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Self-Fertilization, Linkage Disequilibrium, and Strain in *Plasmodium falciparum*

We are impressed by the elegant data presented by R. E. L. Paul *et al.* (*1*) on the high rate of self-fertilization in *Plasmodium falciparum*, the agent of the most malignant form of malaria. This study contributes significantly to our knowledge on this pathogen's basic biology. Nevertheless, we find that there is a major logical gap in the conclusions arrived at by Paul *et al.* They appropriately argue that frequent selfing in *P. falciparum* is a medically relevant feature, for it should favor the maintenance of "multi-locus phenotype associations," in particular those governing virulence, drug resistance, or variant surface antigen polymorphism. This is quite logical: Self-fertilization, by inhibiting genetic recombination, leads to a situation of actual clonality (*2*) (offspring genotypes that are identical to the parental cells), which should help stabilize those multi-locus associations that are elsewhere favored by natural selection. Then they state that "there was sufficient outbreeding to disrupt any linkage disequilibria" (linkage disequilibrium is the nonrandom association between genotypes scored at different loci). These two proposals taken separately are conceivable, but they are incompatible to each other.

If self-fertilization, as evidenced by studying the three loci *MSP-2*, *MSP-1*, and *GLURP*, was unable to maintain any multi-locus association between these loci (as shown by lack of linkage disequilibrium at the three loci), it is not tenable that it could significantly help in stabilizing any other multi-locus combination. Two possibilities can be entertained. First, selfing can play in itself a significant role in maintaining multi-locus association, and this should be observed with the *MSP-2*, *MSP-1*, and *GLURP* loci. Second, the natural selection has the dominant role in stabilizing those multi-locus phenotypes associated with virulence, drug resistance, or "immunologically sensitive" variant antigens (a statement that is consistent with the observation of linkage equilibrium at other loci). In the latter case of variants mainly maintained by natural selection, the role of self-fertilization would appear consequently limited.

Another concern in the approach used by Paul *et al.* lies in the difficulty of evidencing any linkage disequilibrium with their data. Each of the three loci under study exhibits considerable allelic variation. The expected frequency of each possible multi-locus combination is therefore low, which proportionally lowers any possibility of evidencing linkage, even with exact statistical tests. This situation leads to a large type II error risk (to see no significant linkage while linkage does exist). If a conservative model is taken, in which five equiprobable alleles (much less than actually recorded in these data) are segregating at each locus, the probability of any multi-locus combination does not exceed $0.2^3 = 0.008$. This renders difficult to evidence any significant linkage, unless considerable sample sizes are used, which is not the case in this study.

Although the discovery of high-rate self-fertilization in *P. falciparum* is a major breakthrough in our knowledge of the agent of malaria, its actual impact on this parasite's population structure in humans still has to be clarified by classical population genetic means that depend on linkage disequilibrium analysis. The notion of strain in microbiology relies on the existence of stable multi-locus associations (especially, of course, those combinations dealing with medically relevant characters), and if no such multi-locus associations are found in *P. falciparum*, the notion of strain has to be held in abeyance for this parasite. Should this be verified, any efforts for individualizing multi-locus genotype (that is, strain characterization) in *P. falciparum* may not be successful, for these genotypes will appear as most unstable. The only approach that remains possible in this case is the typing of individual genes.

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Response: Tibayrenc and Lal highlight an important issue in genetic analyses of organisms in which sex is obligatory, especially with respect to species of medical importance such as *P. falciparum*. As stated by us and by Tibayrenc and Lal, a high degree of self-fertilization will have important medical consequences by favoring the maintenance of multi-locus phenotypes such as virulence and drug resistance. Assessing the mating structure of populations can be achieved indirectly by measurements of association between loci (linkage disequilibrium) and directly by measurements of heterozygosity. Our direct measurement of the degree of heterozygosity in the oocyst parasite population found that the mating structure was typified by a high degree of inbreeding which was in contrast to that previously found in a region of more intense malarial transmission, Tanzania (*1*). While such a high degree of inbreeding would be expected to result in linkage disequilibrium, in this study (*2*) we found no evidence for linkage, even when using sequence data only (GENEPOP Fisher exact, $P > 0.1$) (*3*). However, linkage analysis may produce misleading results (*4*) and as indeed pointed out by Tibayrenc and Lal, large sample sizes are required to detect linkage (*5*). In this study, linkage analysis was performed for a comparison with the heterozygosity data as malariologists had previously accepted the absence of linkage disequilibrium as evidence for a panmictic population structure (*6*). Our study highlighted the relative insensitivity of linkage analysis in assessing the extent of inbreeding.

A third point raised emphasizes the need to use selectively neutral loci to establish such mating patterns. In our report we used three loci, two of which were parasite surface antigens. The fact that all three loci produced the same inbreeding coefficient would suggest that the result found is real, although there is some evidence that regions of the merozoite surface proteins, other than those amplified, may be under selection (*7*).

The comments made by Tibayrenc and

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Lal are helpful in underlining inherent difficulties in the interpretation of population genetic data. The high rate of inbreeding found by the more direct heterozygosity measurement would not have been predicted by linkage analysis of the data set.

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Faunal Evidence and Sterkfontein Member 2 Foot Bones of Early Hominid

Ronald J. Clarke and Phillip V. Tobias (*1*) state that they have found the "oldest South African hominid" in Sterkfontein Member 2, as the STW 573 foot remains demonstrate a mosaic of ape-like and human-like features. Their dating of the deposit, between 3.5 and 3.0 million years ago (Ma), is somewhat tenuous, as it is based largely on presumed sedimentological rates that could vary dramatically with different cave morphologies and environments (*2*). Considerations of the associated Member 2 faunal assemblage suggest the strong possibility of a more recent age.

Chronological seriations of the southern African faunal assemblages (*3*), however, place Sterkfontein Member 2 just prior to Sterkfontein Member 4 and after the Makapansgat and Taung fauna. All of the Sterkfontein Member 2 species appear in Sterkfontein Member 4 (circa 2.6 to 2.5 Ma) or in later sites, or in both, under a variety of environmental and taphonomic conditions. However, three of the species (namely, *Papio izodi*, *Chasmaporthetes siberbergi*, and *Megantereon cultridens*), indeed genera, which commonly appear at these later sites, do not appear at Makapansgat Members 3 (circa 3.2 to 3.0 Ma) or 4 (circa 3.0 to 2.9 Ma). It is possible that all of these species were missed by the accumulating agents at Makapansgat Member 3, but this seems highly unlikely; Makapansgat is the richest southern African fossil source in terms of biodiversity, with more than 40 large mammal species represented (as compared to approximately 40 such species known historically from the area). It would be unexpectedly idiosyncratic for three species to appear in the fossil record before Makapansgat, be totally absent in the Makapansgat assemblage, and then reappear in the Sterkfontein Member 4 and later sites.

Given the variety of later sites at which these species appear, temporal considerations override ecological or taphonomic explanations of the differences between assemblages.

Thus, on the basis of the associated fossil fauna, the Sterkfontein Member 2 foot bones do not appear to be as old as Makapansgat Member 3 at 3.0 Ma, but fall closer in time to Sterkfontein Member 4. If this dating were correct, then STW 573 may belong to *Australopithecus africanus*, a hominid species long known to have had some apelike features in its postcranial morphology.

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Response: The eroded and disconformable contact between Beds C and B and the interdigitating contact between Beds B and A, of Sterkfontein Member 4; and the thick band (0.5 m) of recrystallized mesocrystalline calcite interposed between the top of Member 2 and Bed B of Member 3 (*1*) represent time lapses in the history of deposition, prior to the laying down of Beds B, C, and D of Member 4 from which most fossil remains emanate (*2*). The upper beds of Member 4 have a probable dating of earlier than 2.6 Ma. As a depth of about 15.0 m separates them from Member 2, and as this depth of deposit includes at least four interfaces or flowstone horizons, our claim that Member 2 must be older than 3.0 Ma and probably nearer to 3.5 Ma is modest.

The sequence of Members, originally worked out by Partridge (*1*), has been confirmed by his analysis of cores extracted during diamond drilling of the full thickness of the six Members (*3*).

With regard to the rate of sedimentation, according to Partridge [note 27 in our report (*4*)], the 6.5-m *average* thickness of Member 3 would probably have taken 0.3 to 0.5 Ma to accumulate. McKee questions this and states (in note 2) that McFadden *et al.* (*5*) postulate that "a similar depth of deposit at Makapansgat [would] have accumulated in under 130 thousand years." This is incorrect. At Makapansgat, the depth of deposit from the Gilbert/Gauss transition in Member 2 to the upper of two intervals of apparently reversed palaeomagnetism (Kaena event) in the lower part of Member 4 is 9.5 m (*6*). The lapse of time between these two levels under the earlier calibrated polarity time scale is 0.52 Ma (*5*), but under the recalibrated scale (*7*) it is 0.54 Ma. This is equivalent to 0.37 Ma for the deposition of 6.5 m (not 130,000 years) and to 0.57 Ma for that of the 10-m upper limit of thickness of Member 3 (*1*). These estimates of 0.37 to 0.57 Ma for the time taken for Member 3 to accumulate are close to the 0.3 to 0.5 Ma we cited [note 27 in (*1*)]. Even if we adhere to the earlier calibrated polarity time scale, the estimated time lapse is 0.36 to 0.55 Ma. Thus, the application of the Makapansgat rate of sedimentation to the Sterkfontein Formation corroborates and strengthens our claim that Member 2 is appreciably older than Member 4: our new calculations on this basis indicate that Sterkfontein Member 2 might have been as much as 0.8 Ma older than Beds B and C of Member 4.

The carnivoran species existed in Africa before 3.5 Ma and persisted to end-Pliocene or later. This long time-span nullifies their use for dating Member 2.

As to the primates, *Parapapio broomi* has hitherto been identified not only from Sterkfontein Member 4, but also from the somewhat older Makapansgat Members 3 and 4. The genus *Parapapio* is known in the African fossil record, according to McKee, from 4 to 2 Ma, while White *et al.* (*8*) have identified cf. *Parapapio sp.* among the fauna from Aramis, Ethiopia, dated to 4.4 Ma. The long range of the genus, and the hitherto known span of the species, render this cercopithecoid of little value for resolving the dating of Sterkfontein Member 2. Its presence in Member 2 does not preclude the assignment of a dating of 3.0 Ma or older.

Papio izodi, which McKee identified from Member 2, is at present represented by remains from only two southern African sites, Taung (Hrdlicka Deposits) and Sterkfontein Member 4 (*9*). Although McKee *et al.*