tachment. The presence of a methyl group in thymine, instead of a hydrogen atom in uracil, appears to be an important factor for the isolated pair structure.

We have demonstrated the feasibility of nondestructive selection of mass and geometrical configuration of both neutral and negatively charged polar complexes. In biology, it is often difficult to distinguish between intrinsic properties and environmental effects. The production of supersonic beams of several unmethylated DNA or RNA bases as well as of some of their pairs allowed the experimental determination of the geometries of these pairs and direct comparison to quantum chemical calculations.

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Mating Patterns in Malaria Parasite Populations of Papua New Guinea

R. E. L. Paul, M. J. Packer, M. Walmsley, M. Lagog, L. C. Ranford-Cartwright, R. Paru, K. P. Day*

Description of the genetic structure of malaria parasite populations is central to an understanding of the spread of multiple-locus drug and vaccine resistance. The *Plasmodium falciparum* mating patterns from Madang, Papua New Guinea, where intense transmission of malaria occurs, are described here. A high degree of inbreeding occurs in the absence of detectable linkage disequilibrium. This contrasts with other studies, indicating that the genetic structure of malaria parasite populations is neither clonal nor panmictic but will vary according to the transmission characteristics of the region.

Sexual recombination, which is an obligate part of the malaria life cycle in the mosquito vector, is believed to play a significant role in the generation of parasite diversity (*1*) observed in natural parasite populations (*2–4*). Although clonal population structures have been proposed for other parasitic protozoa (*5*) and bacteria (*6, 7*), there are conflicting population genetic data for malaria (*8*). Linkage disequilibrium analyses have been unable to resolve the extent to which the genetic structure of malaria parasite populations deviates from panmixia (*3, 8, 9*). The interpretation of such analyses is easily confounded by the choice of sampling scale (*2, 7, 10*), which in the case of malaria must be defined in relation to the spatial heterogeneity of parasite transmission within a given endemic area (*2*).

Measurement of heterozygosity enables detection of any deviation from Hardy-Weinberg equilibrium, which is expected under random mating conditions. *Plasmodium falciparum* is haploid for most of its life cycle except for a brief period in the mosquito vector. Heterozygosity can easily be detected in the oocyst with gene amplification techniques (*11*). This life cycle stage resides in the mosquito midgut wall and contains the haploid products of meiosis. Laboratory experiments have shown no mating incompatibility between different parasite genotypes (*12*), so detection of oocyst heterozygosity should depend on the number of simultaneously infectious genotypes per human host and the feeding behavior of the anopheline vector. Interrupted feeding and consequent sampling of multiple hosts by the mosquito vector may also contribute to the diversity of parasite genotypes in the blood meal (*13*), although this contribution is considered negligible (*2, 14*).

We have measured oocyst heterozygosity and patterns of genetic linkage disequilibrium in parasite populations of rural Madang, on the north coast of Papua New Guinea (PNG), where intense, all-year-round transmission of malaria occurs. Parasite samples were collected from both human residents and blood-fed mosquitoes in three inland (Umun, Bau, and Sah) and three coastal (Agan, Dogia, and Maraga) villages (*15*) where a fivefold difference in transmission has previously been reported (*16, 17*). Parasite DNA was extracted and amplified for three polymorphic loci: *merozoite surface proteins 1* and *2* (*MSP-1*, *MSP-2*) and the *glutamate-rich protein* (*GLURP*), located on chromosomes 9, 2, and 10, respectively, of the 14 chromosomes of the haploid genome (*18*). These loci were amplified by polymerase chain reaction (PCR) techniques, sized, and probed with allele-specific probes (*11, 19–21*).

Considerable allelic diversity was observed in parasite populations from both human blood and mosquitoes (Fig. 1). There were 14 alleles identified for *MSP-2*, 9 alleles for *MSP-1*, and 9 alleles for *GLURP* in human blood samples. There were 13 *MSP-2* alleles, 8 *MSP-1* alleles, and 7 *GLURP* alleles in the oocysts. A comparison of allele frequencies of the human and oocyst parasite populations within a region (coastal or inland) was carried out independently for each locus: The human parasite populations were significantly different from the oocyst parasite populations for *MSP-2* and *GLURP* ($P < 0.01$) (*22*). Mosquito and human parasite populations share most of the alleles, but they are found at very different frequencies. This suggests that not all infections in the human population were simultaneously and equally infectious. The substantial difference in prevalence of infection in the human (20 to 29% inland, 38 to 51% coastal) compared with the blood-fed mosquito population [1% (*20, 23*)] presumably results from this variable infectiousness of the parasite and the source of the bloodmeal [that is, animal compared with human (*24*)].

Heterozygosity after blood-feeding re-

R. E. L. Paul and K. P. Day, Wellcome Centre for the Epidemiology of Infectious Disease, Department of Zoology, University of Oxford, South Parks Road, Oxford, OX1 3PS, UK.
M. J. Packer, M. Lagog, R. Paru, Papua New Guinea Institute of Medical Research, Post Office Box 378, Madang, Papua New Guinea.
M. Walmsley, Department of Biology, Imperial College, London, SW7 2BB, UK.
L. C. Ranford-Cartwright, Institute of Cell, Animal, and Population Biology, Division of Biological Sciences, University of Edinburgh, Edinburgh EH9 3JT, UK.
* To whom correspondence should be addressed.

quires multiple infectious genotypes in the human host. The number of single-locus genotypes per person varied from one to three and was not significantly different between the coastal (1.9) and inland (1.73) villages when data for each locus were pooled [$\chi_1^2 = 0.33$ (*25*)]. The mean number depended on the locus used, reflecting its degree of polymorphism (Table 1). Taking into account all three loci, 72% of the human population (96 of 133) had multiple genotypes, whereas only 15% of the oocyst sample were heterozygous.

Any deviation from random mating will produce a lower than predicted number of heterozygote oocysts (*26*). For all loci, the number of heterozygotes was less than that expected given the allele frequency distribution (Table 2). The extent to which there is deviation from random mating is described by Wright's coefficient of inbreeding, f (*27*). F statistics are a set of tools to account for population subdivision (coastal and inland) and to partition any observed heterozygote deficit appropriately (*28*). The F statistics were evaluated by using the three-locus genotype for the sampled oocyst data (*22*), yielding an F_{IS} of 0.915 [99% confidence interval (CI) of 0.835 to 0.966], which is significantly different from zero ($P < 0.0002$) (*29*) and indicates large deviations from random mating at the human population level.

A second approach to assess the mating structure of a population is by inference from nonrandom associations between physically unlinked loci: linkage disequilibrium. This has been the standard measure to describe the population genetics of a number of microorganisms (*7*, *9*, *10*). Linkage disequilibrium can occur for many ecological and evolutionary reasons (*30*), including nonrandom mating. For the human parasite population, the existence of linkage disequilibrium between pairs of loci was assessed within each region by using only single-genotype infections (*31*). There was no linkage disequilibrium between any of the loci in either region ($P > 0.1$) (*22*). For the oocyst data, analyzed as coastal or inland populations, no linkage disequilibria were observed, each locus being independent of the others ($P > 0.1$) (*22*). Thus, analysis of the parasite in either host at different spatial scales revealed no linkage disequilibrium. The apparent lack of linkage disequilibrium and the occurrence of nonrandom mating means that there was sufficient outbreeding to disrupt any linkage disequilibria (*32*). This observation indicates that a reconsideration of the conclusion of panmixia may be warranted with respect to previous population genetic analyses of Gambian parasite populations (*3*).

The high rate of inbreeding observed in PNG parasite populations was predicted by sex allocation theory. A female sex ratio bias of infectious, sexual stages (gametocytes) has been observed in parasites from PNG, which is consistent with minimal local mate competition (*33*). The experimental corroboration of this theoretical prediction suggests that measurement of gametocyte sex ratios may be a useful indicator of the genetic structure of malarial populations in different endemic areas.

It is not altogether surprising that parasites that exist in a subdivided habitat (human hosts) have restricted breeding partners. In fact, even within humans, limited overlap of the infectious periods of different parasite genotypes might restrict outcrossing. A high rate of inbreeding may be expected given that the number of parasite alleles per locus per person varies only between one and three. Therefore, with our human parasite data (Table 1), the number of homozygous oocysts can be estimated as 62.3% (*34*). The observed proportion of oocysts that were homozygotes was 85%. Although only qualitative, the number of heterozygous oocysts expected from the distribution of genotypes in the human population was less than that found, suggesting that multiple infections may not give rise to equal probabilities of selfing or crossing, because all genotypes are not simultaneously or equally infectious. This contrasts with the result obtained from a predictive model that used the Tanzanian data, which implied that all genotypes were simultaneously infectious (*4*, *35*). This lack of overlap in infectiousness may be purely a consequence of the dynamics of the human host immune response to polymorphic parasite antigens of various life cycle stages.

An oocyst study in Tanzania (*4*), which used comparable techniques but a different sampling strategy (*36*), concluded that mating was random. However, population genetic analysis of this study showed that there was a significant inbreeding coefficient ($F_{IS} = 0.33$) (*35*), albeit less than in PNG ($F_{IS} = 0.915$). This difference in breeding structure, as measured by F_{IS}, is explicable by comparison of the intensity of malaria transmission in the two endemic areas (*23*). A transmission intensity in Tanzania 10-fold that in PNG results in more parasite genotypes per person (3.2 compared with 1.8) and consequently higher rates of oocyst heterozygosity. Thus, the genetic structure of malaria populations will be determined by the number of infectious genotypes per human host, which appears to be a function of transmission intensity. However, the inability to detect differences in the number of genotypes per human host in coastal and inland regions in PNG,

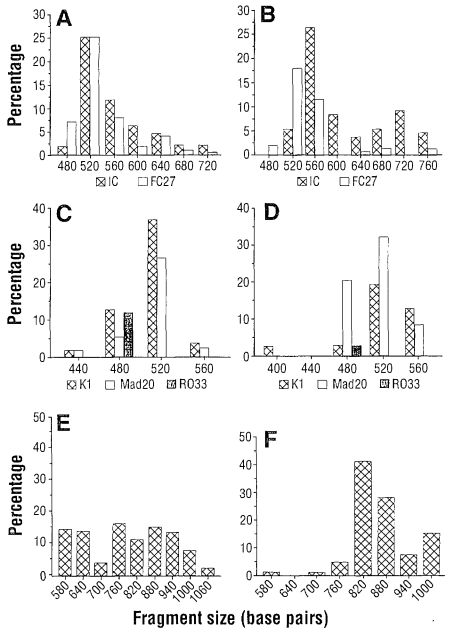

Fig. 1. The frequency distribution of alleles of three polymorphic *P. falciparum* loci in human and mosquito (oocyst) parasite populations: (**A**) *MSP-2* human, $n = 221$; (**B**) *MSP-2* oocyst, $n = 128$; (**C**) *MSP-1* human, $n = 142$; (**D**) *MSP-1* oocyst, $n = 78$; (**E**) *GLURP* human, $n = 116$; and (**F**) *GLURP* oocyst, $n = 82$. Samples from the coastal and inland villages are combined, although they were analyzed separately. The legend designations refer to the allele-specific probes used for *MSP-1* and *MSP-2*.

Table 1. Number of human hosts with single or multiple parasite genotypes. The values for all loci indicate the minimum number of genotypes per person.

Locus	Number of alleles	Number of human hosts with one, two, or three parasite genotypes		
		One	Two	Three
MSP-2	14	49	74	7
MSP-1	9	77	25	5
GLURP	9	97	11	1
All loci		37	83	13

Table 2. Comparison of the observed and the expected number of heterozygote oocysts. The expected heterozygote number was calculated from oocyst allele frequencies with the assumption of panmixia (Fig. 1). Allele classes were pooled where necessary to achieve suitable sample sizes for analysis (allele number: *MSP-2*, 10; *MSP-1*, 6; *GLURP*, 5).

Locus	Observed	Expected	χ^2
	Homozygote oocysts	Heterozygote oocysts	Heterozygote oocysts	
MSP-2	55	9	54*	240
MSP-1	38	1	30*	121
GLURP	40	1	29*	92

*$P < 0.01$.

where there is a fivefold difference in transmission intensity, suggests that this relation may be nonlinear.

Geographical variation in the extent of inbreeding may have consequences for the success of potential global malaria control strategies. Mathematical models have demonstrated an effect of recombination and the degree of outbreeding on the development of multiple-drug resistance with reference to their use as mixtures or sequentially: inbreeding may accelerate the build-up of drug resistance (*37*). One would expect variability in the evolution of multilocus phenotypes such as virulence and drug and polyvalent vaccine resistance to occur in places of differing malaria transmission characteristics purely as a result of parasite mating patterns. The relatively slow spread of chloroquine resistance in Tanzania (*38*) compared with PNG (*16*) lends weight to this prediction, as high degrees of inbreeding may accelerate the spread of multilocus drug resistance (*37*). Furthermore, inbreeding rates may influence the rate of development of any strain-specific component of immunity, if strains are defined by multilocus variant surface antigen genotypes as proposed by Gupta and Day (*39*).

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15. Finger-prick blood samples were taken during the wet season of 1992 from all 387 occupants of 10 randomly chosen houses within each of the villages and placed into 8 M guanidine-HCl [V. M. Marshall and R. L. Coppel, in *Protocols in Molecular Parasitology*, J. E. Hyde, Ed. (Humana, Totowa, NJ, 1993), vol. 21, pp. 223–248)]. Informed consent was obtained from all participants involved in the study, and permission was obtained from the PNG Medical Research Advisory Council. Parasitaemia counts of parasite-infected red blood cells per 200 white blood cells were obtained, and parasite DNA was extracted from the samples of those testing positive for *P. falciparum*. Readings of the blood smears revealed a *P. falciparum* parasite rate of 38% (Maraga), 39.6% (Dogia), 50.9% (Agan), 26.3% (Sah), 28.3% (Umun), and 26.5% (Bau). The coastal villages (Maraga, Dogia, and Agan) had higher overall rates of infection and significantly higher prevalence rates than inland villages [coastal 0.45; inland 0.31, $\chi_1^2 = 11.1$, $P < 0.05$, binomial errors, GLIM (*40*)]. Indoor resting catches of mosquitoes were carried out for the 10 nights after the finger-prick sampling in the 10 study houses of each village. The mosquitoes were maintained for 6 days (to allow growth and microscopic visualization of any oocysts) at ambient, natural conditions at which time they were dissected for the presence of oocysts on the midgut wall. Oocysts were dissected out and placed in lysis buffer (*11*) for subsequent parasite DNA extraction. Approximately 16,000 mosquitoes were dissected. Three anopheline species were present, *Anopheles farauti, A. punctulatus,* and *A. koliensis*, with an oocyst (of any *Plasmodium* species) infection rate of <3%. There were too few oocyst-positive mosquitoes of *A. punctulatus* and *A. koliensis* to enable an analysis of the effect of mosquito species on parasite genotype.
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20. We selected 141 human blood samples on the basis of positive parasitaemia (*15*): 129 were PCR-positive for *MSP-1*, 130 for *MSP-2*, and 109 for *GLURP*; of these 107 were probe-positive for *MSP-1* and 130 for *MSP-2*. All oocysts were analyzed, ~35% of which probed positive for at least two of the three loci. The inability to detect more than 30% of the oocysts as *P. falciparum*–positive by PCR is not surprising, because entomological data (*23*) would suggest that at least half of the oocysts would be *P. vivax*. *Plasmodium ovale* and *P. malariae* are also present. As the PCR is *P. falciparum*–specific, oocysts of the other *Plasmodium* species would not be detected.
21. H. A. Babiker, L. Ranford-Cartwright, A. Sultan, G. Satti, D. Walliker, *Trans. R. Soc. Trop. Hyg.* **88**, 328 (1994). Control PCR experiments were carried out by mixing field samples, and no allele-specific PCR bias was noted. For *GLURP*, the PCR primers used were 5′ (outer) ACATGCAAGTGTTGATCC, 3′ (outer) GATGGTTTGGGAGTAACG, 5′ (nested) (*19*), 3′ (nested) [S. Viriyakasol *et al.*, *Bull. WHO* **73**, 85 (1995)]. PCR was performed in a total volume of 50 μl containing 50 mM KCl, 10 mM tris-Cl, 2.5 mM $MgCl_2$, 100 nM of each primer, 75 μM deoxynucleotide triphosphate, and 1 unit of Taq polymerase (Perkin Elmer Cetus). For both outer and nested PCRs a total of 30 cycles were performed, each cycle consisting of a denaturation step of 25 s (nested 1 min) at 94°C, an annealing step of 1 min at 45°C (nested 2 min at 55°C), and an elongation step of 2 min at 68°C (nested 2 min at 70°C).
22. Data for the allele frequency comparisons and analysis of linkage disequilibrium were analyzed with GENEPOP version 1.0 (1994) (M. Raymond and F. Rousset, Laboratoire de genetique et Environnement, URA CNRS 327, Institut des Sciences de l'Evolution, CC 065, USTL, Place E. Bataillon, 34095 Montpelier cedex 05, France), which uses Fisher exact tests. For all analyses the oocyst data set was sampled, conservatively counting any oocysts from the same mosquito with identical three-locus genotypes as a single datum point, treating every incidence of sex between gamete pairs with a novel combination of genotypes within each mosquito as the unit of analysis. For allele frequency comparisons, the full human parasite data set was used, counting the total incidence of each allele and dividing by the sum of the alleles. This comparison assumes the minimum number of genotypes per person (Table 1), so the human allele frequency is an estimate as pointed out in Babiker (*4*).
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25. For calculation of parasite prevalence and infection number, generalized linear interactive modelling techniques available in the GLIM statistical package were used (*40*).
26. Number of heterozygotes expected under random mating was predicted by applying the Hardy-Weinberg law to the oocyst allele frequencies. This assumes that any selection on the *P. falciparum* loci in the vertebrate host does not influence the amount of heterozygosity.
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28. It is necessary to take into account the effects of population subdivision to be able to partition the overall inbreeding (F_{IT}) to that from within (F_{IS}) and between (F_{ST}) subpopulations. Here F_{IS} measures the reduction in heterozygosity within individual oocysts, with respect to subpopulations (oocysts from either coastal or inland areas); F_{ST} measures reductions within these subpopulations with respect to the total, F_{IT} (*32*).
29. For the calculation of *F* statistics, FSTAT was used, which is a program to calculate Weir and Cockerham's estimators of *F* statistics and bootstrapped confidence limits [FSTAT, 1.0; J. Goudet, Lausanne University, Switzerland (1993); B. S. Weir and C. C. Cockerham, *Evolution* **38**, 1358 (1984)]. The oocysts were sampled as in (*22*).
30. Linkage disequilibrium is the statistical dependence of one locus on another, such that two-locus allelic combinations occur together more often than would be expected by chance (linkage equilibrium). The presence of linkage disequilibrium can be caused by physical linkage on the chromosome, epistatic selection pressures, nonrandom mating, and small population sizes (*32*).
31. For the linkage disequilibrium analysis between each pair of loci, the human parasite data set was sampled, excluding those samples bearing multiple infections as identified by either of the pair of loci compared [*MSP-2* and *MSP-1* (or *GLURP*), $n = 14$ coastal, 11 inland; *MSP-1* and *GLURP*, $n = 38$ and 18]. Small sample sizes led to analysis with sequence data only for *MSP-2* and *MSP-1*. The oocysts were sampled as in (*22*).
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34. Assuming simultaneous infectiousness, the probability of selfing depends on the number of other infections present, that is, 50% with two infections and 33.3% with three infections.
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