

A test of the chromosomal theory of ecotypic speciation in *Anopheles gambiae*

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Edited by Francisco J. Ayala, University of California, Irvine, CA, and approved December 29, 2007 (received for review October 15, 2007)

The role of chromosomal inversions in speciation has long been of interest to evolutionists. Recent quantitative modeling has stimulated reconsideration of previous conceptual models for chromosomal speciation. *Anopheles gambiae*, the most important vector of human malaria, carries abundant chromosomal inversion polymorphism nonrandomly associated with ecotypes that mate assortatively. Here, we consider the potential role of paracentric inversions in promoting speciation in *A. gambiae* via “ecotypification,” a term that refers to differentiation arising from local adaptation. In particular, we focus on the Bamako form, an ecotype characterized by low inversion polymorphism and fixation of an inversion, 2Rj, that is very rare or absent in all other forms of *A. gambiae*. The Bamako form has a restricted distribution by the upper Niger River and its tributaries that is associated with a distinctive type of larval habitat, laterite rock pools, hypothesized to be its optimal breeding site. We first present computer simulations to investigate whether the population dynamics of *A. gambiae* are consistent with chromosomal speciation by ecotypification. The models are parameterized using field observations on the various forms of *A. gambiae* that exist in Mali, West Africa. We then report on the distribution of larvae of this species collected from rock pools and more characteristic breeding sites nearby. Both the simulations and field observations support the thesis that speciation by ecotypification is occurring, or has occurred, prompting consideration of Bamako as an independent species.

chromosomal inversion | ecological speciation | Bamako
chromosomal form | inversion polymorphism | selection

Differences in chromosome number or structure are often found between species, even those that are very closely related. This pattern has stimulated interest in the contribution of chromosomal changes to speciation itself. It has been argued that chromosome mutations are causal by directly inducing some degree of reproductive incompatibility [e.g., sterility (1)]. However, this argument does not hold for paracentric chromosomal inversions (i.e., those not involving the centromere). Such inversions are commonly polymorphic and not associated with reduced fertility in dipteran species like fruitflies and anopheline mosquitoes. Indeed, clinal, microspatial, and seasonal shifts in inversion frequencies associated with environmental conditions testify to the adaptive significance of chromosomal inversions (2). In this light, more recent considerations have hypothesized an indirect causal relationship to speciation, whereby chromosomal rearrangements protect adaptive divergence that may lead to the evolution of reproductive isolation (3). This effect is achieved through suppressed recombination between alternative arrangements bearing sets of genes differentially adapted to environmental heterogeneities. Kirkpatrick and Barton (4) formalized these arguments. They presented a general model showing that chromosomal inversions can become established because they capture sets of genes that confer local adaptations, protecting these gene combinations from recombination with

genes from migrants. This may occur even in the absence of epistasis, the presumed basis of the coadaptation theory of maintenance of inversions (2, 5).

Twenty-five years earlier, Coluzzi (6) (see also ref. 7) presented verbal arguments for a chromosomal speciation process much like that modeled by Kirkpatrick and Barton (4). At its core, it is a conceptual model of ecological speciation in which ecological and adaptive divergence among populations leads to reproductive isolation that promotes speciation (ref. 8 and refs. therein). However, Coluzzi's model was proposed with reference to anophelines, which are particularly good candidates due to ubiquitous inversion polymorphisms within and fixed inversion differences between species. The chromosome number ($n = 3$) and genome recombination length (9) are small, so the probability of an inversion capturing locally adapted alleles is relatively high. In addition, anopheline populations may undergo extreme fluctuations in size and distribution due to seasonal, climatic, or ecological oscillations. Coluzzi (6) hypothesized that during times of high growth rates, populations expand to colonize adaptively marginal habitats at the geographic or ecological periphery of the normal range. During population contractions, such peripheral populations could be stabilized by alleles adaptive to the marginal conditions. Renewed population expansion would reunite peripheral and central populations. Resulting interbreeding would swamp locally adapted alleles, unless they were protected from recombination by chance inclusion in a chromosomal inversion newly arisen in the peripheral population. The process has three possible outcomes: (i) extinction of the peripheral population, (ii) incorporation of inversion polymorphism by the central population, and (iii) speciation of the peripheral population via “ecotypification,” a term that refers to differentiation arising from local adaptation in anopheline mosquitoes (M. Coluzzi, personal communication). New alleles adapted to the recently occupied habitat are expected to accumulate within the inversion. Alleles promoting reproductive isolation can also arise (within or outside the inversion) due to pleiotropy, epistasis, or simply chance and increase through Batson/Dobzhansky/Muller or other processes (10, 11), leading eventually to speciation.

Author contributions: N.C.M., C.E.T., and N.J.B. designed research; N.C.M., M.B.T., A.S., F.E.E., M.B.C., and C.E.T. performed research; F.E.E., M.B.C., S.F.T., and C.E.T. contributed new reagents/analytic tools; N.C.M., C.E.T., and N.J.B. analyzed data; and N.C.M., J.R.P., C.E.T., and N.J.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0709806105/DC1.

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Table 2. Factors that lead to inversion polymorphism based on binomial regression and analysis of deviance of model parameters

Coefficient	Binomial regression				Analysis of deviance	
	Estimate	SE	z	P	RD	Percent
Intercept	192.2	10.8	17.88	<0.001	2713.80	
S_0	-5.4×10^{-4}	5.5×10^{-4}	-0.98	0.33	2713.05	0.03
S_1	-2.7×10^{-3}	1.1×10^{-3}	-2.39	0.02	2710.13	0.11
m_0	-361.0	243.2	-1.48	0.14	2709.60	0.02
m_1	4.4	0.5	9.54	<0.001	2630.48	2.82*
r	38.4	3.9	9.82	<0.001	2536.10	0.04*
s	7.0×10^{-4}	2.4×10^{-2}	-0.29	0.77	2535.00	0.04
t_i	-0.6	3.7×10^{-2}	-18.34	<0.001	1945.47	21.72*
n_i	0.2	1.7×10^{-2}	13.67	<0.001	1714.76	8.50*

Binomial regression: null deviance, 2,714 on 4,999 degrees of freedom (df); residual deviance (RD), 1,715 on 4,991 df. For analysis of deviance, terms were added sequentially in the order presented above. *, $P(\chi^2) < 0.05$.

power, although results were qualitatively the same as for the 2,500-simulation experiment. The inverted karyotype never reached fixation under the conditions explored, so the outcomes reflect establishment of inversion polymorphism ($0 < p_f < 1$) or loss of the inversion ($p_f = 0$) at the end of the simulations.

Binomial regression indicated that several factors affected whether chromosomal inversions persisted. Minimum size of the peripheral population (S_1), migration rate to the peripheral habitat (m_1), time of the year when the inversion occurred (t_i), number of loci captured by the inversion (n_i), and recombination rate (r) all had a statistically significant effect on inversion persistence; core population size (S_0), migration rate from the peripheral habitat (m_0), and relative fitness of the maladapted (migrant) alleles (s) had no such effect (Table 2). An analysis of deviance on the binomial regression model was used to rank the importance of the variables using the R programming language (www.r-project.org). It revealed two classes of significant predictors (Table 2): t_i and n_i explained much more of the deviance, whereas r and m_1 had relatively minor roles. All other factors were not significant at $P = 0.05$. Examination of t_i and n_i individually shows that inversions persisted most often when introduced to a small and growing population (Fig. 2A) and when the inversion encompassed more loci conferring adaptive properties (Fig. 2B).

In a separate analysis restricted to those simulations which ended in inversion polymorphism, we used a conditional model to test which factors were significant determinants of final inversion frequency. These were m_1 , s , and n_i . For detailed results, see SI Table 4.

Field Results. Distribution of the 2Rj inversion was compared in the core (puddle and swamp) and peripheral (rock pool) breeding sites. A total of 459 larvae from collections in September of 2000 and 221 larvae from September 2006 were successfully karyotyped for 2Rj using molecular diagnostics (19). Collections in 2000 were identified by day and puddle/pool; the numbers from each category were too small to permit detailed analysis, so they were pooled into rock pool vs. puddles/swamp. Collections in 2006 were made on the same day from each source; the numbers collected from each puddle/pool were small and not recorded, thus they were also pooled. Table 3 gives the numbers and frequencies of each 2Rj karyotype (i.e., +j/+j, +j/j, and j/j) found in alternative habitats, rock pools or puddles/swamp, for both years. The distribution of larval genotypes in both years was statistically different between samples from rock pools vs. puddles/swamp, with $P < 0.001$ [Fisher's exact test (20)]. Further, in both years, there was an excess of the 2Rj chromosome arrangements in the rock pools relative to the puddles/swamp, just as predicted by the ecotypification hypothesis for origin of the Bamako form. Of 680 larvae analyzed, only four 2Rj/+j heterozygotes were found.

Discussion

We note three particularly important aspects of these results. First, the simulations based on models parameterized specifically to coincide with the population dynamics of *A. gambiae* indicate that establishment of a new chromosomal inversion through local adaptation and recombination suppression in a peripheral

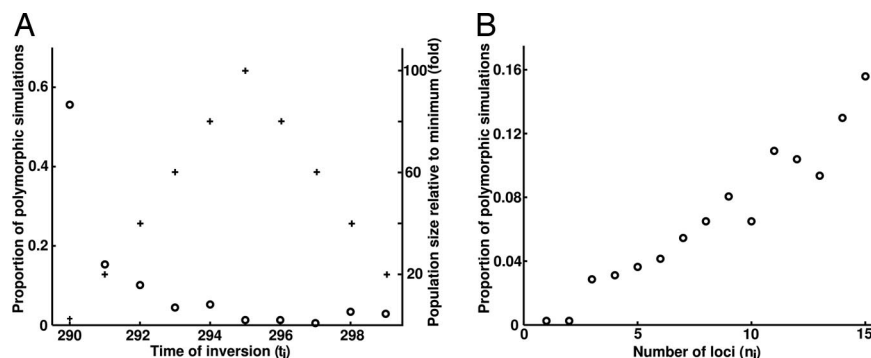


Fig. 2. Main factors affecting inversion polymorphism. Proportion of simulations ending in inversion polymorphism as a function of (A) time of inversion introduction (t_i) and (B) number of loci captured in the inversion (n_i). In A, t_i is given in time steps of the simulation, where each time step is a single generation between step 290 and 299 (1 year). Population size (crosses) is indicated on the right vertical axis as fold-increase relative to the minimum S_1 at step 290. Proportion of polymorphic simulations (circles) is indicated on the left vertical axis.

ture, i.e., the chromosomal forms of *A. gambiae* s.s. Until now, such identification has been possible only for adult females based on polytene chromosomes in ovarian tissue. This has complicated detailed study of ecological and other aspects of immature life stages and males of these taxa. The development of a DNA-based diagnostic test for inversions by Coulibaly *et al.* (19) now allows much more detailed understanding of these forms. Application of this tool allowed us to combine empirical and simulation approaches to address an issue of extreme importance in understanding the population biology, from a medical perspective, of arguably the most important species of insects in the world.

Materials and Methods

Model Simulations. We created a “minimal model” (30) that includes the essentials of the ecotypification hypothesis with regard to chromosomal inversions in *A. gambiae* s.s. All parameters used are summarized in Table 1, along with the ranges simulated based on empirical studies referenced in that table. We simulated two populations of mosquitoes connected by migration. The first population represented the core portion of the range and supported a large number of individuals. The second population represented a marginal habitat supporting fewer individuals. Each mosquito had a diploid genome of 15 equally spaced (recombination distance) biallelic loci under selection. Ten generational steps (≈ 1 year) were simulated with population sizes changing from a low in the dry season (S_0 for core, S_1 for peripheral) to a maximum in the wet season ($100S_0$, $100S_1$), then back to S_0 and S_1 in generations 10, 20, 30, etc. The evolutionary variables were selection (s), recombination (r), and migration between populations (m).

Each simulation started with randomly chosen alleles for all individuals and was run for 289 generations to attain near-equilibrium migration-selection balance. During the next year (generations 290–299), an inversion was introduced into the peripheral population at a single time step, t_i ; note that t_i also determines the population size into which an inversion was introduced. The inversion was assumed to have “captured” n_i alleles at the 15 biallelic loci that have adaptive advantage in the peripheral habitat. The simulation was then run to generation 600, and the frequencies of the inversion in the core and peripheral populations were recorded. The outcome was either polymorphism of the inversion [$(0 < p_f < 1)$] or loss ($p_f = 0$). The range of the parameter space explored was defined by the empirical data or reasonable estimates based on the biology of the mosquitoes as indicated in Table 1. Approximately 5,000 simulations were run exploring this parameter space [for additional details, see *SI Text* (31)].

Field Observations. The sampling locations were near the village of Banambani, Mali. Banambani is in a largely agricultural area of the Sudan-Savanna vegetation belt, ≈ 20 km from Bamako, at $12^\circ 48' N$ and $8^\circ 03' W$. The climate is of the Northern Sudan type, with a rainy season extending

from May to October and a dry season during the rest of the year. Three sources of development for anopheline larvae have been identified in the area, as shown in the map of Edillo *et al.* (17): a field of laterite rock with holes that hold water (“rock pools”) to the south east of the village, puddles primarily on the near west side of the village and a swamp/pond farther west of the village beyond the puddles. Photographs of these are shown in Fig. 1. The distance between them was < 2 km, well within the flight range of individual female *A. gambiae*. For information about dispersal by *A. gambiae* around Banambani and more comprehensive description of the area, see ref. 32.

Our collections were made during the peak of the rainy season in 2000 and 2006. The puddles in subarea 1 (17) had few or no larvae, so we sampled predominantly from subareas 2 and 3. The rock pool collections came from all of the rock pool subareas, predominantly from the lower rock pools, subarea 3. The sampling procedures were slightly different for 2000 and 2006, because the objectives of the original studies differed slightly. In 2000, samples of mosquito immatures were collected every alternate day from 28 July to 25 August, whenever weather permitted. Using a standard aquatic larval dipper, 60 dips (350 ml) were taken from edges of sampling sites within each type of habitat. The larvae were transported to the laboratory in Bamako, where DNA was extracted and sent to the University of Texas Medical Branch, Galveston, for further identification to species (33) and molecular form (34). See Edillo *et al.* (17) for further details. After storage at $4^\circ C$ in individual vials, extracts of 5 form larval DNA were shipped to the University of Notre Dame (UND) for karyotype analysis. The 2006 samples were made by placing a tray into the pool or puddle, then removing individual *A. gambiae* s.l. larvae with a dropper (Fig. 1A). Approximately 10 larvae per bag were stored in small plastic bags with water from that source for transport to the laboratory in Bamako. After drying on filter paper, larvae were placed individually in small vials with 80% ethanol and shipped to UND for molecular characterization. Approximately 90–95% of the larvae were *A. gambiae* (the others were *Anopheles arabiensis*); among the *A. gambiae* s.s., $\approx 95\%$ corresponded to the S molecular form. The data presented here are confined to the S molecular form of *A. gambiae*, which in Mali may be either the Savanna or Bamako chromosomal forms.

The 2Rj inversion that largely distinguishes Savanna from Bamako was molecularly karyotyped using a recently developed PCR diagnostic assay that has been extensively validated in Mali (19).

ACKNOWLEDGMENTS. We thank Mario Coluzzi (University of Rome, Rome) for from his help and encouragement; this paper grew from the seeds of inspiration that he planted in each of us. We thank G. Lanzaro (University of California, Davis) for generous sharing of DNA, T. Collier (University of California, Los Angeles) for help with simulations, and M. Kern and four Notre Dame undergraduate researchers (D. Thaner, M. Belton, J. Liu, and V. Au) for excellent technical assistance. The suggestions of two anonymous reviewers are gratefully acknowledged. Funding was provided by National Institutes of Health Grants 1 R01 AI51633 (to C.E.T.), R01 AI046018 (to J.R.P.), and R01 AI63508 (to N.J.B.).

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