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RECENT RADIATION OF ENDEMIC CARIBBEAN *DROSOPHILA* OF THE *DUNNI* SUBGROUP INFERRED FROM MULTILOCUS DNA SEQUENCE VARIATION

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Abstract.—Studies of island endemism provide a unique opportunity to elucidate fundamental mechanisms of speciation. Here we examine intra- and interspecific DNA sequence variation at four unlinked genetic loci among populations of the *Drosophila dunni* subgroup to provide a detailed genealogical portrait of the process of speciation among these island endemic species. Our data indicate two major rounds of diversification that have shaped the *D. dunni* subgroup. The first occurred 1.6–2.6 million years ago and separated three major lineages, one in Puerto Rico and the Virgin Islands, a second in the northern Lesser Antilles and Barbados, and a third in St. Vincent and Grenada. A second round of diversification occurred in the last 96,000 years in the northern Lesser Antilles and Barbados. The four distinct species that resulted from this recent round of diversification maintain relatively high amounts of genetic variation, similar to that of a closely related mainland species, and share extensive ancestral polymorphism. These data suggest a minimal role for population bottlenecks linked to founder events in the history of the *D. dunni* subgroup. Further, the recent divergence of these island populations highlights the extremely rapid development of reproductive isolation and distinct patterns of abdominal pigmentation that has occurred in these species.

Key words.—DNA sequence variation, *Drosophila dunni* subgroup, island endemism, phylogeography, speciation.

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The phenomenon of island endemism provides a rich source of insights into the operation of adaptation and speciation in nature. Because the formation of novel species on islands is so ubiquitous, involving diverse island systems and taxa of all types, the study of island endemism offers an unparalleled opportunity to identify fundamental genetic and environmental mechanisms that underlie the speciation process. Despite this, genealogical portraits of island speciation events have been relatively scarce, and even fewer have provided resolution from multiple unlinked portions of the genome (but see Kliman et al. 2000). Because different genetic loci can provide dramatically different insights into population history, incorporating multiple markers known to exhibit separate patterns of inheritance (e.g. sex-linked vs. autosomal markers) and markers with varying effective population sizes (e.g. mtDNA vs. autosomes) provides a much more detailed genetic picture of the demography of speciation events than a single or arbitrarily chosen set of markers.

Here we analyze the pattern of speciation that has led to a unique radiation of *Drosophila* species in the eastern Caribbean through an examination of DNA sequence variation at two autosomal loci, one mitochondrial gene, and a Y-linked gene. The system we address is the *D. dunni* subgroup, a monophyletic clade of six species that are endemic to the islands of Puerto Rico, the Virgin Islands, and the Lesser Antilles (Heed 1962; Hollocher 1996). This subgroup is part of the larger *D. cardini* group, which contains 16 species confined to the Neotropics and subtropics (Heed 1962; Heed and Russell 1971). Every major island from Puerto Rico to Grenada is home to only a single species from the *D. dunni* subgroup, with no two species co-occurring on the same island, as shown in Figure 1 (Heed and Krishnamurthy 1959; Heed 1962). Species designations were initially determined on the basis of reproductive isolation, chromosomal inversions, genitalia morphology, and the pattern of abdominal pigmentation (Williston and Aldrich 1896; Heed and Krishnamurthy 1959; Heed 1962). This last trait is most remarkable because pigmentation in the subgroup is arranged in a near-perfect cline, such that the lightest species are in the north and the darkest are in the south (Heed and Krishnamurthy 1959; Hollocher et al. 2000a,b). This pigmentation trait alone has been shown to be diagnostic in identifying each member of the *D. dunni* subgroup (Hollocher et al. 2000a).

Highlighting the dramatic pigmentation variation that exists in the subgroup is a remarkable lack of other distinguishing traits among these species. Each share a similar ecology and habitat preferences, and, apart from pigmentation, they have nearly indistinguishable morphologies. Heed (1962) describes chromosomal inversions that differentiate *D. dunni* (Puerto Rico and St. Thomas) and *D. similis* (St. Vincent and Grenada) from each other and from the remaining members of the subgroup. The remaining four species (termed the *nigrodunni* complex) have homosequential chromosome arrangements. The extremely close genetic relationships among the *nigrodunni* complex have been further confirmed based on similarity among enzyme electrophoretic mobilities (Napp and Cordeiro 1981). Interestingly, the same three lineages are also distinguished by the morphology of the male genitalia. This trait differentiates *D. dunni* and *D. similis* from each other and the *nigrodunni* complex, but does not distinguish any member of the *nigrodunni* complex from one another. The failure of male genital shape to discriminate these taxa indicates that divergence has probably been extremely recent, as this trait evolves quite rapidly among many insects, including *Drosophila* (e.g. Coyne 1983; Eberhard 1985; Arnqvist 1998). In spite of the similarities among the members of the *nigrodunni* complex, these species show significant levels of postmating reproductive isolation ranging

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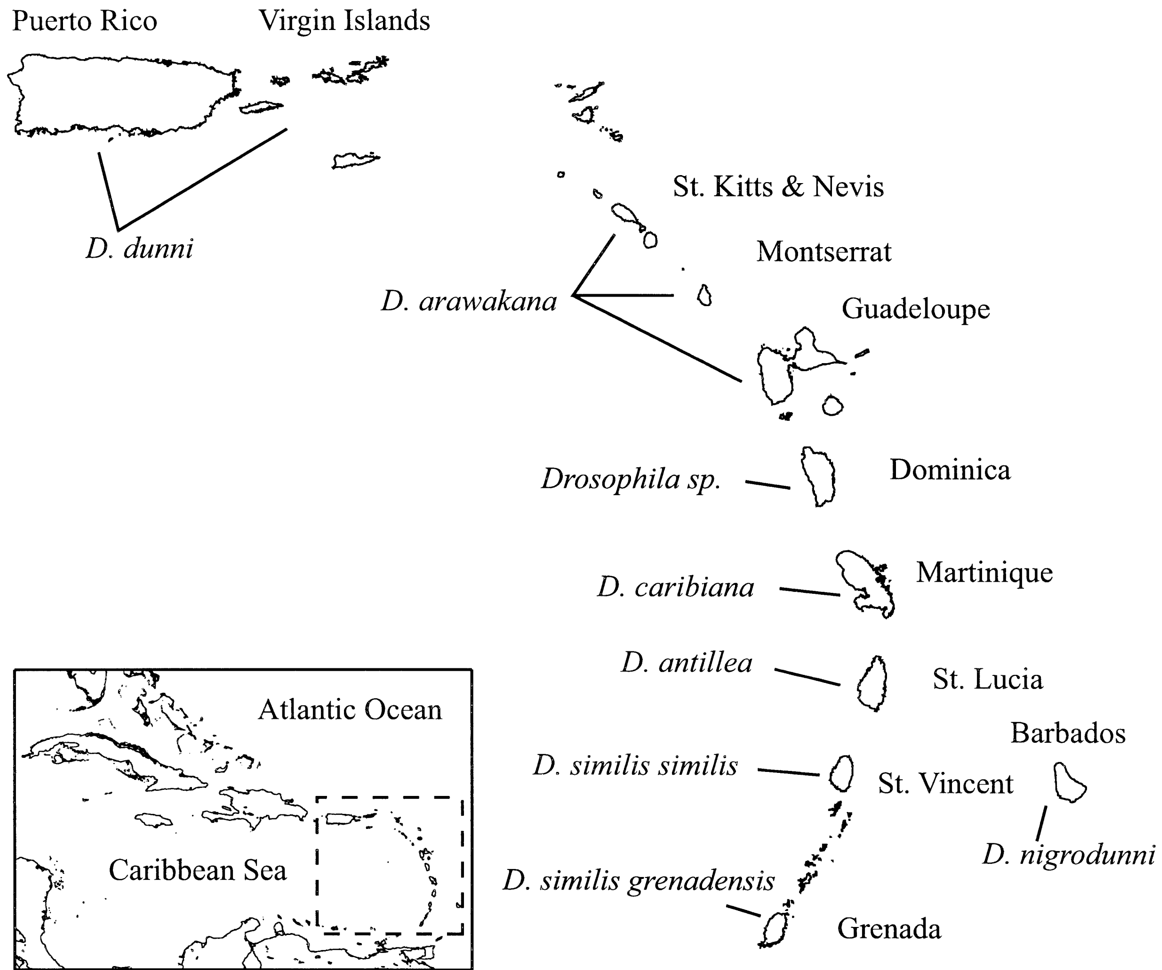

FIG. 1. The distribution of *Drosophila dunni* subgroup species. The species designation of specimens from the island of Dominica is currently unknown.

from complete sterility of hybrids to partial fertility of F_1 offspring with a steep drop in fertility and viability in the F_2 and F_3 generations (Heed and Krishnamurthy 1959; Heed 1962).

The *D. dunni* subgroup provides a fascinating opportunity to examine patterns of speciation among closely related species. This group is especially interesting because of the apparent interplay between natural selection acting on abdominal pigmentation and the divergence of these taxa. Here, we present a genealogical analysis of four genetic loci across nine island populations from the subgroup and one mainland population of *D. acutilabella*, which is the closest relative to the *D. dunni* subgroup with a noninsular distribution. Every species from the subgroup is represented in our study, and we also include specimens from the island of Dominica, which was not sampled when the taxonomy of the group was first established. Because the initial criteria for determining taxonomic status relied heavily on abdominal pigmentation, we believe that island populations are a more natural unit through which to analyze divergence among these species. Thus, our study compares intra- and interisland genetic variation, rather than using the existing species designations as our unit of study. Through this framework we test the hypothesis that divergence in these species has occurred through a pattern of ''island hopping,'' in which new islands were successively colonized by older island populations, with a loss of variation at each hop, as proposed by Heed (1962). Specifically, we quantify the genetic variation within each island, estimate the extent to which variation is shared among islands, and compare the genealogies of each of the genetic loci with reference to patterns of island colonization.

MATERIALS AND METHODS

Drosophila Strains

Specimens used in this study originated from two sources. From each island population for which they are available, specimens from the National *Drosophila* Species Resource Center (NDSRC) in Bowling Green, Ohio (now in Tucson, Arizona) were included. A single specimen was sampled from each of the following strains: *D. dunni dunni* (Puerto Rico, 15182-2291.0), *D. dunni thomasensis* (St. Thomas, 15182-2301.0), *D. arawakana arawakana* (two strains: Guadeloupe, 15182-2261.0; Montserrat, 15182-2261.2), *D. caribiana* (Martinique, 15182-2281.0), *D. antillea* (St. Lucia, 15182-2251.0), *D. nigrodunni* (two strains: 15182-2311.0, 15182-

TABLE 1. Study populations, their location, taxonomy, and number of alleles sampled at the following loci: Cu, Zn *superoxidase dismutase* (*sod*), *engrailed* (*en*), *cytochrome oxidase II* (*COII*), and *male fertility factor kl-5* (*kl-5*). Dashes indicate that the locus was not surveyed in the given population.

Population	Islands	Taxonomic designation	*sod*	*en*	*COII*	*kl-5*
PRT	Puerto Rico and St. Thomas	*Drosophila dunni*	5	5	6	—
SK	St. Kitts and Nevis	*D. arawakana kittensis*	7	6	11	7
GU	Montserrat and Guadeloupe	*D. arawakana arawakana*	7	5	14	6
DO	Dominica	*Drosophila* sp.	6	5	6	5
MA	Martinique	*D. caribiana*	8	7	16	5
SL	St. Lucia	*D. antillea*	8	8	10	6
BA	Barbados	*D. nigrodunni*	8	8	11	8
SV	St. Vincent	*D. similis similis*	5	5	3	1
GR	Grenada	*D. similis grenadensis*	4	3	4	1
FL	Florida	*D. acutilabella*	5	4	5	—

2311.1; both from Barbados), and *D. acutilabella* (Florida: 15181-2171.9). The second source of samples used in this study was wild-caught individuals, or isofemale derivatives of wild-caught individuals. These specimens were collected from the island of Puerto Rico by H. Hollocher in August 1994, St. Vincent and Grenada by H. Hollocher in July 1996, Dominica by H. Hollocher in December 1997, Barbados by H. Hollocher in January 1999, Guadeloupe by J. Wilder in July 1999, and St. Kitts and Nevis, St. Lucia, and Martinique by E. Dyreson and E. Schielke in June and July 2000. Wild-caught and specimens from the NDSRC were pooled according to their island of origin in all analyses. Our results (see below) show no genetic differentiation at the loci we examined between NDSRC and wild-caught specimens. Additional specimens of *D. acutilabella* collected from Broward County, Florida in July 2001 were kindly donated by S. Perlman. Table 1 indicates the total number of alleles sampled from each population at each of the genetic loci in this study.

Our study focused on nine island populations from the *D. dunni* subgroup, and also on a Florida population of *D. acutilabella*. The latter population was primarily used as a representative mainland population for comparisons of intraspecific genetic diversity. Each of the *D. dunni* populations represents a single island, or small island group known to contain conspecific populations, as described by Heed (1962). Island populations, and their existing taxonomic status, are given in Table 1.

Genetic Loci Sampled

DNA sequence variation was examined at four loci for each population: the mitochondrial gene *cytochrome oxidase II* (*COII*), the unlinked autosomal genes Cu, Zn *superoxidase dismutase* (*sod*) and *engrailed* (*en*), and the Y-linked gene *male fertility factor kl-5* (*kl-5*). Primers used to amplify each locus are shown in Table 2. We developed new primers for two of the loci in this study. For the *en* locus, we designed degenerate primers using the program GeneFisher version 1.22 (Giegerich et al. 1996) based on an alignment of publicly available *en* sequences from *D. melanogaster* (M10017), *D. virilis* (X04727), and *Anopheles gambiae* (U42214). For the *kl-5* locus we followed the same protocol as above using aligned sequences from *D. melanogaster* (AF210453) and *D. hydei* (AF031494). For *kl-5,* a first set of degenerate primers was used to isolate the gene from *D. nigrodunni* (primers kl5F1: GCT CCG GCA AGG TGG ARG TNT GGY and kl5R1: TGC GGT TGA ACT CAT CKA ARC ANC CCC). Based on the *D. nigrodunni* sequence, a second set of specific primers were designed (shown in Table 2) and used across the *D. dunni* subgroup. For the two autosomal loci, primers were chosen such that the amplified region spanned an intron (the second intron of *en* and the single intron of *sod*).

DNA was isolated from individual flies using the squish prep of Gloor et al. (1993). In general, a single allele from five to 13 individual specimens per population was surveyed at each genetic locus. The only major exception to this was for *kl-5,* from which fewer samples were obtained from some populations due to amplification difficulties. Additionally, only three to five alleles were sampled across the *sod, en,* and *COII* loci each for the Saint Vincent and Grenada populations. Amplification reactions were carried out using Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA), under the conditions listed in Table 2.

The total number of bases amplified from the *COII* locus encompassed 325 bp of coding region. The amplified *sod*

TABLE 2. Amplification properties of *sod, en, COII*, and *kl-5*. Primers for *sod* are as described in Kwiatowski et al. (1994). *COII* primers are as described in Brower (1994) and Spicer (1995). The remaining primers were developed in this study.

Locus	Location	DNA amplification primers	T_m
sod	Autosomal	N:CCTCTAGAAATGGTGGTTAAAGCTGTNTGCGT C:CTTGCTGAGCTCGTGTCCACCCTTGCCCAGATCATC	56.0
en	Autosomal	EnF:CGCCGCCCCAAGCARCCNAARRA EnR:TGGTGTGGTTGTACAGGCCYTGNGCCA	59.0
COII	mtDNA	LeuA:ATGGCAGATTAGTGCAATGG LysB:GTTTAAGAGACCAGTACTTG	52.0
kl-5	Y chromosome	Kl5F:CAAATTATGTGGACTGCTGAAAC Kl5R:AACTCATCTAAACATCCCCAAGC	56.0

region spanned 344 bp of coding region and the entire first intron of the gene (which was approximately 630 bp in length, with considerable variation due to insertions and deletions). The *en* locus encompassed 251 bp of coding region and the entire second intron of the gene (about 420 bp). Finally, we amplified 514 bp of coding region from *kl-5*.

The amplicons of *COII* and *kl-5* were each sequenced directly using an Applied Biosystems Big Dye Termination Kit and an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA). Amplicons from the two autosomal loci were cloned into the PCR2.1 cloning vector using a TOPO TA Cloning Kit (Invitrogen). A single clone from each individual was then sequenced using the same protocol as above. Estimating population genetic parameters from cloned PCR products is known to be problematic due to the inclusion of Taq-induced mutations that become amplified in the cloning process (Kobayashi et al. 1999). However, the magnitude of this problem can vary widely depending on the protocol and type of Taq polymerase used. To estimate the magnitude of this error in our present study, we re-sequenced approximately 5.4 kb of initial sequence from multiple cloned products. We found no differences in amplification products among clones. A single discrepancy between clones would have corresponded to an error rate of 1.95×10^{-4} mutations per base pair, which we use here as an upper-bound estimate for determining the potential number of spurious mutations in our study. This estimate is very similar to the cumulative Taq error rate determined in previous studies using similar methodology (e.g. Kwiatowski et al. 1991). Based on this estimated error rate, a maximum of 10 of the 267 total mutations observed in our study at *sod* and *en* (or 3.7%), may be caused by Taq error. Due to the nature of Taq-induced error, each of these mutations are likely to be low-frequency autapomorphies, and thus have no influence on phylogenetic inferences from the dataset (Kwiatowski et al. 1991). Further analyses in this study are confined to estimating parameters that are relatively insensitive to mutations introduced through Taq polymerase error and cloning. Sequences were deposited in GenBank under the accession numbers AY173140–AY173369.

Data Analysis

DNA sequences were inspected and edited visually using the program BioEdit version 5.0.9 (Hall 1999). Exon regions were excised from the two autosomal loci and only noncoding intron sequence was used in subsequent analyses. Intron/exon structure was determined by comparing the translated sequences with published *sod* and *en* data from *D. guttifera* (Kwiatowski and Ayala 1999; AF021826) and *D. virilis* (Kassis et al. 1986; X04727), respectively. Alignments were preformed using the program Clustal W (Thompson et al. 1994). The intron region of *en* contains a complex $(AC)_N$ microsatellite in each of the species surveyed. Because of alignment difficulties caused by high levels of apparent slippage mutations, this region was excluded from all subsequent analyses.

The program DNAsp version 3.53 (Rozas and Rozas 1999) was used to quantify levels of intra- and interspecific genetic variability across all loci. Nucleotide diversity for each locus and population was estimated by both the pairwise number of sequence differences, π (Nei and Li 1979), and Watterson's θ (Watterson 1975), which is based on the number of segregating sites in a population. In a stationary population both of these statistics are estimators of the quantity $4N_e\mu$ (where N_e is the effective population size and μ is the mutation rate) for the autosomes and $N_e\mu$ for the mitochondria and Y chromosome (with the additional assumption of an equal breeding sex ratio in the latter two cases). Sites with alignment gaps, which were abundant in the introns of the autosomal loci, were ignored in each analysis. The program Arlequin version 2.000 (Schneider et al. 2000) was used to perform an analysis of molecular variance (AMOVA) on all data from the *nigrodunni* complex. This analysis allows us to estimate the degree to which genetic variation is partitioned among populations at each locus.

Neighbor-joining gene trees for *sod*, *en,* and *COII* were constructed using the program PAUP* version 4.0b10 (Swofford 1998). Genetic distances were calculated for each dataset using the best-fit model of nucleotide substitution, which was estimated for each locus using hierarchical likelihood-ratio tests, implemented through the program Modeltest 3.06 (Posada and Crandall 1998). The same basic model of nucleotide substitution, HKY + G (Hasegawa et al. 1985), described each locus, although parameter estimates varied between loci (*sod*: transition/transversion ratio [ti/tv] = 1.0622, shape parameter of gamma distribution [α] = 0.6279; *en*: ti/tv = 1.0111, α = 0.4420; *COII*: ti/tv = 7.3804, α = 0.0975). The topologies of the resulting trees appeared relatively robust to the choice of substitution model, since very similar results were obtained using Jukes-Cantor genetic distances. Confidence in each tree was assessed by performing 1000 bootstrap replicates. Haplotype networks for the *COII* and *kl-5* loci were estimated with the program TCS version 1.13 (Clement et al. 2000), which uses statistical parsimony to estimate a cladogram, as described in Templeton et al. (1992).

Results

Intra-island Genetic Variation

The autosomal loci surveyed in this study exhibit high levels of polymorphism on nearly every island, as shown in Table 3. The mean value of π for island populations was 0.0184 at the *sod* locus and 0.0164 at the *en* locus. These values are comparable to those of the mainland population of *D. acutilabella*, which has π values of 0.0156 and 0.0183 for the two loci, respectively. Among individual island populations, there was a relatively wide range in the levels of genetic diversity. Nucleotide diversity at the *sod* locus was high in nearly all populations except Barbados (BA) and Grenada (GR). BA had a marginally lower than average value of π (0.0137), whereas that of GR was exceptionally low (0.0061). The *en* locus showed a wide range of variability over the dataset as well. However, only St. Kitts stood out as having a particularly low value of π (0.0068).

Our data for the mitochondrial and Y-linked genes showed much less variation in each population, as expected based on the lower effective population size of these loci. In the case of *COII,* populations contained from one to four haplotypes. In populations where more than a single haplotype was ob-

TABLE 3. Polymorphism data for each population. L, number of nucleotides surveyed; S, number of segregating sites; π, pairwise sequence divergence; θ, Watterson's (1975) estimator.

Population	Locus	L	S	π (SE)	θ (SE)
PRT	*sod*	559	25	0.0207 (0.0028)	0.0215 (0.0113)
	en	333	15	0.0234 (0.0043)	0.0216 (0.0118)
	COII	324	2	0.0027 (0.0008)	0.0027 (0.0021)
SK	*sod*	517	28	0.0196 (0.0033)	0.0221 (0.0105)
	en	323	6	0.0068 (0.0014)	0.0081 (0.0048)
	COII	324	3	0.0017 (0.0007)	0.0032 (0.0021)
	kl-5	514	0	0.0	0.0
GU	*sod*	494	32	0.0225 (0.0030)	0.0264 (0.0125)
	en	323	8	0.0112 (0.0020)	0.0119 (0.0069)
	COII	324	3	0.0017 (0.0008)	0.0029 (0.0019)
	kl-5	514	0	0.0	0.0
DO	*sod*	515	22	0.0172 (0.0021)	0.0187 (0.0094)
	en	323	10	0.0136 (0.0028)	0.0149 (0.0084)
	COII	324	1	0.0009 (0.0006)	0.0013 (0.0013)
	kl-5	514	0	0.0	0.0
MA	*sod*	537	40	0.0304 (0.0028)	0.0287 (0.0130)
	en	323	23	0.0252 (0.0035)	0.0291 (0.0141)
	COII	324	3	0.0014 (0.0005)	0.0028 (0.0018)
	kl-5	514	0	0.0	0.0
SL	*sod*	531	39	0.0230 (0.0030)	0.0283 (0.0128)
	en	302	18	0.0167 (0.0018)	0.0230 (0.0110)
	COII	324	2	0.0012 (0.0006)	0.0022 (0.0017)
	kl-5	514	2	0.0013 (0.0008)	0.0017 (0.0013)
BA	*sod*	544	17	0.0137 (0.0015)	0.0121 (0.0058)
	en	313	13	0.0131 (0.0016)	0.0160 (0.0080)
	COII	324	1	0.0010 (0.0005)	0.0011 (0.0011)
	kl-5	514	0	0.0	0.0
SV	*sod*	501	15	0.0120 (0.0066)	0.0144 (0.0078)
	en	332	10	0.0122 (0.0019)	0.0145 (0.0082)
	COII	324	0	0.0	0.0
GR	*sod*	520	5	0.0061 (0.0017)	0.0052 (0.0034)
	en	330	11	0.0226 (0.0064)	0.0222 (0.0143)
	COII	324	0	0.0	0.0
FL	*sod*	527	16	0.0156 (0.0037)	0.0146 (0.0079)
	en	309	11	0.0183 (0.0037)	0.0194 (0.0115)
	COII	324	0	0.0	0.0

served, levels of polymorphism were very similar between silent third sites at the *COII* locus and the introns of the autosomal loci, when corrected for effective population size (data not shown). The *kl-5* locus showed less variation than *COII* or either of the autosomal loci. Each population surveyed contained only a single haplotype, except for St. Lucia, which contained two haplotypes.

Inter-island Genetic Variation

Autosomal loci

Island populations of the *D. dunni* subgroup fall into three major clades based on phylogenetic analysis of both *sod* and *en*. These clades are shown in the phylograms in Figures 2 and 3. The first clade contains all individuals surveyed from the Puerto Rico/St. Thomas (PRT) population, and is supported by 100% of the bootstrap replicates at the *sod* locus and 96% at the *en* locus. The second clade contains all the St. Vincent (SV) and Grenada (GR) individuals (100% support at *sod* and 99% at *en*). The final clade contains all of the individuals from the remaining populations of St. Kitts, Guadeloupe, Dominica, Martinique, St. Lucia, and Barbados (94% support at *sod* and 76% at *en*). This final group corresponds to Heed's (1962) *nigrodunni* complex.

Although the same major clades are supported by both autosomal loci, the branching order of the clades varies somewhat between *en* and *sod.* The *en* tree suggests a branching order of PRT, followed by SV and GR, and finally the *nigrodunni* complex. However, this branching order is supported by only marginal bootstrap values (62% and 76%, respectively). At the *sod* locus, a different pattern is suggested, with the earliest divergence occurring between the *nigrodunni* complex and a lineage that includes the PRT, SV, and GR populations. A second branch supports the subsequent divergence of PRT from SV and GR. However, this branching order received only weak bootstrap support.

The results of the AMOVA for the autosomal data indicate that the great majority of genetic variation occurs within, rather than among, island populations of the *nigrodunni* complex. These results, shown in Table 4, indicate that 87.01% of *sod* variability occurs within populations, whereas only 12.99% exists among populations. The *en* locus shows a similar pattern, with 91.92% of variation within populations and 8.08% among populations. Although the among-population components of variation are very small at both loci, the Φ_{ST} values for both *sod* and *en* indicate significant population subdivision within the *nigrodunni* complex (Table 4).

Mitochondrial COII locus

The mitochondrial *COII* locus shows somewhat more population structure than the autosomal loci discussed above. In addition to delineating two strongly supported clades encompassing the Puerto Rico/St. Thomas and St. Vincent and Grenada populations, the data at this locus resolve a single island population within the *nigrodunni* complex, Martinique, as being monophyletic (shown in Fig. 4). Interestingly, the *COII* data do not resolve a branching order for any of the clades within the *D. dunni* subgroup, nor do they support a distinct clade encompassing the entire *nigrodunni* complex. It is also interesting to note that, like the autosomal loci, the *COII* data do not show any differentiation of the St. Vincent and Grenada populations, which both contain a single identical haplotype.

We have further explored the *COII* data by using statistical parsimony to construct a haplotype network (Templeton et al. 1992; Clement et al. 2000), shown in Figure 5A. We do not include data from the Puerto Rico/St. Thomas, St. Vincent, or Grenada populations because those samples are uninformative in the sense that there are too many mutational steps between these groups for them to be joined unambiguously with the *nigrodunni* complex (or for St. Vincent or Grenada to be joined to Puerto Rico/St. Thomas). Our data suggest that differentiation exists between four potential clades within the *nigrodunni* complex: one composed of all Martinique haplotypes, one containing a majority of the St. Kitts haplotypes, one containing a majority of the Barbados and all of the St. Lucia haplotypes, and a final group containing most Guadeloupe and Dominica haplotypes. In concordance with the structure indicated by the haplotype network, the AMOVA for the *COII* locus (Table 4) indicates that the great majority of *nigrodunni* complex genetic vari-

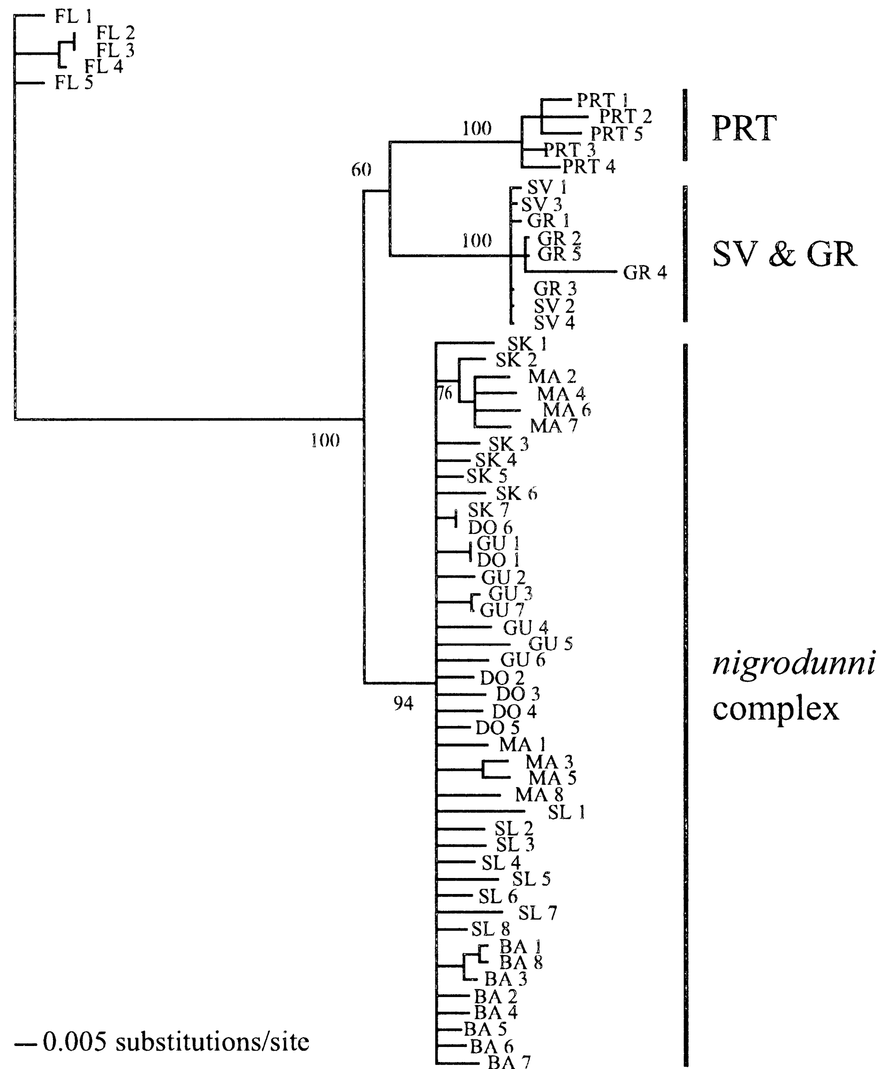

FIG. 2. Neighbor-joining tree from the *sod* intron.

ation is partitioned among populations (85.34%), with a much smaller proportion occurring within populations (14.66%).

The Y-linked kl-5 locus

Divergence of the *kl-5* locus between populations showed a different topology than that described for the autosomal loci or *COII*. A haplotype network for the data is shown in Figure 5B. The haplotypes from the St. Vincent and Grenada populations were highly divergent from those of the *nigrodunni* complex and cannot be joined to the network using our parsimony criteria. Within the *nigrodunni* complex, four populations (Martinique, Guadeloupe, Dominica, and St. Kitts) all share only a single haplotype. This pattern is particularly surprising because the relatively strong divergence of the Martinique population at the *COII* locus is not mirrored at *kl-5*. The two remaining populations, Barbados (BA) and St. Lucia (SL), each contain a unique haplotype, and both are separated from the remainder of the *nigrodunni* complex by two mutational steps. The first step is shared by BA and SL and consists of an isoleucine to methionine replacement mutation, whereas the steps separating BA from SL are silent mutations. Comparison with St. Vincent and Grenada as outgroups indicates that the replacement is a derived trait in the BA and SL populations. The results of the AMOVA (Table 4) show a similar pattern to that of *COII* in that the majority of genetic variation exists among populations (91.21%), with only a small proportion occurring within populations (8.79%).

DISCUSSION

Worldwide, many island archipelagos contain endemic species of *Drosophila*, including well-known examples, such as endemic members of the *D. melanogaster* subgroup on islands in the Indian and Atlantic Oceans and the spectacular *Drosophila* radiation on the Hawaiian archipelago. Studies of these taxa have revealed that the demographic processes occurring during island speciation can be quite variable. An illustrative case is that of the island endemics *D. sechellia*

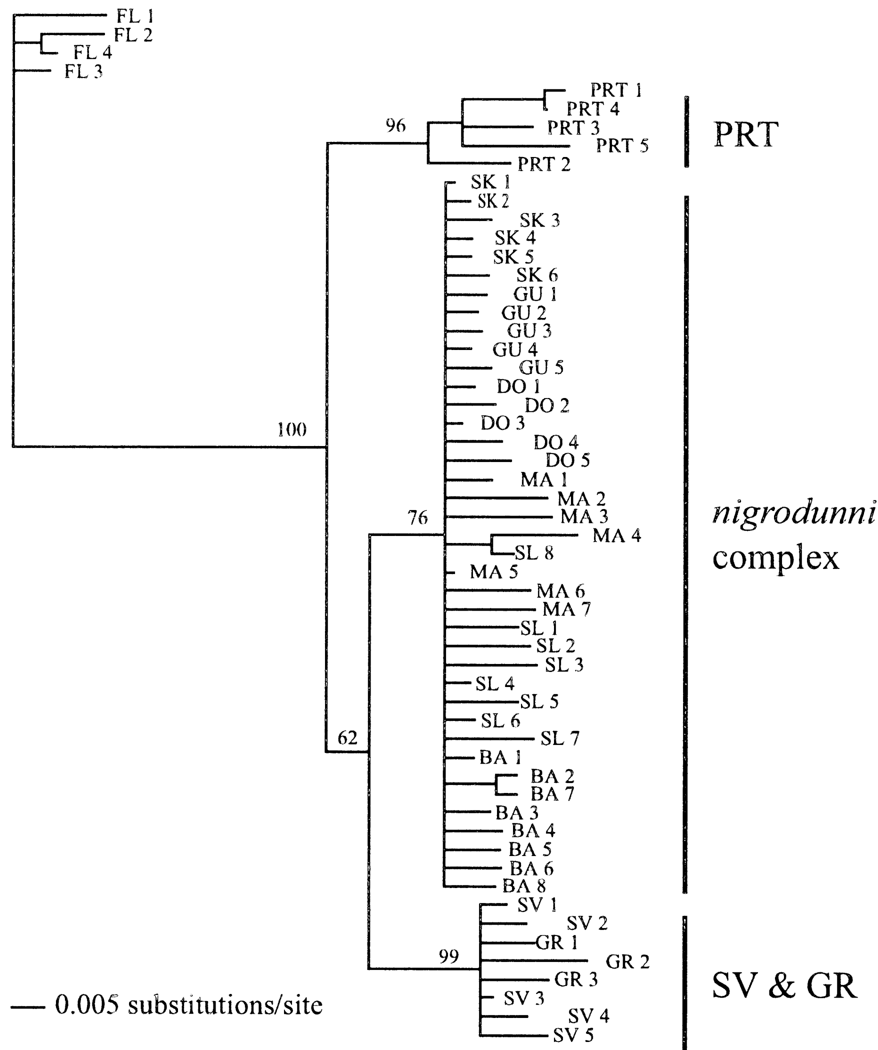

FIG. 3. Neighbor-joining tree from the *engrailed* intron.

and *D. mauritiana*, which are both recently derived from the widespread African species *D. simulans*. Kliman et al. (2000) have shown that these two speciation events took place at approximately the same time. However, *D. sechellia* has a pronounced lack of genetic variation at most genomic loci, whereas *D. mauritiana* has relatively high levels of polymorphism at many loci. Furthermore, *D. mauritiana* shares much variation with *D. simulans*, whereas *D. sechellia* does not. These patterns reveal fundamental differences in the evolutionary history of these species, despite their superficial similarity. Although isolation from the mainland was clearly important in each case, the role of population bottlenecks in shaping the extant genetic variation in each species has been profound. *Drosophila sechellia* has clearly experienced a pronounced bottleneck that has drastically reduced variation, whereas *D. mauritiana* has not. These results indicate that extreme population bottlenecks are one possible mode of *Drosophila* speciation on islands, but not the only one.

The *D. dunni* system differs significantly from the above cases because most (or all) endemic island species in the group are derived from other insular island taxa, as indicated by the monophyly of the subgroup. Although there has been a significant opportunity for loss of genetic variation as it has been filtered through successive colonization events, our

TABLE 4. Analysis of molecular variance within the *nigrodunni* complex for each locus.

Proportion of variation	*sod*	*en*	*COII*	*kl-5*
Among populations (%)	12.99	8.08	85.34	91.21
Within populations (%)	87.01	91.92	14.66	8.79
Φ_{ST} (*P*-value)	0.130 (<0.001)	0.081 (0.007)	0.853 (<0.001)	0.912 (<0.001)

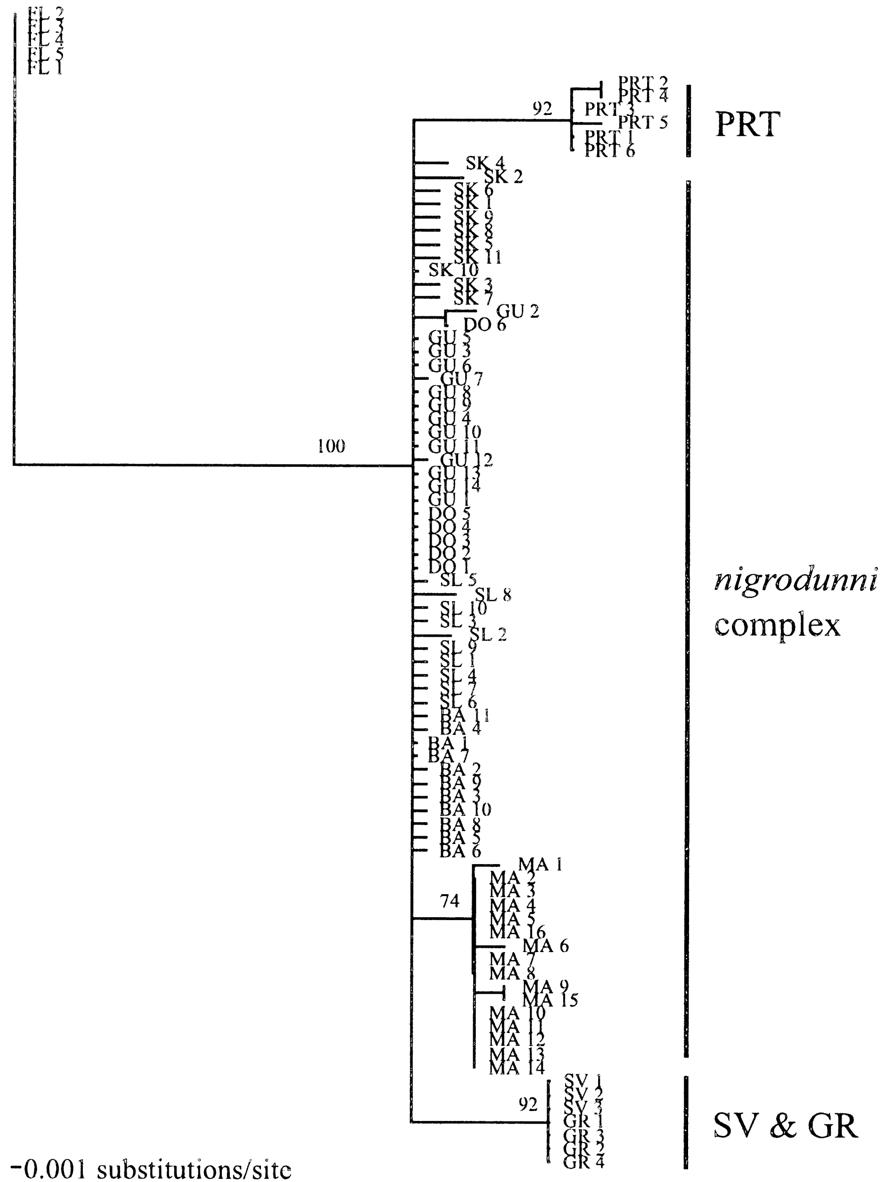

FIG. 4. Neighbor-joining tree from the mitochondrial *COII* gene.

data indicate that this has not had a large impact on the extant variability within *D. dunni* populations. At the three loci that we surveyed in both the *D. dunni* subgroup and the Florida population of *D. acutilabella*, we found that genetic variation is not reduced in the island populations compared to the mainland. In fact, there are numerous island populations where variation actually exceeds that of the mainland at both the mitochondrial and autosomal loci (Table 3), and no island has markedly low levels of variation across all loci. The similar amounts of genetic variation between the mainland species and island members of the *D. dunni* subgroup indicates that bottlenecks played only a weak role, at most, in the history of these populations. Further, our results do not provide support for the serial bottlenecks hypothesized in Heed's (1962) ''island hopping'' model. Still, weak historical bottlenecks cannot be completely ruled out, because relatively little variation may be lost if a reduction in population size is of short duration and followed by a rapid population expansion (Nei et al. 1975; Eyre-Walker et al. 1998; Fay and Wu 1999; Grant et al. 2001).

Interestingly, the only genetic locus we surveyed with markedly low levels of variation was the Y-linked *kl-5* gene. Although we could not compare this locus with mainland members of the *D. cardini* species, our results are consistent with population studies of this gene in the species *D. simulans* and *D. melanogaster*, which also have extremely low levels of polymorphism (Zurovcova and Eanes 1999). To some degree, this is an expected pattern for polymorphism surveys of the Y chromosome, because it is one of the regions of the *Drosophila* genome least affected by recombination. This lack of recombination exposes the entire Y chromosome to genetic hitchhiking through both background selection and

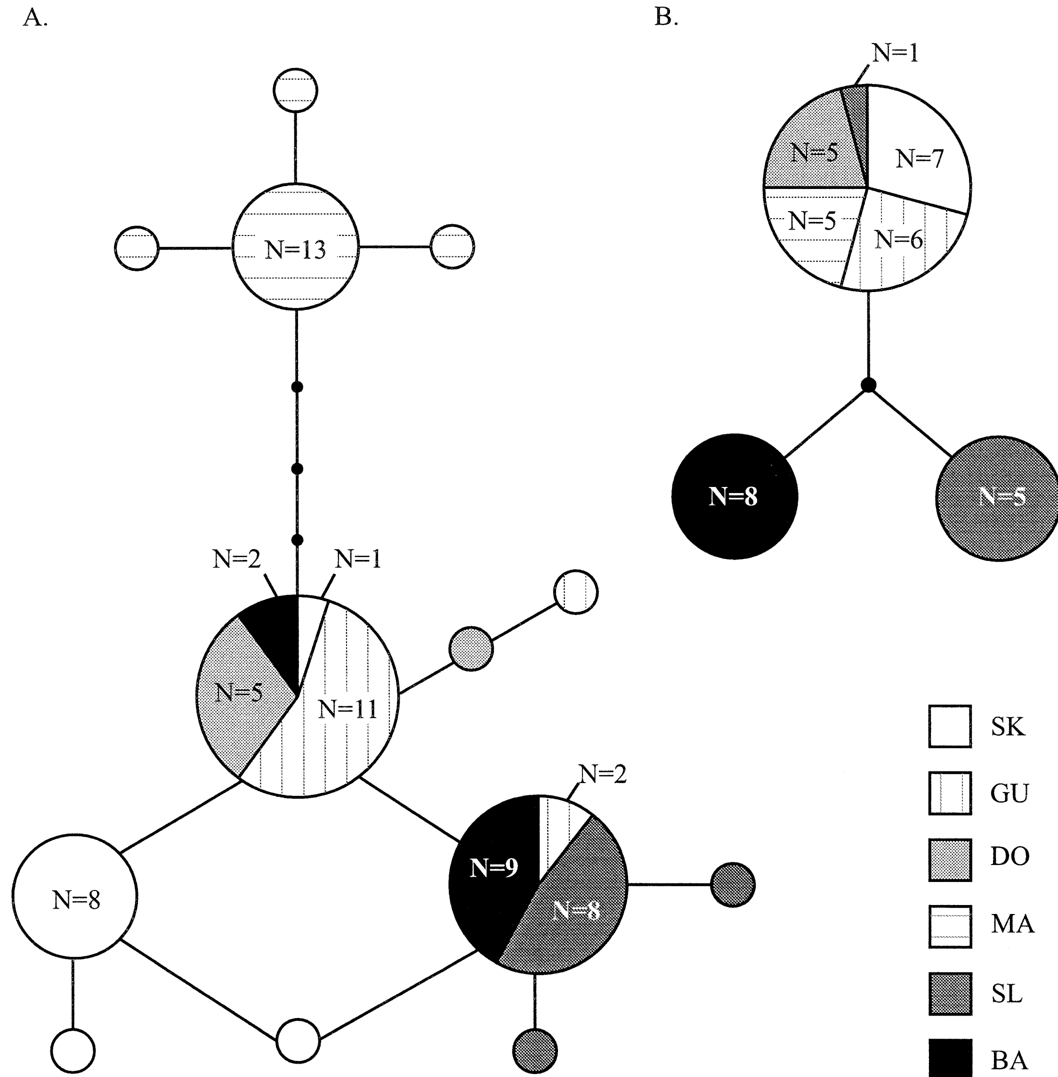

FIG. 5. Haplotype networks for the *COII* (A) and *kl-5* (B) loci. The legend indicates islands of origin for each haplotype (codes as defined in Table 1). Small circles indicate haplotypes observed only once in our sample; dots indicate unobserved intermediate haplotypes.

periodic selective sweeps (Maynard-Smith and Haigh 1974; Kaplan et al. 1989; Stephan et al. 1992; Charlesworth et al. 1993; Jensen et al. 2002). An additional potential cause of the low level of polymorphism at *kl-5* is that the effective size of the *Drosophila* Y chromosome may be even less than one-fourth that of the autosomes (the expected N_e, given an equal breeding sex ratio) if the effective population sizes of the sexes are not equal (Charlesworth 2001). *Drosophila melanogaster* males have been shown to have a much higher variance in mating success than females (Bateman 1948; Crow and Morton 1955). If this is also true in members of the *D. dunni* subgroup, it could contribute to the low levels of observed Y chromosomal variation.

Genetic Differentiation within the D. dunni Subgroup

Our phylogenetic analyses divide the *D. dunni* subgroup into three distinct lineages, in concordance with previous genetic and morphological studies (Heed 1962; Hollocher 1996). One of these lineages contains a single population encompassing Puerto Rico and the Virgin Islands (PRT). A second lineage contains two populations, St. Vincent and Grenada (SV/GR), which are genetically indistinguishable at the loci we have surveyed. The final lineage, the *nigrodunni* complex, is composed of six island populations (St. Kitts, Guadeloupe, Dominica, Martinique, St. Lucia, and Barbados). These three clades received strong bootstrap support in our analyses of *sod* and *en,* whereas only the PRT and SV/GR clades were strongly supported at the *COII* locus. The failure of the *COII* data to delineate a monophyletic group encompassing the entire *nigrodunni* complex or to describe a branching order for the major *D. dunni* clades differs from a previous analysis of mtDNA diversity in the *D. dunni* subgroup (Hollocher 1996). This previous work surveyed a much larger portion of the mtDNA molecule, and thus provided more resolution with regard to these portions of the phylogeny. In the present study we examined many more individuals (although over a shorter sequence), which allowed us to more precisely delineate patterns of haplotype sharing among the

closely related populations. Indeed, this approach leads us to question the earlier phylogenetic inference of a monophyletic clade containing the Barbados and St. Lucia populations, which was based on an apparently fixed difference in the *COII* region. Our more complete sampling indicates that this mutation is actually a shared polymorphism among several *nigrodunni* complex populations.

To estimate the divergence times of the three major clades, we have applied the mitochondrial molecular clock from Hawaiian *Drosophila* of 2.0% divergence per million years (Desalle et al. 1987) to our data. Based on this rate of divergence, the *nigrodunni* complex appears to have diverged from the St. Vincent and Grenada clade 1.6 million years ago (MYA); 3.2% divergence), and from Puerto Rico/St. Thomas 1.9 MYA (3.6% divergence). These latter clades diverged from one another somewhat earlier, at 2.6 MYA (5.1% divergence). These divergence times should be considered only as approximations as they rely on a number of tenuous assumptions, including a constant rate of evolution between the lineages for which the rate was estimated and our study system, as well as uncertainty in the initial estimation of species divergence times based on island formation events in the Hawaiian archipelago (Russo et al. 1995).

Of particular interest in our dataset is whether or not there is any indication of genetic differentiation within the *nigrodunni* complex lineage, because of the dramatic pigmentation divergence and reproductive isolation that exists among many of these populations. Interestingly, there is a great deal of heterogeneity among loci with respect to patterns of genetic divergence. The autosomal loci reveal no significant differentiation of any populations based on our phylogenetic analyses (Figs. 2 and 3). Further, the AMOVA indicates that the great majority of autosomal genetic variation occurs within rather than among these populations (Table 4). This pattern is somewhat surprising, as it indicates a large amount of ancestral variation is shared among islands.

In contrast to the autosomes, *COII* and *kl-5* show significant population differentiation of a number of *nigrodunni* complex populations, and also show a much higher proportion of variation partitioned among populations (Table 4). Our phylogenetic analysis of *COII* indicates complete monophyly of the Martinique (MA) population (Fig. 4). In addition, the *COII* haplotype network (Fig. 5A) shows potential differentiation of a number of island groups, including one containing the Barbados (BA) and St. Lucia (SL) populations, one containing Guadeloupe (GU) and Dominica (DO), and one that contains only the St. Kitts (SK) population. Interestingly, the haplotype network of the *kl-5* locus differentiates a different set of *nigrodunni* complex populations (Fig. 5B). At this locus, the SK, GU, DO, and MA populations all share a single haplotype, whereas BA and SL are each composed of predominantly unique haplotypes.

The lack of congruence between autosomal, mitochondrial, and Y-linked patterns of differentiation among members of the *nigrodunni* complex may simply be the result of each locus being in distinct (and incomplete) phases of lineage sorting. At the autosomal loci, the great majority of genetic variation is shared among populations, indicating that divergence is likely to have occurred more recently than the average coalescence time for autosomal loci, or $4N_e$ generations. We have previously used microsatellite data to independently estimate N_e in the Guadeloupe population at 2.3×10^5 (Wilder et al. 2002). If we assume this value to be similar across all populations, and assume 10 generations per year, then we predict that the populations of the *nigrodunni* complex diverged within the last 92,000 years (i.e. $4 \times 2.3 \times 10^5 \div 10$). We emphasize that this date should be considered only approximate given the great deal of stochasticity inherent in the coalescence process (Hudson and Turelli 2003). Still, the complete lack of autosomal monophyly, and rarity of mitochondrial or Y chromosome monophyly among any members of the *nigrodunni* complex, indicates the very recent divergence of these populations.

The Geography of Divergence

As shown by the phylogenies of the mitochondrial and autosomal loci, the three major lineages of the *D. dunni* subgroup are genetically distinct and we estimate them to have been isolated in allopatry on the islands of the eastern Caribbean for 1.6–2.6 million years. The origin of the three lineages may have coincided with the initial colonization of the Lesser Antilles from the Greater Antilles (the home of the Puerto Rico/St. Thomas population, and the other most closely related members of the *D. cardini* group). Shortly thereafter, the newly colonized islands may have differentiated into northern, middle, and southern populations, representing the modern Puerto Rico/St. Thomas, *nigrodunni* complex, and St. Vincent/Grenada lineages, respectively. Our phylogenetic analysis provides support for this hypothesis in the short internode distances separating the three lineages, indicating that they became isolated contemporaneously. Additionally, if these diversification events occurred over a relatively short time span, lineage sorting may have been incomplete between the first and second divergence events, reflected by the variable lineage branching orders that we observe among loci.

The existence of three highly distinct clades in the *D. dunni* subgroup illustrates several major biogeographic features of the eastern Caribbean. In our data we see a relatively deep phylogenetic division that occurs between St. Vincent and St. Lucia in the south and St. Kitts and the Virgin Islands in the north (Fig. 6). Phylogenetic breakpoints at these points are a remarkably common feature of Caribbean biogeography. In particular, the break between St. Vincent and St. Lucia, two islands that have very similar environments, is seen in an extraordinarily broad range of taxa. The break appears most robust among insects, where it has been observed among organisms with extremely diverse ecologies. These taxa include Hemipterans of the family Lygaeidae (Slater 1988), flightless Carabid beetles of the genus *Platynus* (Liebherr 1988), and the butterfly species *Dryas iulia* (Davies and Bermingham 2002). However, the break is not restricted to insects and has also been observed for frogs of the genus *Eleutherodactylus* (Kaiser et al. 1994), and the bird species *Coereba flaveola* (Seutin et al. 1994). One of the few taxa for which a major phylogenetic breakpoint has been described that does not occur between St. Vincent and St. Lucia is the *Anolis* lizards. In this group a break has been described two islands to the north, between Dominica and Martinique

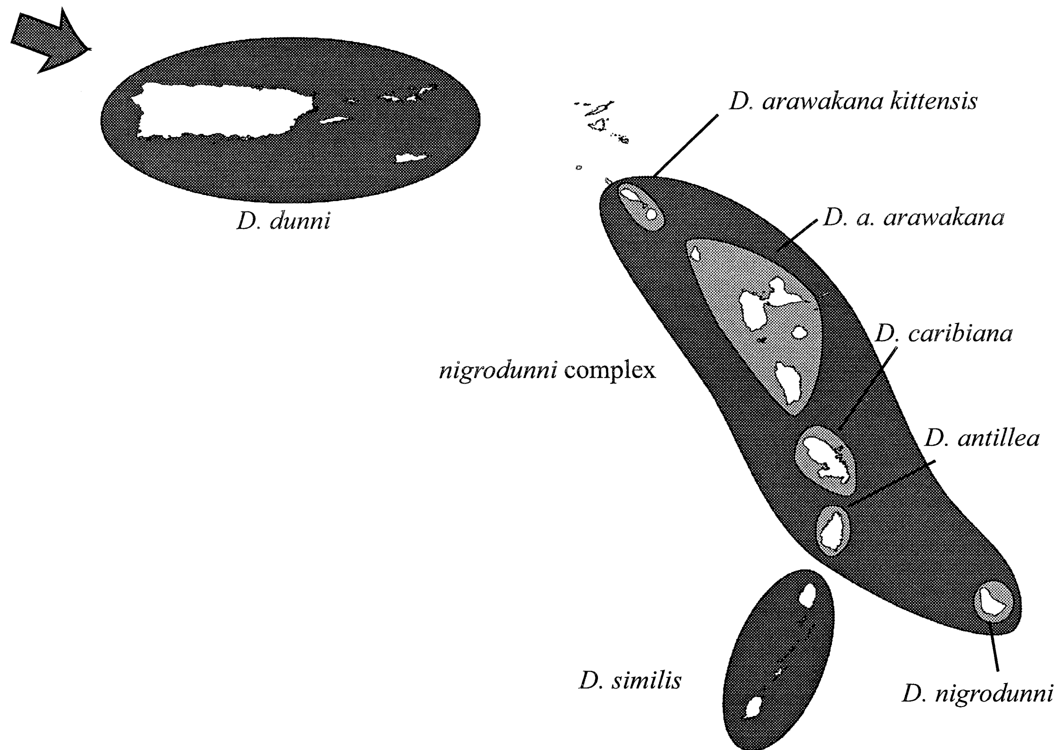

FIG. 6. Three lineages of the *Drosophila dunni* subgroup. Bubbles indicate patterns of divergence among members of the *D. dunni* subgroup. Darker circles indicate older lineages that diverged approximately 1.6–2.6 million years ago, and lighter circles indicate populations that diverged within the last 92,000 years. The arrow indicates the probable origin of the entire subgroup from the Greater Antilles.

(Roughgarden 1995). However, no other taxa, including the *Plasmodium* parasites of the anoles (Perkins 2001), have been observed to share this breakpoint, indicating that it is relatively unique among the *Anolis* lizards. Based on the preponderance of taxa that appear to share the deep phylogenetic split between St. Vincent and St. Lucia, the gap between those islands appears to be a remarkably prominent feature of Caribbean biogeography.

Our data from the *D. dunni* subgroup illustrate an important point regarding this break between St. Vincent and St. Lucia. Among many taxa this phylogenetic split occurs because it is the point where competing dispersal streams from the Greater Antilles and South America meet (e.g. the butterfly *Dryas iulia* and the lygaeids; Slater 1988; Davies and Bermingham 2002). However, all of the populations we describe here appear to have originated in the Greater Antilles, with none having particularly close affinities with any South American species compared to species from the Greater Antilles (Heed 1962; Hollocher 1996). Thus, our data indicate that the barrier to dispersal between these islands is to some extent independent of major north-south dispersal streams. The gap between St. Lucia and St. Vincent, once crossed, has led to the complete isolation of the St. Vincent/Grenada lineage from the *nigrodunni* complex, despite the fact that all *D. dunni* populations originated in the north. This observation strengthens the view that the water gap between St. Vincent and St. Lucia is a real barrier to dispersal for numerous organisms and is not simply an arbitrary point where dispersal streams from the north and south tend to meet.

Populations, Species, or Subspecies: Reconciling Our Data with Taxonomy

In this work we have addressed populations as our evolutionary units of study, without regard to the existing taxonomy of the group. An examination of our data shows that the taxonomic work of Heed (1962) and Heed and Krishnamurthy (1959) has held up extremely well. These studies divided the *D. dunni* subgroup into six species, shown in Figure 6. Two of these encompass single lineages identified in our study, with *D. dunni* composing the Puerto Rico/St. Thomas population and *D. similis* composing St. Vincent and Grenada. The original taxonomy of Heed (1962) considers these latter two islands to be inhabited by two distinct subspecies that are completely interfertile, *D. s. similis* and *D. s. grenadensis*, respectively. At the loci we surveyed, we were not able to detect any genetic differentiation of these populations, indicating that they may only be in the very early stages of divergence, or may in fact be connected by regular gene flow. Unlike any other islands in our study, the St. Vincent and Grenada populations are connected by a dense chain of intermediate islands (the Grenadines), which may facilitate dispersal between them.

The third lineage in our study encompasses the *nigrodunni* complex, or the St. Kitts, Guadeloupe, Dominica, Martinique, St. Lucia, and Barbados populations. These populations are divided into four separate species (Fig. 6). The autosomal loci we surveyed do not identify any genetically isolated populations within this group, but *COII* and *kl-5,* in combi-

nation, show distinct patterns of differentiation for all populations except Guadeloupe and Dominica. Thus, the delineation of four species by Heed (1962) is supported by our genetic data. Furthermore, two populations that were originally described by Heed (1962) as subspecies, Guadeloupe and St. Kitts, (which contain *D. a. arawakana* and *D. a. kittensis*, respectively) are genetically differentiated from one another at the *COII* locus. Thus, despite their complete interfertility in the laboratory, these populations appear to represent populations with independent evolutionarily trajectories.

One notable result of our study is that the Dominica population is genetically indistinguishable from Guadeloupe based on the loci we surveyed. The Dominica population was not sampled in the initial survey of *D. dunni* forms from the eastern Caribbean, and was thus not included in initial taxonomic descriptions (Heed and Krishnamurthy 1959; Heed 1962; Heed and Russell 1971). Based on the genetic similarity with the Guadeloupe population, our results indicate that the Dominica population should be classified as *D. arawakana*.

Synthesis

The *D. dunni* subgroup exhibits a unique pattern of endemism on the islands of the eastern Caribbean. Our analyses describe populations that are divergent to greatly varying degrees, ranging from an ancient split between three lineages at the northern, central, and southern regions of the eastern Caribbean, to extremely recent differentiation among the islands of the central Lesser Antilles, summarized in Figure 6. Thus, the system we describe represents an opportunity to examine the process of speciation among island endemics at multiple stages of divergence, from older well-established insular species, to young species that have only recently been isolated from neighboring populations.

Among the most intriguing patterns that we see are those in the *nigrodunni* complex. It is interesting to consider the mode of speciation in such a recent and rapid divergence, particularly in light of the dramatic differences in pigmentation between these species. Our data do not indicate that either ''island hopping'' or bottleneck effects associated with founder events have greatly influenced the genetic diversity of these populations. Instead, we hypothesize two scenarios that may have led to the observed pattern. First, members of the *nigrodunni* complex may have once been a panmictic multi-island population, but have recently undergone a cessation of inter-island gene flow, which has allowed for the divergence of separate populations. Alternatively, the data are consistent with islands having been genetically isolated since colonization, so long as bottlenecks accompanying colonization were sufficiently weak not to cause a reduction in genetic variation. Geological evidence does not provide any obvious evidence to support either hypothesis of the *nigrodunni* complex origin. With the exception of Barbados, which has only been above sea level for approximately 700,000 years, the Lesser Antilles are a series of volcanic islands that have occupied their current position, and have remained unconnected to one another, for approximately 20 million years (Mesolella 1967; Bender et al. 1979; Donnelly 1988). Thus, the events that have shaped the evolution of the *nigrodunni* complex (and indeed the entire *D. dunni* subgroup) appear to vastly postdate the formation of the eastern Caribbean islands.

Whatever the exact mode of differentiation, the radiation of the *D. dunni* subgroup presents a fascinating system in which to study speciation. Despite episodic genetic divergence among members of the subgroup, variation in the remarkable morphological cline in pigmentation is regular across both ancient and recent lineage divisions, indicating the almost certain role of natural selection in causing the pigmentation differences. Natural selection may be acting on other traits as well. For instance, ethanol tolerance and Adh activity have been found to be different between *D. arawakana* and the remainder of the *nigrodunni* complex (Colón-Parrilla and Pérez-Chiesa 1999). However, we know of no other selective force that appears to be acting across the entire *D. dunni* subgroup and to have played so dramatic a role in shaping the morphological evolution of these species. Our study reveals important questions regarding the nature of this cline. Based on our divergence estimates, it appears that the morphological evolution that has taken place has done so at a very rapid pace, especially among members of the *nigrodunni* complex. Thus, it may represent a recent change in the selective regime to which these species are exposed, possibly a change that has caused speciation as well as morphological differentiation. Elucidating the molecular pathways responsible for the abdominal pigmentation traits and the functional role of the pigmentation differences among these species will reveal important clues into the process of adaptation and perhaps the process of speciation as well.

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