Population Genetics of *Ochlerotatus eatoni* (Diptera: Culicidae) Endemic Species to Two Macaronesian Islands

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ABSTRACT Analyses of 11 isoenzyme loci of *Ochlerotatus eatoni* (Edwards, 1916), endemic to two Macaronesian Islands (Madeira and Tenerife, Canary Islands), revealed substantial genetic structure in the study populations. Samples from sites on the south and north of Madeira displayed a significant reduction of variability compared with those from central Madeira and Tenerife. The Tenerife population exhibited a severe deficit of heterozygosity with similar magnitude across all the loci examined. The complex pattern of variation in *Oc. eatoni* is because of interplay of breeding structure, genetic drift, and geographical and historical factors. From these findings, we concluded that island colonization by *Oc. eatoni* was not marked by founder effect.

KEY WORDS *Ochlerotatus eatoni*, endemism, population structure, Macaronesian Islands

Ochlerotatus eatoni (Edwards, 1916) (Diptera: Culicidae) was until recently classified as *Aedes eatoni* (Reinert 2000, Schaffner et al. 2001). Some members of the *Aedes* genus [such as *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse)] have been intensively studied because of their efficiency in transmitting arbovirus diseases such as dengue and yellow fever (Powell et al. 1980, Tabachinck and Wallis 1985, Apostol et al. 1996, Paupy et al. 2004). These successful colonizers have spread worldwide from their native origin in tropical and subtropical areas (Smith 1956, Forattini 1986, Hawley 1988, Rai 1991), and their biology, ecology, population genetics, and evolution are well known. Recently, members of the subgenus *Stegomyia* also have been studied (Taafe Gaunt et al. 2004). In contrast, species of the genus *Ochlerotatus* are relatively poorly studied (Cook et al. 2005), and intraspecific population studies are particularly rare (Rey et al. 2001). Endemic to islands from two Macaronesian archipelagos (Madeira and the Canary Islands), *Oc. eatoni* has a very limited geographical distribution. First described by Edwards (1916) in Monte (Madeira) and then by Clavero (1947) in Monte de las Mercedes (Tenerife, Canary), it was later found in other localities of the two islands (Capela 1981, Baez 1987). *Oc. eatoni* normally occurs in rural, urban, and forested areas and is only occasionally found inside houses. *Oc. eatoni* has a catholic choice of breeding sites, including both natural sites (tree holes and rock pools) and artificial sites (tires and plastic or metal bottles) (Y.C., personal communication). The host it feeds upon is unknown. However, *Oc. eatoni* does not show anthropophilic behavior and probably uses other vertebrates as blood sources (Y. Gonçalves).

The evolutionary biology of *Oc. eatoni* is of special interest because it represents two fundamental events: speciation and colonization within two islands. This study outlines a first attempt to shed light on evolutionary history of *Oc. eatoni*, by analyzing isoenzyme variations in five populations from Madeira and one population from Tenerife (Canary Islands).

Materials and Methods

Sampling Areas. *Oc. eatoni* populations were sampled from Madeira and Tenerife islands. The size of the islands is 740 and 2,034 km^2, respectively.

Samples and Electrophoresis. Adult mosquitoes were collected from five localities in Madeira: Babosa (Bab) and Campanário (Cam) in the south, Queimadas (Que) and Chão do Ribeira (ChR) in the north, and Chão dos Louros (ChL) in the center (Fig. 1). Adults also were collected from one locality, Monte de las Mercedes, in Tenerife. Individuals were identified and kept at −80°C until analysis. Populations were screened for their isoenzyme polymorphism, and 11 enzymes loci were tested: phosphogluconate dehydrogenas (Phi), adenylate kinase (Ak), phosphoglucoisomerase (6Pdh), glutamate oxaloacetate transaminases (Got), phosphoglucomutase (Pgm), α-glycerophosphate dehydrogenase (α-Gpd), isocitrate dehydrogenase (Idh), malic dehydrogenase-NADP (Me), malic dehydrogenase-NAD (Mdh), esterase (Est), and peptidase (Pep). Starch gel electrophoresis was carried out according to Loukas and Krimbas (1980). Ak, Got, Idh, Est, and Pept were screened in Tris-boric-EDTA, pH 8; Pgm, Me, and Mdh were screened in Tris-maleic-EDTA-$MgCl_2$, pH 7.4; and 6Pdh and α-Gpd were screened in Tris-citrate-

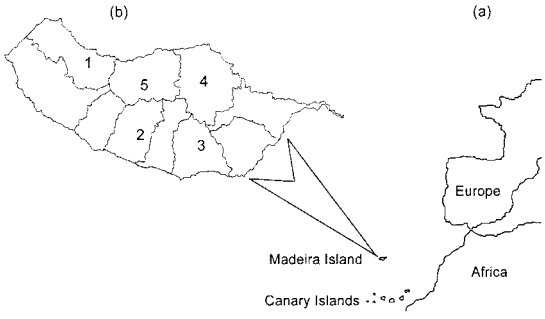

Fig. 1. (a) Geographical position of Madeira and the Canary Islands in relation to Africa and Europe. (b) Map of Madeira showing the five sampling localities: 1, Chão da Riberira (ChR); 2, Campanário (Cam); 3, Babosa (Bab); 4, Queimadas (Que); and 5, chão dos Louros (ChL).

EDTA, pH 8.5, buffer systems. Alleles were numbered according to their mobility relative to that of the most common allele.

Data Analysis. GENEPOP (version 3.3) software (Rousset and Raymond 1995) was used to analyze population parameters. F_{IS} and F_{ST} were calculated according to Weir and Cockerham (1984) and deviation from Hardy–Weinberg (HW) equilibrium caused by heterozygote deficiency or excess was estimated using Markov-chain algorithm of Guo and Thompson 1992. Linkage disequilibrium was estimated by Fisher test. Ohta SD statistic was used to determine the role of selection acting on pairs of loci (Ohta 1982). Fisher (1970) exact test was used to assess genetic differentiation between pairs of populations or groups. Number of effective migrants per generation (Nm) was estimated by private allele method (Barton and Slatkin 1986) and by using the equation $Nm = [(1/F_{ST}) - 1]/4$ (Wright 1969).

Results

Variability and Tests of Disequilibrium. Of the 11 enzymes analyzed, only Pep was monomorphic in all the populations. The remaining 10 enzymes segregated between two (6-Pdh and Got) to seven (Pgm) alleles in the six geographical populations studied (Table 1). The level of variability measured by average number of alleles (a), percentage of polymorphism (p), and level of mean heterozygosity (H) differed among the populations Based on the endemic nature of the species studied and published data on genetic variability of nonendemic mosquito species (Giming 2000, Taaf et al. 2004), we divided the Madeira populations of *Oc. eatoni* into three groups: 1, populations Ch R, Cam, and Que that exhibited low level of variability (p = 18.2–27.3 and H = 0.044–0.074); 2, population Cam with intermediate level of variability (p = 63.3 and H = 0.118); and 3, population Ch L with high level of variability (p = 77.7 and H = 0.194). The single population from Tenerife had high level of variability (p = 72.7 and H = 0.346), similar to the central population (ChL) of Madeira (Table 1)

The inbreeding coefficient (F_{IS}) was used to measure any departure from HW equilibrium. Of 33 tests, 12 departed significantly from equilibrium, with Bonfferoni correction (Table 1), because of heterozygote deficiency. In the Tenerife population, all polymorphic loci had significant heterozygote deficiency, indicating the influence of inbreeding. In Madeira, six of the 24 polymorphic population/locus combinations were not at HW equilibrium. Me was the only locus with deficiencies in all the populations, except ChL and Bab. Considering all the loci, only one population (Que) did not show any departure from equilibrium.

The null hypothesis (genetic equilibrium) was rejected ($P < 0.05$) in five of 50 pairwise comparisons after analysis of nonrandom combinations of genotypic association between pairs of loci. These were between Phi-Pgm, Got-Pgm, and Ak-Mdh in the Tenerife population and between Phi-Pgm in ChR and ChL. High heterozygote deficiency in the Tenerife population may have been the cause of disequilibrium. Disequilibrium was detected between Phi-Pgm, 6Pdh-Got, and Got-Pgm. The Ohta test (Ohta 1982) was performed, and the total linkage disequilibrium (D_{IT}) was divided between the indices of disequilibruim within populations (D_{IS}, D'_{IS}) and between populations (D_{ST}, D'_{ST}). For all pairs of loci, D_{IS} was lower than D_{ST} and D'_{IS} was greater than D'_{ST} (data not presented), indicating a role of genetic drift but not of selection in causing the observed disequilibrium in the populations of *Oc. eatoni.*

Genetic Differentiation. The level of genetic differentiation between pairs of geographical populations, estimated by F_{ST} and N_m values (Table 2), clearly shows the differentiation of the Tenerife population from all of the Madeira populations. In the latter group, the population of ChL (from the center) was significantly differentiated from the southern populations (Cam and Bab) but not from the northern populations (ChL). Pairwise comparisons between populations from the south and north were nonsignificant, except between Bab and Cam.

To measure the overall level of genetic differentiation, the populations were arranged into three groups: 1, all populations (from Canary and Madeira); 2, Madeira populations; and 3, Madeira populations, except the central population (ChL). In group 1, F_{ST} values were significant in eight of 10 loci studied whereby the total F_{ST} value in this group was 0.206 ($P < 0.001$). In group 2, only four of 10 loci had significant F_{ST} values, and although the total value was less than the value observed in group 1, it still was highly significant ($F_{ST} = 0.050$, $P < 0.001$). Idh locus had a preponderant weight in differentiating Madeira populations from the Tenerife population (Tables 1 and 2). In group 3, the total F_{ST} value was not significant ($F_{ST} = 0.026$, N.S.), and only two loci showed significant (with less magnitude) F_{ST} values (Table 3; Fig. 2).

The number of effective migrants (Nm) for all loci by F_{ST} method and Barton and Slatkin (1986) method was 0.941 and 0.567, respectively. The estimated Nm

Table 1. Allelic frequencies and F_{IS} values at 10 enzymes loci in six populations of *Oc. eatoni*

Locus	Pop					
	ChR	Cam	Bab	Que	ChL	Ten
Phi						
(*n*)	36	27	32	23	23	27
100	0.944	0.944	1.000	1.000	0.739	0.815
102	0.042	0.056	0.000	0.000	0.196	0.185
105	0.014	0.000	0.000	0.000	0.065	0.000
F_{IS}	+0.487	−0.048			+0.694	**+1**
Ak						
(*n*)	26	20	21	20	27	28
100	0.981	0.889	0.971	0.900	0.963	0.875
106	0.019	0.111	0.029	0.100	0.018	0.107
110	0.000	0.000	0.000	0.000	0.018	0.018
F_{IS}		−0.097		−0.059	−0.010	**+0.845**
6-Pdh						
(*n*)	23	30	39	23	23	24
100	1.000	1.000	0.897	0.956	0.870	0.607
102	0.000	0.000	0.103	0.043	0.130	0.393
F_{IS}			**+1**	+1	**+1**	**+0.860**
Got						
(*n*)	25	27	21	21	23	34
98	0.020	0.000	0.000	0.000	0.044	0.235
100	0.980	1.000	1.000	1.000	0.956	0.768
F_{IS}					+1	**+1**
Pgm						
(*n*)	26	27	27	21	21	26
95	0.038	0.093	0.000	0.000	0.143	0.019
98	0.019	0.000	0.000	0.000	0.024	0.077
100	0.923	0.907	1.000	1.000	0.762	0.577
102	0.019	0.000	0.000	0.000	0.071	0.096
104	0.154	0.000	0.000	0.000	0.000	0.154
105	0.000	0.000	0.000	0.000	0.000	0.039
107	0.000	0.000	0.000	0.000	0.000	0.039
F_{IS}	+0.227	−0.083			+0.533	**+0.401**
α-Gpd						
(*n*)	28	33	32	21	27	28
98	0.000	0.030	0.000	0.000	0.018	0.036
100	1.000	0.845	1.000	1.000	0.910	0.964
103	0.000	0.121	0.000	0.000	0.056	0.000
115	0.000	0.000	0.000	0.000	0.018	0.000
F_{IS}		**+0.777**			+0.373	−0.019
Idh						
(*n*)	34	23	29	20	23	31
90	0.000	0.000	0.000	0.000	0.000	0.323
95	0.000	0.000	0.000	0.000	0.000	0.549
100	1.000	1.000	1.000	1.000	1.000	0.129
F_{IS}						**+1**
Me						
(*n*)	39	57	43	23	25	27
95	0.012	0.026	0.023	0.087	0.100	0.185
97	0.128	0.175	0.058	0.130	0.020	0.019
98	0.038	0.017	0.012	0.000	0.020	0.000
100	0.897	0.781	0.907	0.783	0.860	0.796
103	0.026	0.000	0.000	0.000	0.000	0.000
105	0.013	0.000	0.000	0.000	0.000	0.000
F_{IS}	**+0.608**	**+0.904**	+0.206	**+0.769**	+0.376	**0.892**
Mdh						
(*n*)	43	26	31	26	20	32
97	0.012	0.019	0.000	0.000	0.029	0.406
100	0.965	0.942	1.000	0.961	0.941	0.594
103	0.023	0.019	0.000	0.038	0.029	0.000
105	0.000	0.019	0.000	0.000	0.000	0.000
F_{IS}	+0.664	−0.020		+1	−0.016	**+1**
Est						
(*n*)	31	26	35	26	21	21
98	0.016	0.019	0.014	0.019	0.048	0.000
100	0.984	0.981	0.985	0.981	0.881	1.000
102	0.000	0.000	0.000	0.000	0.024	0.000
103	0.000	0.000	0.000	0.000	0.024	0.000
105	0.000	0.000	0.000	0.000	0.024	0.000
F_{IS}					+0.372	
F_{IS} total	**0.439**	**0.554**	**0.574**	0.678	**0.541**	**0.845**
a	2.4	2.2	1.6	1.6	3.1	2.5
p	27.3	63.3	18.2	18.2	77.7	72.7
H	0.057	0.118	0.044	0.074	0.194	0.346

n, sample size. a, mean number of alleles per locus; p, percentages of polymorphic loci; H, mean heterozigousity. F_{IS}, inbreeding coefficient indicating the reduction of heterozygosity due to nonrandom mating. Probability of rejecting HW equilibrium in the case of heterozygotes deficiencies when significant by Bonefferoni correction is shown as corresponding F_{IS} in bold.

Table 2. Pairwise comparison of population differentiation

Pop	ChR	Cam	Bab	Que	ChL	Ten
ChR	—	7.814	25.25	12.25	5.702	0.500
Cam	0.031 (0.186)	—	4.75	49.75	6.893	0.643
Bab	0.012 (0.846)	**0.050** (0.016)	—	27.528	3.128	0.642
Que	0.020 (0.768)	0.005 (0.464)	0.009 (0.864)	—	3.987	0.630
ChL	0.042 (0.257)	**0.035** (0.027)	**0.074** (0.004)	0.059 (0.161)	—	0.975
Ten	**0.333** ($<10^{-6}$)	**0.280** ($<10^{-6}$)	**0.334** ($<10^{-6}$)	**0.284** ($<10^{-6}$)	**0.204** ($<10^{-6}$)	—

F_{ST} estimates with corresponding *P* values (in parentheses). Significant values are in bold and presented below the diagonal. Nm estimates are above the diagonal.

and F_{ST} values among the populations are given in Fig. 2.

Discussion

Analysis of genetic variations in *Oc. eatoni* reveals a substantial and complex population structure across its distribution range. The following sections consider the role of the factors contributing to the observed patterns of genetic variation in this species.

Population Structure and Migration. The F_{ST} tests and Nm estimates identify three subsets of *Oc. eatoni* populations: 1, north and south of Madeira; 2, center of Madeira; and 3, Tenerife. The genetic differentiation data support the clustering of the samples from the north and the south of Madeira into a single panmictic population, distributed across an extended geographical area, and, unusually, displaying a decrease of genetic variation compared with the central population. The geographical distances between the sampling localities in Madeira are small (between 8 and 20 km), and no correlation was detected between geographical distances and the level of differentiation. The population from Tenerife was also highly differentiated from the Madeiran populations. N_m values were very low (less then 1) between the populations of the two islands (Table 3). We expect either no or very little gene flow between the populations of the two islands and more gene flow among populations of the same island depending, primarily, on the dispersal rate of the adults. In *Oc. eatoni*, this rate is unknown, but it has been estimated as 104 m in *A. albopictus* (Bonnet and Worchester 1946, Mori 1979) and 580 m in *Ae. aegypti* (Reiter et al. 1995). If the dispersal rate in *Oc. eatoni* is similar to these two species, its role in homogenizing the populations of the island will depend on the availability of breeding sites.

Human activity and trade also have played an important role in spreading mosquitoes from their place of origin to other parts of the world (Smith 1956, Craven et al. 1988, Peyton et al. 1999). However, the ability of species to colonize a new or a different ecological condition should be the reason behind their geographical distribution.

Population Fragmentation, Seasonal Changes, and Human Activities. Madeiran habitats are highly heterogeneous; annual precipitation and temperature vary greatly from area to area because of the presence of microclimates around the island. The central peaks of the island register the lowest annual temperature and highest annual precipitation. Southern Madeira has a drier climate, compared with the northern and central areas. The lack of genetic differentiation among the southern/northern populations may result from population fragmentation and reduced breeding site availability in less humid areas during the dry seasons. Conversely, breeding sites in the more humid areas are expected to remain almost intact throughout the year. This fragmentation may cause local isolation, decreasing effective population size and permitting drift to influence genetic structure, resulting in the fixation of the most common alleles in some populations and reduction or elimination of the less common alleles in others. This may explain the observed pattern of genetic variation for the majority of loci (AK, 6-Pdh, Got, α-Gpd, Got, Me, Mdh, and Est). However, it is unlikely that drift alone determines the observed allelic frequencies in the Phi and Pgm. Also, it is unlikely that random processes fixed only the same allele in each of the two loci in the samples from the north and south. Phi and Pgm were the two loci showing linkage disequilibrium, although Ohta's test revealed that drift and not selection was the cause of disequilibrium.

Sampling period is another factor that can contribute to perceived differences in genetic variability among the populations. Temporal control of genetic structure has been reported for populations of *Ae.*

Table 3. F-statistic as a measure of population differentiation

Group	Phi	Ak	Pgd	Got	Pgm	Gpd	Idh	Me	Mdh	Est	Total
1	0.100 ***	0.028	0.183 ***	0.151 ***	0.122 ***	0.050 ***	0.620 ***	0.037 ***	0.238 ***	0.033	0.206 ***
2	0.111 ***	0.013	0.054	0.024	0.074 ***	0.055 **		0.031 ***	0.011	0.012	0.050 ***
3	0.011	0.017	0.032	−0.001	0.033 *	0.094 *		0.022	−0.009	−0.017	0.026

Group 1, all populations; group 2, all Madeira populations; and group 3, Madeira populations except ChL.

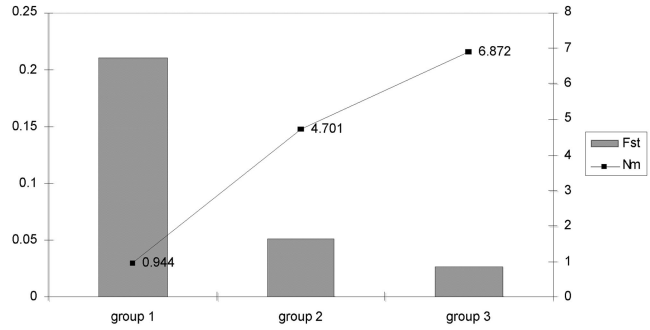

Fig. 2. Comparison of FST and Nm values in three groups of the populations. Group 1, all populations; group 2, populations from the Madeira Island; and group 3, Madeira populations except ChL.

albopictus and *Ae. aegypti* (Kambhampati et al. 1990, Huber et al. 2002). Samples of *Oc. eatoni* from the central population of Madeira were collected in winter (average monthly precipitation of 12.5 mm), whereas all other populations were collected in the summer (average monthly precipitation of 1.5 mm) (Portela et al. 2002). The Tenerife population was sampled in January. Thus, the two structured populations were sampled in the wetter and colder seasons from humid areas.

The application of insecticides also may contribute to the genetic variation observed in *Oc. eatoni.* The indigenous laurisilva forest of Madeira is protected. Some areas are pristine and undisturbed, whereas small agricultural villages are found in other areas, particularly on the periphery of the forest. The central population and one of the northern populations (Que) are located in protected areas of laurisilva with no nearby human habitation. The remaining three populations were situated in unprotected areas with high or moderate levels of habitation and agricultural activity. Extinction and recolonization can play important roles in determining the genetic structure of the populations, especially in small geographic areas (Yan et al. 1998). Therefore, insecticide application may contribute to reduced variability in some of the populations because suitable niches are first vacated and then reoccupied by mosquitoes after insecticide application. The population from Tenerife was collected from a mountainous area of laurisilva forest (much less dense than that found on Madeira) with no apparent human activity. Therefore, seasonal variation and insecticides seem to be important in shaping the pattern of genetic variation.

Dispersion and abundance of the blood source have an important influence in mosquito population structure. The source of blood that *Oc. eatoni* feeds upon remains unknown, and the influence of this phenomenon on *Oc. eatoni* populations merits further study.

Breeding and Genetic Structures. All populations, except Que, showed total heterozygote deficiencies measured by F_{IS} values (Table 1). Inbreeding can cause heterozygote deficits and is expected to affect all the loci. Our study indicated that inbreeding acts strongly upon the Tenerife population but was less marked in the Madeiran populations. Heterozygote deficiency also could be explained by Wahlund's effect in subdivided populations. This effect is expected to occur in case of population admixture with differentiated loci but not with nondifferentiated loci. Therefore, Wahlund's effect does not seem to be consistent with the present data.

Confined to two Macaronesian Islands, *Oc. eatoni* has a small population size but displays exceptionally high genetic differentiation not only between island populations but also within island populations (Madeira). A higher level of genetic variability would be expected if molecular markers other than the ones used in the current study were applied. For example, genetic differentiation between populations of *Ae. aegypti* from Cambodia was found to be 3 to 5 times higher using amplified fragment length polymorphic compared with isoenzyme markers (Paupy et al. 2004).

Speciation and Colonization. *Oc. eatoni* is an example of one speciation event followed at least by one colonization event. The geographical origin of this species cannot be inferred from this study. However, based on the detected genetic variability, we can conclude that the colonization process was not marked by founder effect. Had this been the case, migration would have continued between the populations of the two islands partially facilitated by Man via the trade routes between Madeira and Canary Islands, established since the fifteenth century (Ribeiro 1997). This is unlikely, however, given the patchy distribution of *Oc. eatoni*, particularly in the Canary Islands, where it is confined to two distant localities (Baez 1987).

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