Letter to the Editor

When One Is Not Enough: Introgression of Mitochondrial DNA in *Drosophila*

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The specific purpose of this study is to investigate alternate processes that may have resulted in the formation of the two distinct mitochondrial haplotypes (*ma*I and *ma*II) of *Drosophila mauritiana.* The most plausible explanation for the observed data is that there has been introgression of *Drosophila simulans* mtDNA into *D. mauritiana.* More generally, this article addresses three important issues concerning resolution of species relationships. First, pooling data from distinct process partitions has the potential to obscure biologically informative patterns of substitution (Bull et al. 1993; Ballard et al. 1998). Second, hypothesizing a species phylogeny from a single linkage partition may be problematic (reviewed by Doyle 1992; Avise 1994). Third, phylogenetic hypotheses may be influenced by the inclusion of a single representative of a terminal taxon (Omland, Lanyon, and Fritz 1999). Multiple unlinked loci and multiple individuals from within species that exhibit extensive population subdivision should be included to maximize the potential to fully resolve species relationships.

Slowinski and Page (1999) identified two levels of potential error when inferring species phylogenies from molecular sequence data. First, a gene tree for a set of molecular sequences may be incorrectly inferred if there is sufficient systematic or random error (Swofford et al. 1996). Second, even if a gene tree is correctly inferred, deep coalescence, retention of ancestral polymorphism, gene duplication, and horizontal gene transfer among different species can produce a gene tree that differs from the species tree (Doyle 1992; Avise 1994). This second source of error may be confounded if the nucleotides are linked such that there is a single gene tree, or genealogy. As an example, genes in organelle genomes such as metazoan mtDNA are historically linked with no recombination (but see Awadalla, Eyre-Walker, and Maynard Smith 1999; Eyre-Walker, Smith, and Maynard Smith 1999; Hagelberg et al. 1999). In this case, phylogenetic hypotheses from different mtDNA genes can only infer the mitochondrial genealogy, which may or may not be the same as the true species tree.

The well-corroborated mitochondrial genealogy of the *Drosophila melanogaster* subgroup implies that *D. mauritiana* is paraphyletic relative to *D. simulans* and *Drosophila sechellia* (Solignac and Monnerot 1986; Satta and Takahata 1990; Ballard 2000). One explanation for this result is that the mtDNA genealogy does not reflect the species relationships. The phylogenetic relationships of the *D. melanogaster* subgroup are not clear (Caccone, Amato, and Powell 1988; Hey and Kliman 1993; Kliman and Hey 1993; Hey 1994; Caccone et al. 1996). One hypothesis is that *D. sechellia* and *D. mauritiana* are derived from an ancestral *D. simulans* population and that many ancient polymorphisms are shared between *D. mauritiana* and *D. simulans* (Kliman and Hey 1993). An alternate explanation for the nonmonophyly of the *D. mauritiana* mtDNA haplotypes is that *D. mauritiana* is a complex of sibling species. This alternate explanation is not supported by the morphological data. The consistent morphological differences between members of the *D. melanogaster* subgroup include the shape of the posterior process of the male genital arch and the number of bristles on the sex comb (Coyne 1983; True et al. 1997). The male genital arch from the distinct mitochondrial lineages of *D. mauritiana* is a slender, fingerlike process that is very different from that observed in all other species. Furthermore, isofemale lines of both *D. mauritiana* haplotypes have more bristles on the sex comb than do all lines of *D. simulans* examined.

The paraphyly of *D. mauritiana* mtDNA may have resulted from the retention of ancestral polymorphism or introgression. There is no evidence of recombination among the mtDNA genomes of *Drosophila* (Ballard 2000). To test these alternatives, intron 1 of the nuclear *Alcohol dehydrogenase-repeated* (*Adhr*) locus was sequenced from the same eight isofemale lines that Ballard (2000) considered in his complete mtDNA analysis. The autosomal and mtDNA data are taken from distinct linkage partitions (Slowinski and Page 1999); however, it is likely that there is recombination at the *Adhr* locus (Ballard et al. 1996). Intron 1 of *Adhr* was sequenced because it had considerable variation, and the pattern was consistent with a neutrally evolving locus (Sumner 1991).

This is the first study, to my knowledge, that specifically includes autosomal data from representatives of each species and distinct mitochondrial haplotype in the *D. melanogaster* subgroup. Inclusion of representatives from each lineage permits the investigation of how incomplete sampling of a species may influence phylogenetic interpretations gleaned from the data. Two isofemale lines of *D. melanogaster* are included as outgroup taxa—Oregon R and Zimbabwe 53. The Oregon R line was collected in Roseburg, Oreg., around 1925 by D. E. Lancefield. The *D. melanogaster* Zimbabwe 53 line, referred to here as *D. melanogaster* Zimbabwe, was collected in 1990 at the Sengwa Wildlife Preserve in Africa by R. R. Ramey and L. Brown. Isofemale lines taken from North America and Zimbabwe have clearly differentiated mtDNA (Rand, Dorfsman, and Kann 1994), and many nuclear variants are not shared between the two localities (Begun and Aquadro 1993). *Drosophila mauritiana* has been collected only from Mauritius

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(Tsacas and David 1974; Lachaise et al. 1988). Two isofemale lines of *D. mauritiana* are included, one from each distinct haplotype (*ma*I and -II). O. Kitagawa (Tokyo Metropolitan University, Japan) collected *D. mauritiana ma*I BG1 in Mauritius in 1981 and *D. mauritiana ma*II G52 in 1985. There are three geographically subdivided *D. simulans* haplotypes (Solignac, Monnerot, and Mounolou 1986; Baba-Aïssa et al. 1988; James and Ballard 2000). The *si*I type is known to occur in New Caledonia, Hawaii, Tahiti, and the Seychelles Islands. The *si*II type has a worldwide distribution but has not been collected on the Pacific Islands of New Caledonia, Hawaii, and Tahiti. The *si*III haplotype has been collected only from Madagascar and Reunion Island, where it is sympatric with the *si*II type. Three isofemale lines of *D. simulans* are included. I collected *D. simulans si*I TT01 in Papeete on Tahiti Nui in 1998. *Drosophila simulans si*II DSR was collected in 1984 from Riverside, Calif. (DSR) by A. A. Hoffman (LaTrobe University, Melbourne, Australia). I collected *D. simulans si*III MD199 in Joffreville, Madagascar, in 1998. *Drosophila sechellia* has been collected only in the Seychelles Islands (Lachaise et al. 1988). One isofemale line of *D. sechellia* is included, because intraspecific variation is low (Hey and Kliman 1993; Kliman and Hey 1993; Hey 1994; unpublished data). H. M. Robertson (University of Illinois, Urbana, Ill.) collected this line in 1980 at Tsacas on Cousin Island, Seychelles.

DNA from all lines was extracted using the PureGene Kit (Gentra) following the isolation from fixed tissue protocol. In all cases, the DNA was extracted from individuals less than 14 days of age. The 501-bp region of intron 1 of *Adhr* was amplified (Ballard et al. 1996) and cloned, and a single copy was sequenced (Ballard 2000). Two to four cycle sequencing reactions were employed to sequence this region. Sequences were imported into the Sequencher program, and the chromatograms were investigated. These sequences have been submitted to GenBank (accession numbers AF201423–AF201427, AF201429, AF201436, and AF201447).

The *Adhr* data were aligned without ambiguity against the previously published sequence of Cohn et al. (1984) and Ballard et al. (1996) in CLUSTAL X (Higgins and Sharp 1988). The eight insertion/deletion events ranged in size from 2 to 10 nt. The mtDNA data were obtained from GenBank (accession numbers AF200828–AF200832, AF200834, AF200841, and AF200852) and aligned following Ballard (2000). The mtDNA sequences were not easily alignable at positions 5535–5584 and 6022–6047. Consequently, 74 bp of mtDNA were deleted from the analyses. The region between positions 5535 and 5584 forms the majority of an intervening sequence between COIII and the glycine tRNA. The region between positions 6022 and 6047 is located in an intervening sequence between ND3 and the alanine tRNA. A single data set consisting of the aligned autosomal and mitochondrial data was constructed in PAUP* (Swofford 1998). In both the mitochondrial and the autosomal data sets, each presumed

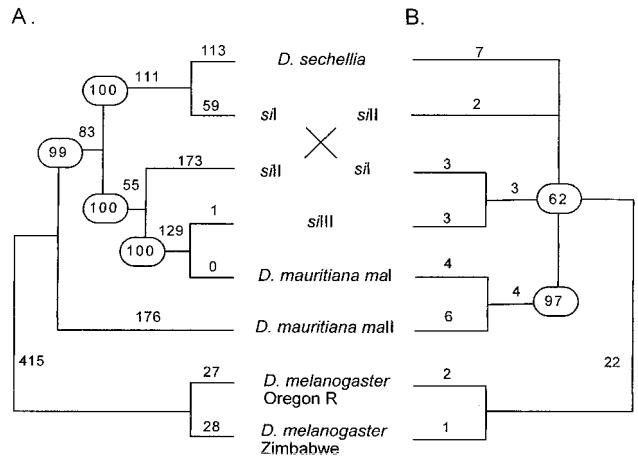

FIG. 1.—Parsimony analysis using PAUP* of the eight taxa included in this study. *Drosophila melanogaster* is the designated outgroup. The *Drosophila simulans* haplotypes are shown as *si*I, *si*II, and *si*III, respectively. *A,* The mitochondrial data. The mtDNA sequences are A+T-rich (mean base frequency of A = 0.39, C = 0.13, G = 0.10, and T = 0.38) and have sequence divergences that are in the range of 0%–4%. The chi-square test of homogeneity of base frequencies across taxa does not suggest that there is any heterogeneity among taxa ($\chi^2_{21} = 2.62$, $P = 1.0$). Of the 14,958 characters included, 385 variable characters are parsimony-uninformative, while 736 are parsimony-informative. A single most-parsimonious tree of 1,370 steps was found (consistency index [CI] = 0.86). *B,* The *Adhr* data. The aligned sequences are A+T-rich (mean base frequency of A = 0.30, C = 0.19, G = 0.18, and T = 0.33) and have sequence divergences that are in the range of 8%–19%. The chi-square test of homogeneity of base frequencies across taxa does not suggest that there is any heterogeneity among taxa ($\chi^2_{21} = 1.406$, $P = 1.0$). Of the 501 characters included, 23 variable characters are parsimony-uninformative, while 27 are parsimony-informative. Three equally parsimonious trees of 55 steps were found (CI = 0.93). The bootstrap proportions from 1,000 pseudosamples are shown in circles. Branch lengths are recorded above each line.

insertion/deletion event was parsimoniously scored by inserting a ''1'' into the matrix at appropriate sites.

The most appropriate method for analyzing independently acquired data sets, in this case the mtDNA and *Adhr* data, is vigorously debated in the phylogenetic literature. Kluge (1989) suggested that all data sets should be analyzed simultaneously according to a total-evidence or simultaneous-analysis (Nixon and Carpenter 1993) procedure to maximize informativeness. This approach seeks a single best-fitting hypothesis that, in cladistics, involves maximizing character congruence. If the *Drosophila* mtDNA and autosomal data are pooled, parsimony analysis generates a single tree that has the same topology as that generated by the mtDNA data set alone (see fig. 1*A*). The procedure of simultaneous analysis does not consider whether each data set may be subject to distinct biological forces. Bull et al. (1993) suggested that subsets of characters that are evolving under demonstrably different rules should be defined a priori, and the null hypothesis that each partition is evolving under homogeneous processes should be explicitly tested. In this study, the mitochondrial and autosomal data are distinct linkage partitions (Slowinski and Page 1999), and distinct evolutionary processes may be acting. To investigate whether these linkage partitions are significantly associated, the incongruence length dif-

ference (ILD) test (Farris et al. 1995) was employed to test the null hypothesis that the mitochondrial genome and *Adhr* are evolving under homogeneous biological processes. This random-partitioning test is an extension of a measure originally reported by Mickevich and Farris (1981) and is based on the null hypothesis of congruence. The mtDNA and intron 1 of *Adhr* are not evolving under the same processes, as determined by the ILD test (1,425 steps; $P = 0.01$) implemented in PAUP* (Swofford 1998). There is a single difference between the mitochondrial genomes of *D. simulans si*III and *D. mauritiana ma*I (fig. 1*A*): an insertion/deletion event at position 13252 of the aligned sequence. In contrast, the data from the nuclear locus suggest that the two isofemale lines of *D. mauritiana* are sister taxa (fig. 1*B*). This result is robustly supported, with 97% of bootstrap pseudosamples (Efron 1982; Felsenstein 1985) supporting this node. Kliman and Hey (1993) and Hey and Kliman (1993) also found that the *D. mauritiana* alleles are monophyletic at the *period*, *yolk protein* 2 and *zeste* loci.

These data suggest that there has been introgression of *D. simulans si*III mtDNA into *D. mauritiana.* This hypothesis is supported by the known distribution of the maternally inherited alpha-proteobacteria *Wolbachia* and by introgression experiments. James and Ballard (2000) noted that both *si*III and *ma*I may be infected with the *w*Ma strain of *Wolbachia.* However, no *Wolbachia*-infected lines of the *ma*II haplotype were detected. Aubert and Solignac (1990) introduced single virgin *D. simulans* females (initial frequency 0.03) into *D. mauritiana* populations. The flies were allowed to mate freely and were then transferred to a new bottle. Populations were followed for at least 6 generations, and some were reexamined after 20, 25, and 33 generations. *Drosophila simulans* females were usually inseminated (39/40) and, on average, the F_1 progeny accounted for 14% of the total population. As the F_1 males are sterile (Robertson 1983), the F_1 hybrid females must have backcrossed to the pure *D. mauritiana* males in the bottles. The offspring of this backcross accounted for about 63% of the population. In subsequent generations, the *D. simulans* mtDNA went to fixation in virtually all populations. In the reciprocal experiments, only a few *D. mauritiana* females were inseminated, and in all cases the mtDNA of the immigrant *D. mauritiana* went to extinction. In Mauritius, *D. mauritiana* is a generalist, domestic species with a broad ecological niche similar to that of the cosmopolitan *D. simulans. Drosophila simulans* has not been collected from Mauritius, but is present on other nearby islands in the Indian Ocean (David et al. 1989). As a consequence of the observed distribution of *D. simulans,* Aubert and Solignac (1990) suggested that *D. simulans* may have been introduced in Mauritius, but the nuclear and mitochondrial gene pools have been absorbed in *D. mauritiana* by introgressive hybridization. Although less likely, three lines of evidence support the alternate possibility that there has been introgression of *D. mauritiana* mtDNA into *D. simulans* following paternal leakage. First, Kondo et al. (1990) backcrossed 331 lines for 10 generations and observed that four lines from the interspecific cross *D. simulans* (*si*II female) × *D. mauritiana* (*ma*I male) showed clear evidence of paternal leakage of mtDNA. Second, Satta et al. (1988) observed that 2 of 25 wild-caught lines from Reunion Island were heteroplasmic for *si*II and *si*III mtDNA. Third, the *ma*I lineage is in the majority in *D. mauritiana* (88%; Solignac, Monnerot, and Mounolou 1986), while the *si*III lineage is in the minority in *D. simulans* (about 33% in Madagascar and Reunion; James and Ballard 2000).

An alternative to introgression across species boundaries is the retention of an ancestral mitochondrial genome. This alternative is considered less likely because of the recent divergence of the *D. simulans si*III and *D. mauritiana ma*I genomes compared with nuclear genes. If there has been introgression of *si*III mtDNA into *D. mauritiana,* it is predicted that the estimated divergence time of the mitochondrial data will be more recent than the divergence time estimated from nuclear data. This presupposes that the nuclear locus was not subject to recent introgression, deep coalescence, retention of ancestral polymorphisms, or strong selection. If it assumed that members of the *simulans* clade diverged from *D. melanogaster* about 2.5 MYA (Powell 1997; Li, Satta, and Takahata 1999) the divergence times can be estimated from the number of silent changes. The estimated divergence time of the *si*III and *ma*I genomes is about 4,500 years, while the estimated divergence time of *si*II and *ma*II mtDNA is around 1.75 Myr. To place an upper bound on the divergence of *si*III and *ma*I with 95% confidence, i.e., the time at which the probability that one or fewer changes has occurred (recall that there is a single nucleotide difference in 15,034 bp) is $0.05 = \exp(-2knt) + 2knt \exp(-2knt)$, where k is the substitution rate per site per year, n is the number of silent sites, and t is the time since divergence. The calibration of substitution rates in *Drosophila* is difficult, but if $k = 1.6 \times 10^{-8}$ for silent sites (Li, Satta, and Takahata 1999) and $n = 6{,}279$, the divergence time of the *si*III and *ma*I mitochondrial haplotypes is unlikely to be longer than 24,000 years ago. In contrast, the divergence time of *D. simulans* and *D. mauritiana* estimated from *Adh* is 0.86–1.45 Myr (Stephens and Nei 1985), and that estimated from the *zeste* and *yolk protein* 2 loci is 580,000–860,000 years ago (Hey and Kliman 1993). Thus, even if the *si*III and *ma*I haplotypes diverged as long as 50,000 years ago and *D. simulans* and *D. mauritiana* diverged as recently as 500,000 years ago, there is still an order of magnitude difference between the divergence times. The simplest explanation for this difference is introgression between the mitochondrial haplotypes.

In this study, multiple individuals of *D. mauritiana* and *D. simulans* were included. Inclusion of multiple individuals within a species is common for morphological studies but rare in systematic studies based on molecular data. Data presented here show that it is important to sample multiple representatives of terminal taxa when there is population subdivision. Inclusion of a single representative of a species may directly influence the phylogenetic hypotheses by affecting (1) evidence for introgression, (2) species relationships, (3) inferred sup-

port for monophyly of an assemblage, and (4) inferences of evolutionary rate. For example, if the *ma*I line were not sampled, there would be no evidence of introgression between *D. mauritiana* and *D. simulans*. As a second example, consider the effect of sampling the *ma*I isofemale line and just one of the three *D. simulans* haplotypes. If the *si*I line were sampled, *D. simulans* and *D. sechellia* would be sister taxa. However, if either the *si*II or the *si*III line were sampled, *D. simulans* and *D. mauritiana* would be sister taxa. Omland, Lanyon, and Fritz (1999) also found that it was important to sample more than one individual to accurately infer species relationships in New World orioles. Moreover, they noted that adding subspecies, even to monophyletic groups, increased the bootstrap support for a clade.

Data presented here address three important issues concerning resolution of the phylogenetic relationships of closely related species. First, pooling data from distinct process partitions has the potential to obscure biologically informative patterns of substitution (Bull et al. 1993; Ballard et al. 1998). In this study, pooling the autosomal and the mtDNA data would obscure the fact that the topologies derived from the two process partitions were not congruent. Second, hypothesizing a species phylogeny from a single linkage partition may be problematic, because the effects of reticulation may be retained through subsequent generations. Including unlinked loci can corroborate phylogenetic hypotheses (Slowinski and Page 1999). Third, inclusion of single representatives of a taxon may directly influence the phylogenetic hypotheses of closely related species. Adding taxa and characters may break long branches and result in a more robust phylogeny (Hillis 1996; Poe and Swofford 1999). Furthermore, the inclusion of multiple individuals from within a species opens the door to the wealth of statistical tests that have been developed in a population genetic framework (reviewed in Ballard and Kreitman 1995).

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