speciation (Mettler & Gregg 1969).

The aim of this work was to measure gene flow between different populations of *An. bellator* collected in the northern and southern extremes of the geographic distribution of this species.

MATERIALS AND METHODS

Mosquito sampling - All insects included in this study were captured in the Atlantic forest borders of the states of Santa Catarina, São Paulo, and Bahia, Brazil, and in the island of Trinidad in Republic of Trinidad and Tobago (Figure).

Recently captured females were identified using Consoli and Lourenço-de-Oliveira key (1994) and im-mediately stored in liquid nitrogen for isoenzyme analysis.

Isoenzyme electrophoresis was performed in agarose gels as previously described (Hjèrten 1961) with modifications proposed by Salles et al. (1986). Briefly, females were individually crushed in 40 µl of 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 ice bath.

A total of sixteen enzyme systems was analyzed by agarose gel electrophoresis using 2 µl of the lysate for approximately 2 h at 4°C in appropriate buffers. Detection of each enzyme was carried out 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 for 20-60 min at room temperature, in darkness, the enzymatic reactions were stopped by addition of 5% acetic acid. Gels were then dried at room temperature and analysis of genotypic frequencies was performed using BYOSIS software (Swofford & Selander 1981).

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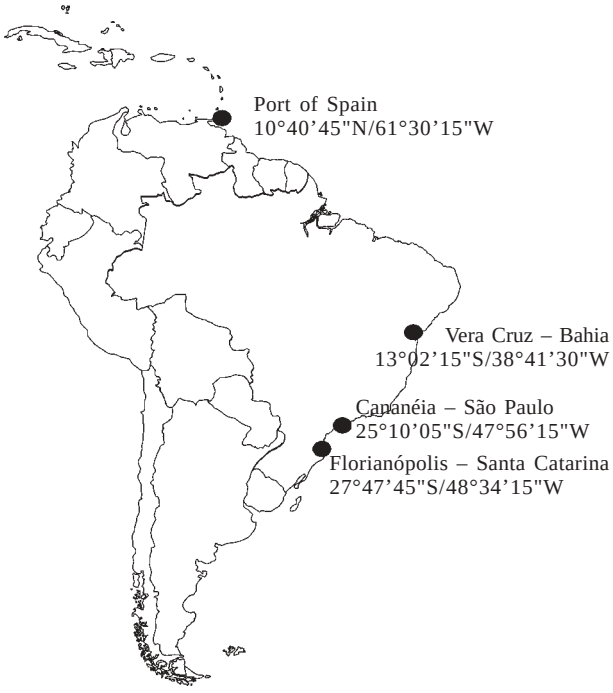

Source of specimens of *Anopheles bellator* used in isoezyme analyses.

RESULTS

From a total of 16 enzymatic systems tested, 11 revealed clear banding patterns [phosfoglucomutase - EC 5.4.2.2 (PGM), hexokinase - E.C. 2.7.1.1 (HK), mannose-6-phosphate isomerase EC 5.3.1.8 (MPI), malic enzyme EC 1.1.1.40 (ME), malate dehydrogenase - E.C. 1.1.1.37 (MDH), esterase - EC 3.1.1.1 (EST), 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, thus allowing the distinction of a total of 12 loci. Allelic frequencies of all *An. bellator* loci analyzed are presented in Table I. A monomorphic pattern was observed for HK, ME-2, and GPI enzymes in all samples. MPI was monomorphic for Bahia and Trinidad populations, and IDH for Santa Catarina, São Paulo, and Trinidad (Table I).

The mean number of alleles per locus ranged from 2.1 to 2.6 and the percentage of polymorphic loci ranged from 58.3 in Trinidad to 66.7% for Santa Catarina, São Paulo, and Bahia populations. The mean heterozygosity among the studied samples ranged from 0.210 to 0.319 (Table II).

The genetic distance calculated according to Nei (1978) varied between 0.076 and and 0.680. Genetic identities varied from 0.386 to 0.927 (Table III).

The values of Fis and Fit were 0.090 and 0.330, respectively. The F_{ST} value was 0.263 (Table IV) and the inferred migrants per generation were 0.70 between all *An. bellator* populations studied. By excluding the Trinidad population from the analysis, the Fst value was reduced to 0.151 and the number of migrants per generation increased to 1.4.

TABLE I

Allelic frequencies of *Anopheles bellator* populations Santa Catarina (SC), São Paulo (SP), Bahia (BA), Trinidad (TR), n = individuals

	Populations			
Locus	SC	SP	BA	TR
PGM				
n	30	30	30	31
A	0.000	0.000	0.033	0.016
B	0.900	0.617	0.717	0.774
C	0.100	0.367	0.217	0.161
D	0.000	0.000	0.033	0.048
E	0.000	0.017	0.000	0.000
HK				
n	30	30	30	30
A	1.000	1.000	1.000	1.000
MPI				
n	36	30	30	30
A	0.000	0.000	0.000	1.000
B	0.375	0.483	1.000	0.000
C	0.625	0.517	0.000	0.000
ME-1				
n	30	31	30	30
A	0.000	0.161	0.167	0.983
B	0.200	0.258	0.600	0.017
C	0.800	0.484	0.233	0.000
D	0.000	0.097	0.000	0.000
ME-2				
n	30	30	30	30
A	1.000	1.000	1.000	1.000
MDH				
n	24	30	29	30
A	0.104	0.000	0.034	0.367
B	0.250	0.333	0.345	0.317
C	0.042	0.000	0.466	0.000
D	0.604	0.667	0.103	0.317
E	0.000	0.000	0.052	0.000
EST				
n	30	29	30	30
A	0.067	0.259	0.000	0.050
B	0.617	0.138	0.167	0.000
C	0.317	0.500	0.350	0.950
D	0.000	0.103	0.483	0.000
GPI				
n	30	30	30	30
A	1.000	1.000	1.000	1.000
IDH				
n	30	30	30	30
A	0.000	0.000	0.067	0.000
B	1.000	1.000	0.750	1.000
C	0.000	0.000	0.183	0.000
PEP-1				
n	30	30	30	30
A	0.333	0.050	0.000	0.100
B	0.000	0.617	0.333	0.000
C	0.567	0.300	0.667	0.450
D	0.100	0.033	0.000	0.450
PEP-2				
n	32	30	30	32
A	0.000	0.017	0.050	0.109
B	0.531	0.750	0.683	0.516
C	0.469	0.233	0.017	0.313
D	0.000	0.033	0.250	0.063
PEP-3				
n	29	32	24	30
A	0.328	0.281	0.208	0.267
B	0.586	0.500	0.771	0.067
C	0.086	0.219	0.021	0.667

TABLE II

Mean of sample per locus, mean of alleles per locus, percentage of polymorphic loci, and mean heterozygozity for four *Anopheles bellator* populations Santa Catarina (SC), São Paulo (SP), Bahia (BA), Trinidad (TR)

Population	Mean sample size/locus	Mean number of alleles/locus	Percentage of polymorphic locus	Mean heterozygosity	
				Ho	He
SC	30.1 ± 0. 8	2.1 ± 0.3	66.7	0.274 ± 0.066	0.309 ± 0.073
SP	30.2 ± 0.2	2.4 ± 0.4	66.7	0.319 ± 0.078	0.362 ± 0.081
BA	29.4 ± 0.5	2.6 ± 0.4	66.7	0.312 ± 0.074	0.334 ± 0.75
TR	30.3 ± 0.2	2.2 ± 0.3	58.3	0.210 ± 0.072	0.241 ± 0.083

TABLE III

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

Population	SC	SP	BA	TR
SC	-	0,927	0.829	0.721
SP	0.076	-	0.876	0.764
BA	0.187	0.132	-	0.680
TR	0.327	0.269	0.386	-

SC: Santa CAtarina; SP: São Paulo; BA: Bahia; TR: Trinidad

TABLE IV

Wright fixation indicies (Fis, Fit, and Fst) for four *Anopheles bellator* populations

Locus	F_{IS}	F_{IT}	F_{ST}
Phosfoglucomutase	-0.100	-0.041	0.054
Mannose-6-phosphate isomerase	0.248	0.716	0.622
Malic enzyme-1	-0.002	0.417	0.418
Malate dehydrogenase	0.150	0.288	0.162
Esterase	0.066	0.311	0.262
Isocitrate dehydrogenase	0.082	0.228	0.159
Leucyl aminopeptidase-1	0.180	0.345	0.202
Leucyl aminopeptidase-2	0.057	0.142	0.090
Leucyl aminopeptidase-3	0.119	0.303	0.208
Mean	0.090	0.330	0.263

DISCUSSION

Interest in better morphological characterization and in intrapopulational comparisons of Neotropical anopheline species has increased during the last few years, mostly focusing on characterization of cryptic species complexes (Conn 1993, Linley et al. 1996, Lounibos et al. 1998, Rosa-Freitas et al. 1998, Manguin et al. 1999, Calle et al. 2002, Sallum et al. 2002, Santos et al. 2003). Most of these studies concern subgenus *Nyssorhynchus* species due to their major role in malaria transmission in South and Central America. Insufficient data are available concerning species of the subgenus *Kerteszia*, probably due to the difficulty of maintaining these species under laboratory conditions and for many areas, the low number of captured mosquitoes.

According to Thorpe and Solé-Cava (1994), genetic distances greater than 0.35 are observed for individuals belonging to cryptic species complexes. Despite the genetic distance observed between Bahia and Trinidad populations, genetic distances under 0.35 were observed between samples from Trinidad and the southernmost extreme of *An. bellator* distribution (São Paulo and Santa Catarina).

Fst values observed for 4 populations of *An. bellator* were relatively high. Fst values higher than 0.05 indicate the existence of a moderate genetic structured population (Wright 1978). A low number of migrants per generation (0.7) reveal the existence of different genetic structured *An. bellator* populations. When only the Brazilian populations are analyzed there is a slightly increase in the number of migrants per generation (1.4). These results indicate the existence of low-level gene flow between Brazilian populations of *An. bellator*, and an even lower gene flow between those and the Trinidad population. Despite these findings, we cannot explain the apparent interruption in the geographical distribution of *An. bellator* in Northern South America, where with exception of Bahia, Paraíba, and Trinidad the species has never been collected. According to Aragão (1964), *An. bellator* originated in the Atlantic coastal formations of the Guyanas, from were it has spread throughout the continent. Considering that there is no report of *An. bellator* along hundreds of kilometers in the Northern Brazilian coast, we may infer that this species possibly did not spread along the coast, but reached Northeastern Brazil through inland routes. Thus, the greater genetic distance observed among Bahia and Trinidad populations can be explained if we consider these areas the extremes of *An. bellator* geographic distribution. Moreover, we must consider the possibility of a recent geographical split of these populations, not allowing enough time for speciation.

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