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AFRICAN MORPHOLOGY, BEHAVIOR AND PHERMONES  
UNDERLIE INCIPIENT SEXUAL ISOLATION BETWEEN US  
AND CARIBBEAN *DROSOPHILA MELANOGASTER*

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## Abstract:

Understanding incipient sexual isolation and speciation is an important pursuit in evolutionary biology. The fruit fly *Drosophila melanogaster* is a useful model to address questions about the early stages of sexual isolation occurring within widespread species. This species exhibits sexual isolation between cosmopolitan and African flies, especially from Zimbabwe populations. In addition, we have recently described another example of partial sexual isolation between some US and Caribbean populations. This and other phenotypic data suggests that Caribbean flies might be segregating African traits. In the present work we study the geographical variation at the pheromone locus desaturase-2, as well as morphology and courtship behavior across the US-Caribbean region. We find that US and Caribbean populations show sharp geographical clines in all traits and demonstrate that Caribbean traits are more similar to those of Africa than to US populations. Further, African traits in the Caribbean are associated with sexual isolation and best explain variation in sexual isolation when all traits are considered together. These results imply that Caribbean mating preferences are likely to be based on African traits and that even at such early stages of sexual isolation, individuals may already cue in on several traits simultaneously during mate choice.

## *Introduction*

Sexual or behavioral isolation is common in nature and plays a central role in maintaining genetic and phenotypic differences between sibling species (Dobzhansky 1937; Mayr 1942; 1963; Coyne and Orr 2004). Understanding how sexual isolation evolves requires that we capture the process before it has reached completion. Of particular interest are natural populations that occupy different environments and that have diverged in traits involved in assortative mating (Dobzhansky and Streisinger 1944; Koref-Santibáñez 1972; Verrell and Arnold 1989; Tilley et al. 1990; Endler and Houde 1995; Johannesson et al. 1995; Noor 1995; Funk 1998; Tregenza et al. 2000; Tregenza 2002; Jiggins et al. 2001; Nosil et al. 2002; Pfennig and Simovich 2002; Jiggins et al. 2004; Boughman et al. 2005; Ortiz-Barrientos and Noor 2005; Nosil et al. 2007). These and other examples of incipient sexual isolation have helped to elucidate the geographical, ecological and historical context of early stages of speciation.

Recent evidence suggests that incipient sexual isolation may occur multiple times in various parts of the species range, especially among geographically widespread species (e.g. Rundle et al. 2000; Nosil et al. 2002; Tregenza 2002; Boughman et al. 2005). It thus becomes important to understand the similarities and differences between multiple cases of sexual isolation within species. For instance, how much genetic and phenotypic differentiation accompanies each case of incipient sexual isolation, which phenotypic traits typically diverge, and what is the genetic and phenotypic basis of assortative mating in each case (e.g. Panhuis et al. 2001; Hendry 2001; Boughman et al. 2005; Nosil 2005; Hendry et al. 2007).

If multiple cases of sexual isolation were to show unique genetic and phenotypic bases, it would indicate that species segregate different types of variation associated with assortative mating in nature. On the other hand, if different cases of sexual isolation share common phenotypes and occupy similar environments, it would imply that there might be adaptive constraints on the conditions and the type of variation underlying these processes. For instance, it is likely that assortative mating may evolve as a byproduct of adaptation to similar environments (see Schluter and Nagel 1995; Rundle and Schluter 2004; also see artificial selection evidence: Killas et al. 1980; Dodd 1989, but see Rundle

2003). This is known as “parallel speciation” and has been recently documented in benthic versus limnetic forms of sticklebacks (e.g. Rundle et al. 2000; McKinnon et al. 2004; Boughman et al. 2005; Vines and Schluter 2006), host-associated races of phytophagous insects (e.g. Funk 1998; Nosil et al. 2002) and possibly in color morphs of cichlids (Allender et al. 2003). In these examples, parallel cases of sexual isolation within species have been argued to evolve independently of one another since populations with parallel phenotypes are typically genetically distant (e.g. Taylor and McPhail 1999, 2000; Rundle and Schluter 2004; but see Coyne and Orr 2004 pg. 406-7). More direct tests of independent genetic basis of parallel evolution of assortative mating behaviors and cues have yet to be performed.

On the other hand, if the same alleles are involved in multiple cases of sexual isolation within species it is most likely that this is a result of common genetic history (e.g. Rundle and Schluter 2004). Migration of alleles responsible for parallel cases of adaptation has recently been described in several species (Colosimo et al. 2004, 2005; also see Schluter et al. 2004). Similarly, it is possible for assortative mating behaviors and associated traits that initially caused sexual isolation in one geographical region to be introduced into other populations, recreating the phenomenon in whole or in part. This scenario may also involve ecological adaptation. For instance, if assortative mating originally evolved as a byproduct of ecological adaptation, it is likely that these behaviors would establish in places where they are preadapted to similar environments. Thus, assortative mating behaviors and associated traits may migrate in a non-random way throughout the species range.

This “historical migration” scenario would imply that once certain population pairs evolve incipient sexual isolation, assortative mating could spread into other populations and thus accelerate the process of speciation across the species range. This would likely lead to multiple cases of incipient sexual isolation more quickly, compared to independent or parallel speciation since migration of relevant alleles is likely to occur on ecological rather than evolutionary time scales. Presently, it is unclear whether there are any biological candidates of this scenario in nature.

The fruit fly *D. melanogaster* is a powerful genetic model species to address questions about the genetic and phenotypic basis of incipient sexual isolation in nature. In

this species, matings between Zimbabwe females and males from outside this region (known as “cosmopolitan”) are typically very rare (Wu et al. 1995; Hollocher et al. 1997a, 1997b). Cosmopolitan females also tend to prefer to mate with their own males, but in this case the preferences are often weaker (Wu et al. 1995). It has been postulated that sexual isolation may have originated as a byproduct of adaptation to temperate versus tropical environments of cosmopolitan and Zimbabwe populations, respectively (Greenberg et al. 2003; but see Coyne and Elwyn 2006).

This species also segregates for mating preferences in other parts of the world, such as the case of “microhabitat” sexual isolation in the West African Brazzaville populations (Capy et al. 2000; Haerty et al. 2002, 2005). Moreover, we have recently discovered that Caribbean and West African populations mate randomly with each other, but show partial sexual isolation against US cosmopolitan flies and against Zimbabwe flies (Yukilevich and True 2008). It is also noteworthy that crosses between Caribbean and Zimbabwe populations show weaker sexual isolation compared to crosses between US and Zimbabwe populations. These results are consistent with previous findings that some Caribbean populations have African-like morphology and pheromones (David and Capy 1988; Capy et al. 1993, 1994; Rouault et al. 2001; Takahashi et al. 2002; but see Fang et al. 2002; Coyne and Elwyn 2006). This has led some to suggest that Caribbean populations may ultimately be of African, rather than European origin, perhaps having come directly via the Trans-Atlantic slave trade from various parts of Africa, especially from West Africa (e.g. David and Capy 1988; Caracristi and Schlotterer 2003).

The Caribbean island populations of *D. melanogaster* have been little studied. Not much is known about pheromonal, phenotypic and behavioral variation across US and Caribbean populations and the extent to which African-like phenotypes segregate in the region. Also, it is unknown what geographical relationships exist between this case of sexual isolation and phenotypic variation that may be involved in mate choice (e.g. Bastock and Manning 1955; Bastock 1956; Ewing 1961, 1964; Partridge et al. 1987; Taylor and Kekic 1988; Pitnick 1991; Grillet et al. 2006; Coyne and Elwyn 2006).

In the present paper we study variation in several traits that are of particular interest for sexual isolation in *D. melanogaster*. Thus we address whether the case of incipient sexual isolation between US and Caribbean flies shares phenotypic similarity to

that found between cosmopolitan and African flies. First, we focus on the *desaturase-2* (*desat-2*) locus that is solely responsible for the difference in a major female cuticular hydrocarbon between African and non-African flies (Coyne et al. 1999; Dallarec et al. 2000; Takahashi et al. 2001; Greenberg et al. 2003). It has been previously reported that non-African populations carry a derived deletion allele at this gene, which results in a high 5,9-heptacosadiene (HD) female phenotype, whereas African populations have retained the ancestral allele, which confers much less 5,9-HD, but high levels of 7,11-HD (see e.g. Takahashi et al. 2002; Coyne and Elwyn 2006). The only non-African populations known to segregate the African ancestral allele are Caribbean populations (Rouault et al. 2001).

This locus has recently been proposed to play a major role in local adaptation to tropical versus temperate environments and as a byproduct to cause sexual isolation between cosmopolitan and Zimbabwe strains (Fang et al. 2002; Greenberg et al. 2003). However, the view that *desat-2* contributes to environmental adaptation has recently been challenged (Coyne and Elwyn 2006). Nevertheless, Coyne and Elwyn still provided some support for the claim that *desat-2* may be partially responsible for sexual isolation. Thus it is of interest to characterize geographical variation at this gene across the US and Caribbean region and to test for any associations with sexual isolation.

Second, we study male morphology. Previous research on a few isofemale lines from the Lesser Antilles revealed that these individuals have smaller body size than those from temperate populations of Europe and US and are thus most similar to African flies, which are also relatively small (Capy et al. 1993, 1994). Male body size is known to be associated with male mating success in both laboratory and field conditions, with females typically preferring to mate with large males (Ewing 1961, 1964; Partridge et al. 1987; Taylor and Kekic 1988; Pitnick 1991). Here we present geographical patterns of male morphology and its relationship to sexual isolation.

Finally, we investigate male courtship behavior since it is known to be under direct sexual selection in *D. melanogaster* (e.g. Bastock and Manning 1955; Bastock 1956). In particular, these studies have demonstrated that mutants with lower frequencies of certain courtship elements have reduced mating success compared to wildtype males. Even though little is known about variation in courtship behaviors in *D. melanogaster*,

including courtship behavior of African males, we have characterized this variation because of its potential role in mate choice.

Our results show that most Caribbean populations are divergent from US populations at the *desat-2* pheromone locus and in morphology and courtship behaviors and that these traits in the Caribbean populations are more similar to both West Africa and Zimbabwe than to US populations. We also demonstrate that these African traits are positively geographically correlated with the strength of sexual isolation, indicating that incipient sexual isolation between US and Caribbean flies and between cosmopolitan and African flies are associated with similar phenotypic trait differences. Thus our results reveal many phenotypic parallels between these different cases of incipient sexual isolation and raise further questions about the genetic makeup of Caribbean populations relative to West Africa and Zimbabwe.

## *MATERIALS AND METHODS*

### **STUDY SYSTEM**

In the summers of 2004 and 2005, R. Yukilevich collected and established isofemale lines from 39 different locations in the southeastern US and various Caribbean islands including the Bahamas Archipelago, Cuba, Haiti, and St. Lucia in the Lesser Antilles (see Fig. 1 for map and Appendix Table A1 for details). African isofemale lines were acquired from J. Pool and C. Aquadro in 2005, which included West African populations as well as southeastern African lines from Zimbabwe (Appendix Table A1). All lines have been maintained at Stony Brook University laboratory on instant *Drosophila* food (Carolina Biol. Supply Inc., Burlington, NC) in a 25°C temperature incubator with a 12h light:12h dark regime. Our analyses were performed on lines that were generally less than two years old.

The sampled populations of *D. melanogaster* represent a 10-degree change in latitude and a 15-degree change in longitude, with about 2,500 km separating the most distant populations (Fig. 1). Preliminary collections in 2003 by R. Yukilevich suggest that these populations are fairly stable within and between years, although episodic regional events, such as droughts and hurricanes, may potentially affect the demography

of the populations. Climate differs across the localities. Cooler and more humid conditions prevail on the mainland, where pines are the dominant vegetation, and hotter and dryer conditions occur on the islands, which are dominated by mangrove and scrub (Correll and Correll 1982; Baker 2001). The number of *Drosophila* species also varies across locations ranging from about fifteen sympatric species in the U.S. to zero sympatric species on many of the far-flung islands. Human population size declines from mainland to islands with a few thousand to a few hundred people on the islands (Baker 2001). *D. melanogaster* abundance tends to parallel this decline.

### **DESATURASE-2 LOCUS VARIATION**

The *desaturase-2* locus is responsible for variation in a major female cuticular hydrocarbon (CH) in *D. melanogaster* (Dallarec et al. 2000; Takahashi et al. 2001). A single 16-bp indel at site 12,552 in the 13-kb region of *desat-2* shows complete concordance with CH profiles, with the 16-bp *insertion* allele conferring the ancestral (African) phenotype of high 5,9/7,11 CH ratio and the 16-bp *deletion* allele conferring the derived (Cosmopolitan) phenotype of low 5,9/7,11 CH ratio (Takahashi et al. 2001). We developed a 200-bp marker with primers flanking the 16-bp 12,552 site indel (forward primer sequence: 5' gct cgc cag cta tct acg ac 3'; reverse primer sequence: 5' ata caa tcc ggc agc ttt tg 3'). We then amplified the sequence region using polymerase chain reaction (PCR) (annealing temp. 59°C, 50<sub>mm</sub>MgCl concentr.1/20 ml., 34 cycles) and ran the PCR products on 1% agarose gels for genotyping. We genotyped a single first generation (F1) lab individual from each isofemale line (see Appendix Table 1 for number of isofemales sampled per locality). We then calculated the percentage of *desat-2* locus homozygotes and heterozygotes and overall frequency of *insertion* and *deletion* alleles in each population and calculated the inbreeding coefficient (*F<sub>is</sub>*) as 1 - (observed heterozygote freq./expected heterozygote freq. based on HWE).

### **MORPHOLOGICAL MEASUREMENTS AND UPGMA CLUSTERING**

All morphological measurements were performed on individuals from single isofemale lines maintained in the laboratory for less than two years. Earlier measures of thorax length and pigmentation of males maintained for only one year showed virtually

identical patterns. We used a randomized block experimental design, where each block represented a single day for a total of thirteen day-blocks. On each of the thirteen days, we setup three well-maintained lines (defined as having over 50 adults) from each available location. For each line, we transferred approximately 50 parental adults into experimental narrow glass vials (25 x 95 mm) with standard corn meal/molasses/agar fly food and with a piece of paper for pupation and let females lay eggs for about two hours, at which time the adults were cleared. This ensured low egg laying density in experimental vials (about 30 eggs per vial) so as to create ideal growing conditions for the next generation. We then placed the experimental vials into a 25°C chamber until adult emergence (approximately 12-13 days).

For each block, we aged the emerging adults to 4-5 days before imaging. We randomly chose on average 56 isofemale lines to image on each of the thirteen-day blocks (on average 1 line per locality). For each isofemale line, we placed live adults onto a CO<sub>2</sub> pad under a LEICA MZ7 stereomicroscope with a fiberoptic light source at fixed light level and randomly picked one male and one female to image. Images were taken using a Zeiss AxioCam video camera attached to the microscope with AxioVision (Rel. 4.3) software on adults standing upright, with the dorsal view facing the camera. This approach increases the speed of imaging and avoids any surgical manipulation of the fly. We repeated this procedure for each experimental block for 13 days.

Images of flies were measured (with the worker blind to the line of origin of each fly) using the ImageJ software (<http://rsb.info.nih.gov/ij/>). We measured: 1) thorax length (measured along the dorsal midline from the most anterior point of the thorax to the most posterior point of scutellum), 2) thorax width (measured between posterior supraalar macrochaetae), 3) head width (between the left and right postvertical bristles), 4) wing length (from the intersection of the L4 vein and the anterior crossvein to the distal intersection of the L1 and L3 veins), 5) wing width (from the distal tip of the axillary vein to the intersection of the posterior crossvein and the L5 vein), and 6) thorax pigmentation (measured as the luminosity of digital pixels of the total thorax area using ImageJ software; presence and absence of thorax trident pigmentation was also scored with qualitatively similar results; data not shown). Luminosity is inversely related to the degree of pigmentation. In total, 1,463 individuals were measured (on average 17

isofemale lines per locality; see Appendix Table 1). Location means for each variable were used for spatial analyses and UPGMA clustering (ANOVAs are available upon request).

To determine how population structure was associated with morphological variation, a standardized matrix of location means for each of the six variables was created (NTSYSpc 2.20; Rohlf 2004). We then calculated a distance matrix based on all six variables (see below). Finally, the morphological distance matrix was used to create a phenogram using the UPGMA clustering method (NTSYSpc 2.20; Rohlf 2004).

## **NO-CHOICE COURTSHIP BEHAVIOR ASSAYS**

### ***Male courtship behavior with standard Canton-S isofemale line***

Male courtship behavior was first studied using no-choice tests with females from the standard isofemale line Canton-S (obtained from W. Eanes). Since it is possible that females may influence the courtship behavior of males, it was important to standardize the tester female (Cook 1979; Tompkins et al. 1982; O'Dell 2003, but see below). The assays were performed from January to May of 2005 using a randomized block experimental design. Each experimental block was initiated by picking two stock lines from each represented locality, totaling about 32 lines per block for a total of ten blocks. These lines were then set up simultaneously using standard cornmeal/molasses/agar media in a 25°C incubator with a 12h light: 12h dark cycle. For each line we placed seven fertilized females into an experimental narrow glass vial (see above) and let them lay eggs for four days. We then cleared the vials, inserted paper for pupation and waited about 12-13 days until eclosion of next generation adults. We then collected 10 virgin males from each line using light CO<sub>2</sub> and aged them for 4-5 days.

We randomly picked an isofemale line and aspirated a random male from that line into a plastic Petri-dish (35 x 10 mm) containing a small piece of *Drosophila* food (since individuals typically mate on food in the wild) through a hole on its side that was then closed using a piece of cotton. The Petri-dish was then placed under a stereomicroscope. Our tests were always performed within 1-5 hours of “lights on” at 25°C to 27°C. We let the male habituate for one min. and then aspirated a random virgin female, 4-5 days old, from the Canton-S isofemale line into the chamber. Upon introducing the female into the

mating chamber we began timing. *Courtship latency* was measured as the time from introduction of the female to the initiation of male courtship behaviors. We studied standard male courtship behavior elements of *D. melanogaster* (as defined by Spieth 1952; Bastock and Manning 1955; Bastock 1956; Welbergen et al. 1987; Greenspan and Ferveur 2000; O'Dell 2003). These included: 1) orienting toward the female by either following her or standing facing her, 2) wing vibration and extension, 3) wing scissoring, 4) licking female ovipositor with proboscis, and 5) attempted copulation. We also measured acts of no courtship, defined when a male ignored the female by not orienting towards her (running away or standing on the opposite side of the chamber). Variation in some of these courtship elements has a known genetic basis. For instance the heritability of wing vibration frequencies in *D. melanogaster* is around 7% in the lab (e.g. McDonald 1979). Mutations at many well-known genes affect these behaviors (e.g. Bastock 1956; Greenspan and Ferveur 2000; Billeter et al. 2002; Drapeu et al. 2003).

These behaviors were recorded using voice-recognition software IBM Via Voice that entered one of the male behavioral acts every 1.5 seconds into a Microsoft word document in a sequential manner (video recordings are available upon request). We studied each male-female pair until successful copulation or when 10 minutes passed from the initiation of male courtship. We then determined the proportion of acts during the courtship ritual showing a particular courtship element (as in Bastock 1956; Welbergen et al. 1987). This proportion is simply the number of instances of each element (e.g. wing vibration) out of the total number of male courtship acts during the courtship period. In total, we were able to study 327 mating pairs (on average 9 isofemale lines per locality).

### ***Courtship in homotypic and heterotypic matings of US and Bahamas flies***

We also studied courtship behavior of males and females from Columbus, Mississippi, US (#23) and from Port Nelson, Rum Cay Island, Bahamas (#41). The setup of isofemale lines and the protocol for studying courtship behavior were identical to the above procedures. On each day of the experiment, we studied an equal number of homotypic and heterotypic matings from both US and Bahamas isofemale lines. In this study we focused on the rate of a particular behavior during courtship, determined by counting all acts of a particular behavior (defined above) that occurred during the

courtship period and then dividing this number by the total time of courtship in seconds. Video recordings were used to assay courtship (available upon request).

## **SPATIAL STATISTICAL ANALYSES**

### ***Distance matrices***

For each measured variable, we created a  $n \times n$  distance matrix, where  $n$  is the number of data points. Each value in the matrix,  $d_{ij}$ , is a measure of the distance between the  $i$ th and  $j$ th data values ( $x_i$  and  $x_j$ ). We used standard Euclidean distances to calculate  $d_{ij}$ . For a single variable (such as geography and *desat-2* locus allele frequency),  $d_{ij} = \text{abs}(x_i - x_j)$ . For distance matrices that include multiple variables, (morphological distances, courtship distances, and combined trait distances)  $d_{ij} = \sqrt{\sum (x_{iv} - x_{jv})^2}$ , where  $v$  represents each included variable. Before distances with multiple variables were calculated we standardized the variables using NTSYSpc 2.20 software (Rohlf 2004). All distance matrices were created using PASSAGE software (Rosenberg 2004).

### ***Spatial Correlograms***

We used a correlogram to describe the average spatial autocorrelation between pairwise localities as a function of their geographical distance (e.g. Sokal and Oden 1978a; 1978b; Epperson 2003). Location pairs were grouped into different distance classes and the average correlation for each distance class was determined using PASSAGE (Rosenberg 2004). We used Moran's  $I$  coefficient which ranges from -1 to +1. Ten distance classes were created based on the geographical distance matrix by assuming an equal number of location pairs per distance class. Our connection matrix assumed that all location pairs within a certain distance range are connected and all others are not (binary weight matrix; a Gabriel connection gave same results). We used PASSAGE (Rosenberg 2004) to determine the significance of individual autocorrelation coefficients and the entire correlogram. Linear clines are revealed when short distance classes show positive Moran's  $I$  and long distance classes show negative Moran's  $I$  (Sokal and Oden 1978a, 1978b; Sokal 1979a; Sokal et al. 1987; Barbujani 2000; see Epperson 2003 for further details).

### ***Design Matrices***

We created design matrices to test explicit hypotheses about the relationship between geography and phenotypic variation. A design matrix describes the relative distances among populations *expected* under a particular geographical hypothesis (Waddle et al. 1998). To construct a design matrix we used a binary assignment of distances of 0's and 1's, where 0 means relatively short distance and 1 means relatively long distance (for an example, see Appendix Fig. A1). We developed six design matrices with specific geographical delimitations across the US-Caribbean region (Appendix Fig. A2). A significant correlation between the design matrix and the actual phenotypic distance matrix indicates that phenotypic differentiation is described well by a particular geographical clustering of localities in the design matrix.

### ***Mantel tests***

Correlations between different distance matrices were calculated using the standard Mantel test of matrix correlation using PASSAGE (Mantel 1967; Sokal 1979b; see Epperson 2003). We tested the significance of the correlation by permuting the rows and columns of one of the matrices 499 times and comparing observed to permuted correlations (Rosenberg 2004). We also used a partial Mantel test, which tests the correlation between two matrices, while holding the third constant (Smouse et al. 1986; Epperson 2003; but see Castellano and Balletto 2002; Rousset 2002).

Finally, we developed a 'sparse' Mantel test to test the significance of geographical associations between matrices where one of the matrices was sparse (i.e. not all pairwise comparisons have data available, such as for our sexual isolation matrix; see below). As in the standard Mantel test, we permuted the rows and columns of one of the matrices, but only calculated the product-moment correlation for values that existed in both matrices. This is valid as long as only one of the matrices is sparse (program available upon request).

## Results

### **DESATURASE-2 LOCUS**

Fig. 1 shows geographical variation at *desat-2* locus across the US-Caribbean localities. We found that the African insertion allele at this locus is widespread throughout the Bahamas and exhibits a sharp geographical cline in the area as indicated by location-specific pie charts and the corresponding spatial correlogram (Fig. 1). This generates a significant *Fst* value of 0.221 across the region. US populations north of Alabama and Mississippi are nearly fixed for the deletion allele, while St. Lucia in the Lesser Antilles shows a similar insertion allele frequency ( $\text{freq}_{\text{insertion}} = 0.55$ ) to the southern Bahamas islands. Compared to African populations in Cameroon (West Africa) and Zimbabwe (Sengwa and Kariba), which are either fixed or nearly fixed for the insertion allele (see inset in Fig. 1), the insertion allele in the Caribbean is intermediate in frequency between US and African populations. These data suggest that the CH pheromone frequencies determined by *desat-2* locus will follow the same pattern.

We also found that this gene shows a very different pattern of observed to expected heterozygosity as determined by the inbreeding coefficient (*Fis*) across Bahamas versus US populations (Fig. 2). Across the islands and southern Florida, there is a strong clinal pattern with the southern islands showing a positive *Fis* or deficit in heterozygotes and northern islands showing a negative *Fis* (heterozygote excess). This pattern may be a result of either: 1) general inbreeding, 2) within-island assortative mating or 3) selection against heterozygotes at this locus (Hedrick 2000). The excess of heterozygotes in the northern islands and Florida indicates either outbreeding in general or specifically based on this locus. No clinal pattern exists across US localities as typically very small deviations in heterozygote frequencies from expectations were seen (Fig. 2). In general, our data indicate that most Bahamas islands are more similar to African populations at this locus than to US populations.

### **MORPHOLOGY**

To investigate how morphology varies across the region, we first performed a Principal Component Analysis (PCA) on five morphological variables (head width,

thorax length, thorax width, and wing length and wing width; Table 1). The PCA revealed that the first Principal Component (PC1) explained 61.61% of total variance. All traits loaded positively on PC1, indicating that it is associated with general body size. PC2 explained 13.84% of total variance, which primarily corresponded to a tradeoff between: a) head width and b) wing length and width (Table 1). Despite large variation among individuals, there is a clear difference between US and Caribbean flies with respect to these first two Principal components (Fig. 3a). US flies tend to be typically larger (positive PC1), but have more narrow heads with longer and wider wings (negative PC2).

To see how this individual variation translates to location-specific patterns, we pooled individuals based on their location. We find that both location means of PC1 and PC2 show steep clines across the US-Caribbean region with PC1 having a much steeper cline compared to PC2, as revealed by spatial correlograms (Fig. 3B). Similarly, PC1 means are highly correlated with both longitude and latitude (Fig. 3C). Virtually all US localities exhibit positive PC1 means and all Bahamas localities exhibit negative PC1 means. Further, US and Caribbean flies significantly differ in thorax pigmentation, with dark flies in US and lighter flies in the Caribbean (see clinal differentiation in Figs. 3B and 3D). Similar  $R^2$  values as in Fig. 3 were observed among females. This extends previous results of clines in morphology among eastern US populations (e.g. Coyne and Beecham 1987).

To illustrate how these morphological patterns relate to African populations, Fig. 4 shows regional means of morphological traits of US and Caribbean flies relative to West and Southeast African flies. Caribbean populations exhibited an intermediate body size between US and African flies, but showed smaller wing size and lighter pigmentation than even in West or Southeast African populations. The UPGMA cluster analysis on all morphological traits resulted in two major phenotypic clusters, one with only US populations and the other containing all Caribbean and African populations with a few US populations from Florida and southern Alabama (Fig. 5). For example, West African populations from Niger and Guinea were morphologically most similar to Haiti and Long Island in the southern Bahamas. Note also that Caribbean islands from northern and southern parts of the archipelago cluster in different groups. In general, our results

indicate that Caribbean flies are morphologically more similar to African populations than to US populations.

### MALE COURTSHIP BEHAVIOR

Below we describe results from no-choice mating tests with a single male and a tester-female from Canton-S isofemale line. First, the initiation of male courtship or latency was not significantly different between US and Caribbean populations (avg. latency<sub>US</sub> = 81.2 secs. n = 159; avg. latency<sub>Carib.</sub> = 75.9 secs. n = 168; Z-score = 0.332;  $P = 0.74$ ; Kruskal-Wallis test). However, upon initiation of courtship, males differed in the frequency of their courtship behavior elements towards the tester female. Table 2 summarizes a PCA of all courtship elements. PC1 explained 42.28% of the variance and corresponded to a tradeoff between: a) wing vibrations/extensions and ovipositor licking behavior and b) following/orienting toward the female and ignoring the female during courtship. This indicates that the most variable male behavior was the degree to which males were active in attempting to stimulate females using courtship song and licking behavior. PC2 explained 20.32% of total variance, which primarily corresponded to a trade-off between: a) ignoring the female during courtship and b) following/orienting toward the female. PC3 explained 16.17% of the variance and primarily corresponded to a trade-off between: a) wing scissoring and attempting copulation and b) wing vibrations/extensions and ovipositor licking. Wing scissoring is rare in *D. melanogaster*, but more common in the sibling species *D. simulans* (e.g. Welbergen et al. 1987). PC3 shows that males emphasize different courtship elements to stimulate females across the region.

To see if male courtship varies with respect to geographical location, we averaged principal components across all isofemale lines in each population. First, we found a significant cline in PC1 (Fig. 6). PC1 cline indicated that the proportion of courtship acts displaying wing vibrations and ovipositor licking behaviors relative to ignoring and simply following the female was significantly higher among US males relative to Caribbean males (Fig. 6 and inset). Most notably, Caribbean males perform wing vibrations significantly less often than US males.

PC2, which is related to ignoring versus following the female during courtship, was not significantly different between US and Caribbean males, indicating that both types of males pursued females with equal perseverance (Fig. 6). Finally, PC3 also showed a significant but weaker cline, with US males typically stimulating the female with wing vibrations and ovipositor licking, while Caribbean males displayed more wing scissoring followed by attempted copulations (Fig. 6). Thus US and Bahamas males seem to attract females using different behavioral strategies.

Interestingly, the Cuban population was more similar to US populations in courtship behavior than it was to Bahamas populations (Fig. 6). Recent evidence indicates that Zimbabwe males also tend to exhibit lower wing vibration frequencies relative to cosmopolitan males (C.-T. Ting, unpublished data; pers. comm.). This suggests that Caribbean and African courtship behaviors are likely to be more similar to each other compared to cosmopolitan populations.

## **GEOGRAPHICAL PATTERNS OF DIFFERENTIATION**

It is of interest to extend the above clinal descriptions to explicitly study geographical patterns of differentiation for each variable. This allowed us to determine if different African-like traits show similar or different distributions across the US and Caribbean populations. To begin to address this question, we created six design matrices, each representing a particular geographical hypothesis (see Table 3, Appendix Fig. A2, and *Materials and Methods*).

The *desat-2* locus was significantly correlated with design matrix #5 (Divergent Family Island Hypothesis), which considers locations south of Nassau, New Providence Island to be different from all northern localities (Table 4). The finding that *desat-2* was not significantly correlated with any other geographical hypothesis shows that most differentiation occurs between the northern and middle Bahamas islands.

On the other hand, the morphological distance matrix (based on all six traits) was most significantly correlated with design matrix #1 (Divergent Northern US Hypothesis) and with design matrix #2 (Florida-Islands Connection Hypothesis; Table 4). These hypotheses group Florida and southern parts of Georgia and Alabama with Caribbean island populations rather than other US mainland localities. This indicates that

individuals from most northern parts of US localities in the study system have different morphology from all southern localities and that African-like phenotypes are spread well across the Florida peninsula. This result is consistent with morphological UPGMA clustering of localities (see Fig. 5 above).

Finally, we found that the courtship behavior distance (based on all six courtship elements in Table 2) was most significantly correlated with design matrix #3 (Mainland and Cuba Hypothesis) and to a lesser degree with design matrix #5 (Divergent Family Islands Hypothesis; Table 4). This shows that most differentiation in courtship behavior occurs between mainland and island populations with the exception that Cuba is included with US. Thus although all traits show parallel clines across the region, the specific geographical patterns of differentiation vary between traits. This indicates that African-like traits differ in their distribution across the US-Caribbean region. Further analyses of the detailed shapes of these phenotypic clines and their relationship to genetic markers (e.g. Barton and Hewitt 1985; Szymura and Barton 1986, 1991; Mallet et al. 1990) is described elsewhere (Yukilevich In Prep).

## CORRELATIONS BETWEEN VARIABLES

Using the pairwise Mantel test of matrix correlations we found that *desat-2* locus alleles, morphology and courtship behavior traits were positively correlated with geographical distance between locations and with each other (see Table 5 and Appendix Table A2). Geographical distance is best correlated with morphological distance, followed by *desat-2* locus distance, and least with courtship behavior distance (Table 5). Since variables could be correlated simply because all are correlated with geographical distance, we used the partial Mantel test to determine whether residual variances remain correlated when holding geographical distance constant (see *Materials and Methods*).

Table 5 shows that male morphology and *desat-2* locus distances are still significantly correlated when geographical distance is accounted for. However, the courtship behavior distance is no longer significantly correlated with either the morphology or *desat-2* locus distances (Table 5). Therefore the positive association between courtship behavior and the other two variables comes only from their common association with geography.

## PATTERN OF SEXUAL ISOLATION

Recently, we performed multiple-choice mating tests between various populations across the US and Caribbean region (Yukilevich and True, 2008). This included 11 mating tests between populations either within US or within Bahamas regions and 18 mating tests between US and Bahamas populations. Only between-region tests revealed significant deviation from random mating with substantial variation in the presence and strength of sexual isolation. We test whether these sexual isolation indices are geographically associated with divergence at the *desat-2* locus, morphology, or courtship behavior. First, we included both within and between-region pairwise mating tests in the analysis. To make a fair comparison of associations between different traits and sexual isolation we only included those mating tests for which there was location-specific data for all three major traits in our study (for a total of 22 pairwise tests). We used Mantel test of matrix correlations to test for significance (see *Materials and Methods* and Fig.7). We found that sexual isolation was significantly correlated with geographical distance between localities (Fig. 7A). This occurred because tests between US and Caribbean populations have higher sexual isolation indices than tests within regions (compare gray and black points in Fig. 7). Given that all variables were positively associated with geographical distance (see Table 5 above), we found that these were also significantly correlated with sexual isolation, with *desat-2* locus distance having the highest correlation, followed by morphology and then by courtship behavior distances (see Fig. 7B-D). However, upon removing the effect of geographical distance on sexual isolation using the partial Mantel test, none of the variables remained significantly associated with sexual isolation. Therefore, none of the phenotypic variables explained variation in sexual isolation beyond what has already been explained by geographical distance.

Since individuals across the region varied in all of these traits (see above), we asked how these variables combine together to influence the association with sexual isolation. We created a combined distance matrix based on standardized values of all thirteen variables in our dataset (see *Materials and Methods*). Interestingly, the combined distance matrix had the highest significant correlation with sexual isolation ( $R^2 = 0.388$ ;  $P = 0.001$ ; Fig. 8), which was substantially higher than any other  $R^2$  seen previously (see

Figs. 7). Moreover, the relationship between the combined distance matrix and sexual isolation remained significant even when geographical distance was held constant (Partial Mantel test:  $P = 0.014$ ). Combining variance across multiple variables could have possibly resulted in a worse or a similar fit to sexual isolation data compared to individual variables. Since we found the fit to be better, this result suggests either that individuals utilize multiple traits for mate choice or that the combined matrix from several variables is best correlated to other unmeasured traits that best explain sexual isolation (see Discussion).

Finally, we asked if there are any associations between trait divergence and sexual isolation for only between-region tests (i.e. between US and Bahamas locations), since these have the highest sexual isolation indexes (black points in Figs. 7 and 8).

Interestingly, we found that sexual isolation between US and Caribbean populations was not significantly associated with geographical distances ( $R^2 = 0.049$ ;  $P = 0.71$ ), *desat-2* locus allele frequency distances ( $R^2 = 0.051$ ;  $P = 0.16$ ) or morphological distances ( $R^2 = 0.0002$ ;  $P = 0.28$ ). Thus sexual isolation between US and Caribbean populations exhibits more of a mosaic geographical pattern. It also supports the above conclusion that relationships of the *desat-2* locus and morphological variables with sexual isolation were significant only because each variable was correlated with geography.

However, we did find that sexual isolation is marginally significantly correlated with differentiation in courtship behavior between US and Caribbean males ( $R^2 = 0.146$ ;  $P = 0.08$ ; see black points in Fig.7D). This suggests that US and Caribbean females may directly cue in on courtship behavior differences of males when choosing mates. Finally, sexual isolation between US and Caribbean flies was significantly correlated with the combined distance matrix of *desat-2* locus alleles, morphology and courtship behavior ( $R^2 = 0.161$ ;  $P = 0.03$ ; see black points in Fig.8). Since the significant correlation occurred regardless of whether all comparisons or only between US and Caribbean comparisons were considered, we conclude that variation in sexual isolation is best explained when multiple traits are considered together. It is thus likely that US and Caribbean flies utilize multiple phenotypic variables when choosing mates.

**NO-CHOICE MATING TESTS BETWEEN US AND BAHAMAS FLIES**

To further understand how male courtship behavior influences mating preferences in US and Bahamas females, we performed homotypic and heterotypic mating tests with US individuals from Columbus, Mississippi (#23) and Bahamas individuals from Rum Cay Island (#41). These populations showed significant partial sexual isolation in multiple-choice mating tests (Yukilevich and True 2008). First, we found that US males had significantly higher rates of wing vibrations compared to Bahamas males towards wildtype females from both populations (Fig. 9A). This is consistent with our previous no-choice mating results using Canton-S females (see Fig. 6). Second, male wing vibrations were associated with male mating success (Fig. 9B). However, upon comparing matings with US versus Bahamas females, we observed that only US females mated successfully with males that had significantly higher wing vibration rates (Fig. 9C). Bahamas females mated randomly with respect to male wing vibration rates. These results suggest that Bahamas females have weaker mating preferences for male wing vibrations than US females, consistent with the lower wing vibration rates of Bahamas males (see Figs.6 and 9A). Despite weaker mating preferences for wing vibration rates in males, we found that Rum Cay females actively rejected US males significantly more often than their own local males by decamping during courtship (Fig. 9D). We did not see the reciprocal rejection behavior in US females (Fig. 9D). These results suggest that Bahamas females have weaker mating preferences for male wing vibrations, but actively reject US males presumably based on other phenotypic cues.

## *Discussion*

Previously we found a mosaic pattern of sexual isolation between US and Caribbean populations. Some Caribbean populations exhibited partial mating discrimination against US populations, while other US-Caribbean pairwise tests did not deviate from random mating (Yukilevich and True 2008). Further mating tests revealed that West African flies from Cameroon mated randomly with Caribbean flies, but also showed mating discrimination against US strains. Also, Caribbean and West African populations were both partially sexually isolated from the behavioral race of Zimbabwe,

although Caribbean flies showed weaker sexual isolation. This suggested that Caribbean flies might share mating preferences and other traits involved in mate choice with those of African flies, especially those from West Africa. In the present paper we study variation in several traits that are of particular interest for sexual isolation in *D. melanogaster*. Thus we address whether the case of incipient sexual isolation between US and Caribbean flies shares phenotypic similarity to that found between cosmopolitan and African flies.

We found that Caribbean populations are divergent from US populations in *desat-2* pheromone locus allele frequencies and in various morphological and courtship behavior traits. These traits exhibit steep parallel clines across the US and Caribbean region, with Caribbean flies being more similar to African flies than to US flies. Therefore, this incipient sexual isolation is accompanied by pheromonal, phenotypic, and behavioral differentiation. Further, we found that despite parallel clines across the region, these African-like traits exhibit different geographical patterns. Some African-like traits were predominantly restricted to southern islands of the Bahamas, while others were distributed well into the Florida peninsula. This indicates that African-like traits are distributed unevenly across US and Caribbean populations.

Finally, we revealed that these African-like traits are geographically associated with incipient sexual isolation between US and Caribbean flies. All traits and sexual isolation were positively correlated with geographical distance. Thus all traits were correlated with sexual isolation, with the *desat-2* locus having the highest correlation, followed by morphology and lastly by courtship. However, when only comparisons between US and Caribbean were considered, courtship was then best correlated with sexual isolation. These results illustrate that different phenotypes vary in the extent to which they may predict variation in sexual isolation in this region.

Interestingly we found that when all traits were considered together, they explained variation in sexual isolation much better than either geographical distance or any individual trait. This correlation between sexual isolation and combined trait divergence remained significant when we controlled for geography. Using no-choice mating tests we showed that only US females preferred males with significantly higher wing vibration rates (i.e. courtship song). However, Bahamas females actively rejected

US males presumably based on some other trait(s). Therefore, even though courtship behavior is likely to play an important role in sexual isolation, our total results suggest that multiple variables are likely to fully explain female mating preferences. This may occur because preferences are weak when based on each particular trait, but are stronger when based on several traits together. Future experiments are necessary to test these predictions.

### ***Relationship to sexual isolation between Cosmopolitan-Zimbabwe populations***

Our results relate to recent findings that the *desat-2* locus may be responsible for climatic adaptation and sexual isolation between cosmopolitan and Zimbabwe strains (Fang et al. 2002; Greenberg et al. 2003, 2006; Coyne and Elwyn 2006). The observation that *desat-2* exhibits a steep geographical cline across the US-Caribbean region is suggestive of its role in climatic adaptation, but future work is necessary to determine the exact nature of selection across these localities.

The observation that *desat-2* contributes to explaining sexual isolation in the Caribbean supports previous results that this locus is at least partially involved in sexual isolation (Fang et al. 2002; Coyne and Elwyn 2006). We found that most southern Bahamas islands exhibit a deficit in heterozygotes at this locus. Aside from other potential explanations, such as inbreeding, one interesting possibility may be that southern Bahamas islands carry genetically distinct subpopulations that partially discriminate against each other based on this locus. Further, as Coyne and Elwyn (2006) point out, this locus may epistatically interact with other loci of African genetic background in causing sexual isolation. This view is supported by our observation that sexual isolation between US and Caribbean flies is best explained when the African *desat-2* insertion allele is combined with African-like morphology and courtship behavior.

These findings support the idea that the US-Caribbean case of sexual isolation may also be partially determined by *desat-2* locus variation. However, we emphasize that because previous mating tests indicated that both Caribbean and West African populations are significantly sexually isolated from Zimbabwe populations (Yukilevich and True 2008), *desat-2* cannot explain sexual isolation fully since all of these

populations harbor the African insertion allele. It is thus likely that the basis of mating preferences in Caribbean and West Africa flies may turn out to be somewhat different from that of Zimbabwe flies. The focus of the present work is on the differences between US flies versus both Caribbean and African flies. Future research should address how Caribbean and West African mating preferences and their associated traits differ from those of Zimbabwe populations.

### ***The role of male morphology and courtship behavior in sexual isolation***

The observation that both male morphology and courtship behavior elements contributed to explaining patterns of sexual isolation is of particular interest. Cosmopolitan females typically discriminate against males with smaller body and wing sizes as well as against males with lower frequencies/rates of wing vibration and ovipositor licking behaviors (Bastock and Manning 1955; Bastock 1956; Ewing 1961, 1964; Partridge et al. 1987; Taylor and Kekic 1988; Pitnick 1991). It is fascinating that these are the very traits that are widespread throughout the Caribbean islands. This suggests that US and Caribbean females differ in mating preferences for these traits, which was partially confirmed by our no-choice mating experiments (see above). Mating preferences for male body size and pigmentation among US and Caribbean females have not been analyzed.

Our study does not exclude other potentially divergent traits between US and Caribbean populations from being involved in sexual isolation. Indeed, it has been previously noted that West African and Caribbean *D. melanogaster* populations harbor unique 7-tricosene and 7-pentacosene male pheromones (Rouault et al. 2001). These pheromones may differ between US and Caribbean males and may be potential targets for female mate choice.

### ***Parallel evolution versus historical preadaptive migration of sexual isolation alleles***

Our major finding is that Caribbean populations harbor both West African and Zimbabwe-like phenotypes and behaviors, and that these traits are geographically associated with incipient sexual isolation between US and Caribbean flies. It is possible that Caribbean populations, independently of Africa, have converged onto various traits

associated with adaptation, perhaps to similar tropical conditions, and that this has led to partial sexual isolation from more temperate US flies. Artificial selection experiments in *Drosophila* suggest that this scenario is plausible (Kilias et al. 1980; Dudd 1989). We would then expect that the genetic basis of each case of incipient sexual isolation would be different and that Caribbean flies would be genetically divergent from African flies. This would then be a very incipient case of independent parallel sexual isolation in the New and Old World populations of this species, akin to recent examples in *Timmema* walking sticks (e.g. Nosil et al. 2002) and in sticklebacks (e.g. Schluter and Niguel 1995; Rundle et al. 2000).

However, it is also possible, and perhaps more likely, that these tropical traits first evolved in Africa and subsequently spread into the Caribbean islands with the historical human slave trades several hundred years ago (e.g. David and Cappy 1988). This “historical migration” scenario is supported by the observation that Caribbean and West African flies mate randomly with each other (Yukilevich and True 2008), and by the fact that the same African insertion allele at *desat-2* locus is segregating in the Caribbean and is associated with sexual isolation.

The few genetic studies of Caribbean populations in relation to Non-African and African populations have so far provided mixed results. First, using ten microsatellite loci, Schlotterer et al. (1997) had shown that Lesser Antilles Caribbean populations were genetically closer to European flies than to African flies (US populations were not included). However, more recent work with 48 microsatellite loci revealed that US populations are genetically more similar to Africa than are European populations and segregate many putative African alleles, suggesting recent admixture of African alleles in the New World (Caracristi and Schlotterer 2003). Further genetic analyses of Caribbean populations are necessary to determine which of these two historical scenarios are more likely.

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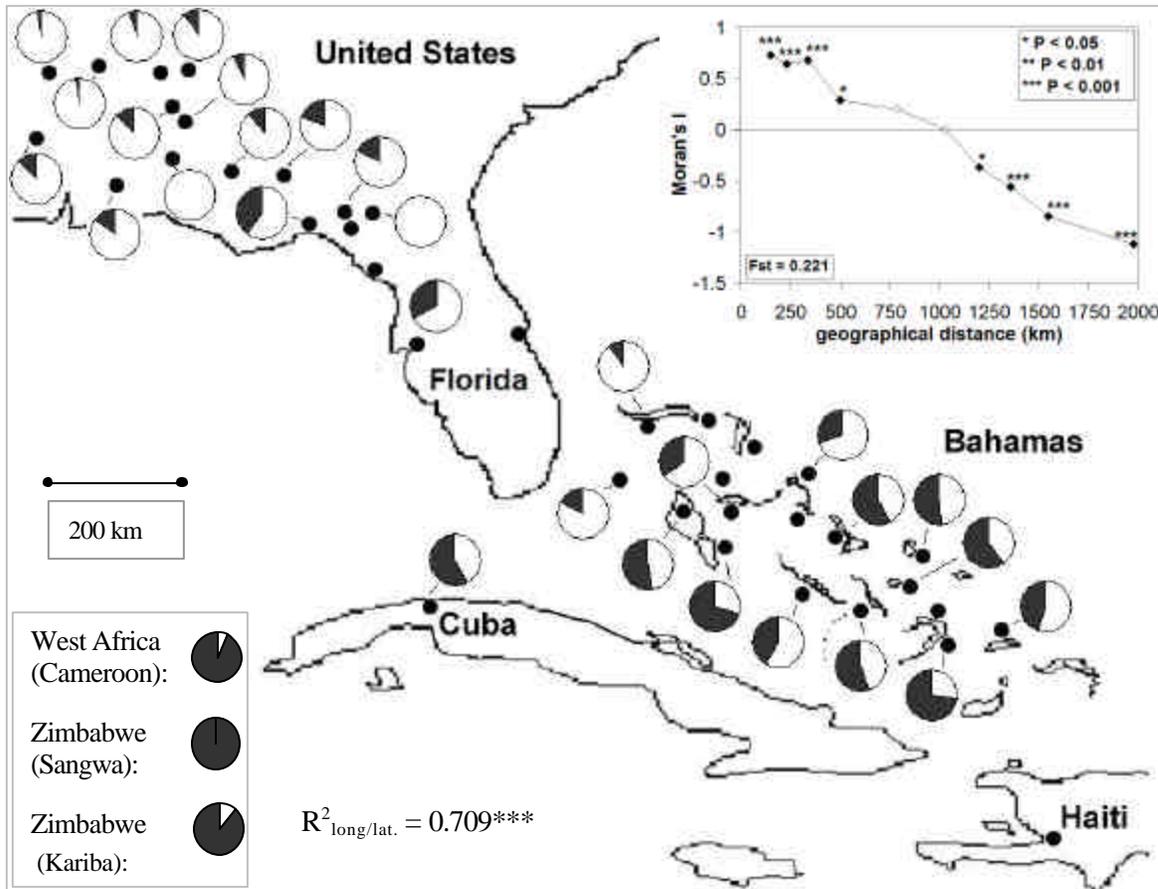


Figure 1. Map of 38 collection sites (shown as black circles) of *Drosophila melanogaster* iso-female lines and *desaturase-2* locus allelic frequency data (per sampled locality) and its geographic correlogram across the system (top-right corner). See Appendix Table 1 for number of isofemale lines used to determine allelic frequency per location. *Desat2* allelic frequencies are described as pie charts across the region, with black representing the African insertion allele frequency and white representing the cosmopolitan deletion allele frequency. Also shown at left bottom corner are allelic frequencies of African locations at *desat2* locus for comparison. St. Lucia allelic frequency is not shown (insertion allele = 55%). Multiple regression of longitude and latitude on *desat2* allelic frequency is shown. Partial  $R^2_{\text{long}} = 0.007$  and  $R^2_{\text{lat}} = 0.057$  ( $P = 0.605$  and  $P = 0.003$ , respectively). The *desat2* locus correlogram shows how Moran's I coefficient of correlation changes as the distance between paired populations increases in km from US to Bahamas. The overall correlogram is significant at  $P$ -value < 0.05 and a Moran's I value for each distance class is designated either as significant (black with a given significance value) or not significant (white). The  $F_{st}$  for *desat2* locus across US - North Caribbean region is 0.221.

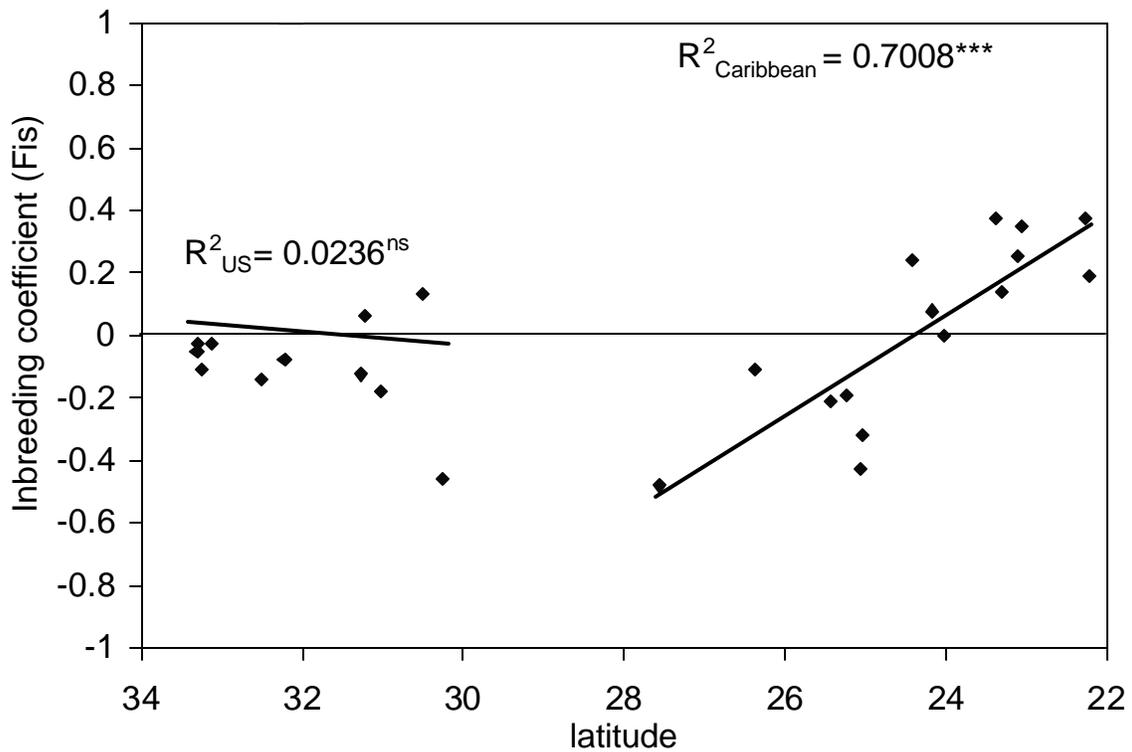


Figure. 2. Relationship between observed and expected frequency of heterozygotes at the *Desaturase-2* described by the inbreeding coefficient ( $F_{is}$ ) across US and Caribbean (Bahamas and Cuba) island populations as a function of latitude.  $R^2$  values were determined independently for each regional test. Non-significant  $R^2$  in US corresponds to  $F = 0.242$ ,  $P = 0.633$ . Asterisks for Caribbean populations signify  $F = 30.45$ ,  $P < 0.0001$ . Note that only for this analysis the Tampa Bay Florida population (latitude 27.56) was included in the Caribbean regional test since this locality is geographically distant from other studied US populations and is more consistent with the pattern across northern Bahamas islands. Including Tampa Bay population in US still results in non-significant  $R^2 = 0.14$ ,  $F = 1.8$ ,  $P = 0.21$  across US and its exclusion from Caribbean still results in a significant  $R^2 = 0.617$ ,  $F = 19.29$ ,  $P < 0.0009$  across Caribbean populations.

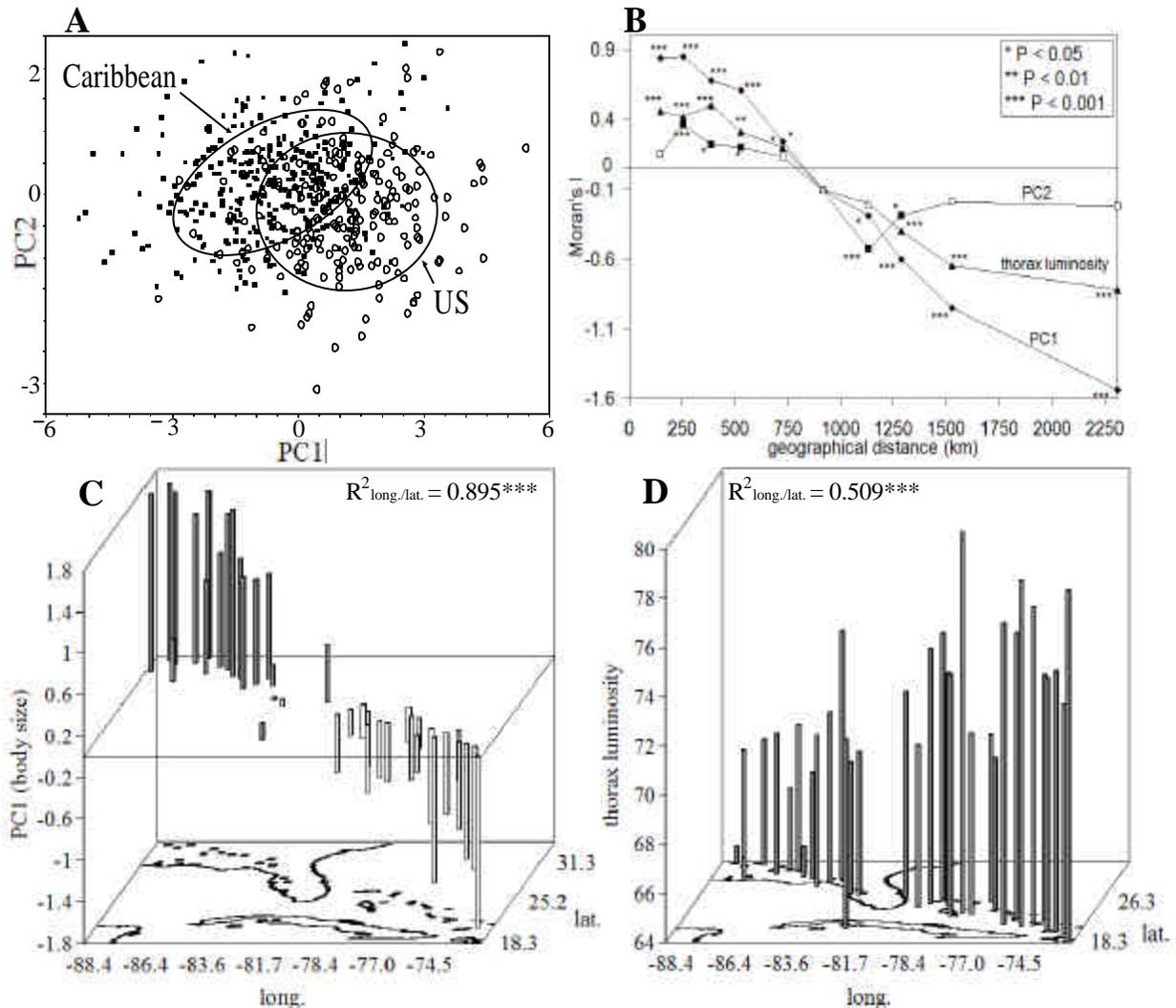


Figure 3. Geographical differentiation in morphological traits across US and Caribbean populations (only male data shown). A) Principal Component values (PC1 and PC2) for US and Caribbean individuals (shown as circles and squares, respectively) from Table 1. Binary normal ellipses for US and Caribbean individuals are shown, representing 65% variation in each region. B) Geographic correlograms for location-means of PC1 and PC2 and thorax luminosity (performed in PASSAGE 1.1; M. Rosenberg 2004). All 3 correlograms are significant at  $P < 0.05$ . Black symbols represent significant Moran's I values (each value for each distance class) and white symbols represent non-significant Moran's I values. C) Clinal differentiation of location means of PC1 from Table 1. Multiple regression of longitude and latitude on PC1 shown. Partial  $R^2_{\text{long.}} = 0.089$  and  $R^2_{\text{lat.}} = 0.125$  are both significant at  $P = 0.009$  and  $P = 0.004$ , respectively. Black bars represent positive values and white bars represent negative values (standard errors not shown). D) Clinal differentiation of thorax luminosity (inverse of pigmentation; see *Materials and Methods*). Partial  $R^2_{\text{long.}} = 0.214$  and  $R^2_{\text{lat.}} = 0.440$  are not significant ( $P = 0.433$  and  $P = 0.205$ , respectively).

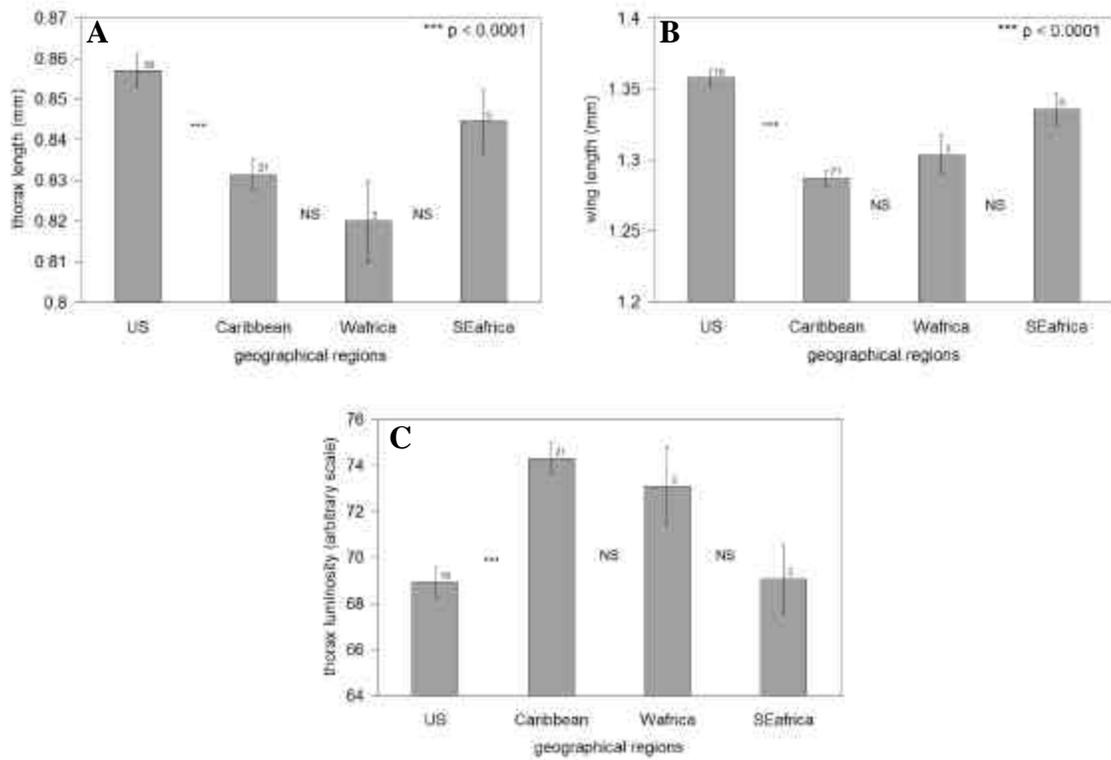


Figure 4. Morphological differentiation between US, Caribbean, West African and Southeast African populations in thorax length (A), wing length (B), and thorax luminosity (C) (only male data is shown). Numbers above bars represent the number of locations being averaged and error bars designate standard error. Significance was determined using the Kruskal-Wallis test.

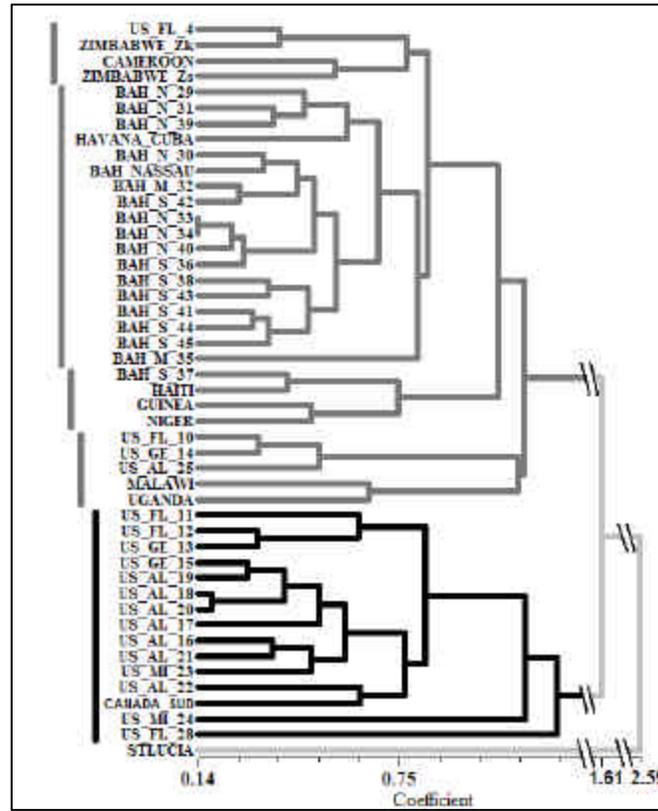


Figure 5. UPGMA Cluster Analysis of males based on six standardized morphological variables: thorax length and width, head width, wing length and width and thorax pigmentation. Analysis performed using NTSYS (Rohlf 2004). Labels consist of the regional name, location area in the region, and id # (REGION\_LOCATION\_ID#). In United States, FL is for Florida, GE is for Georgia, AL is for Alabama, and MI is for Mississippi. In Bahamas, N is for north, M is for middle and S is for south Bahamas islands (see Appendix Table A1 for location details).

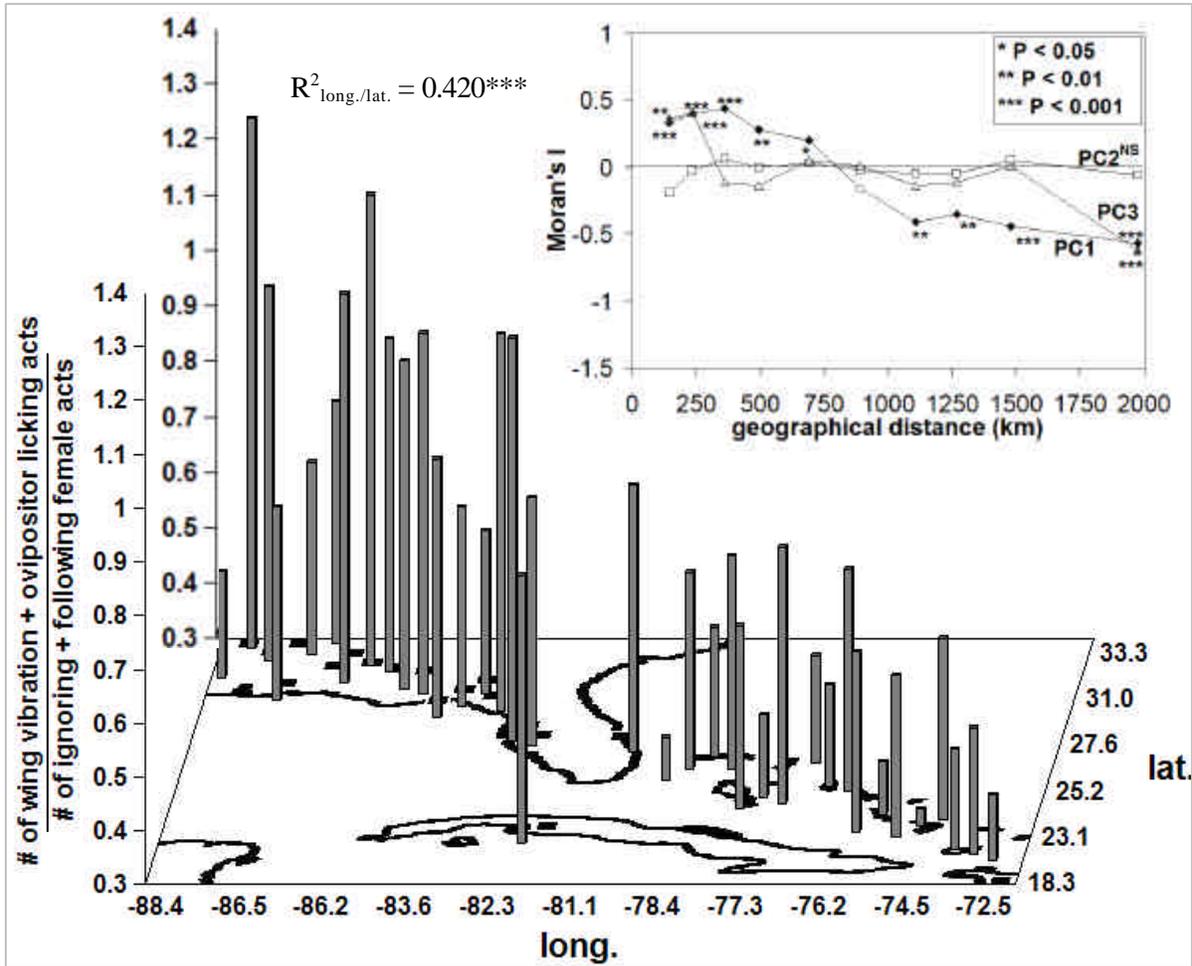


Figure 6. Male courtship behavior across US and Bahamas locations. Bar graph represents a relationship described by first Principal component in Table 2, which is predominantly a tradeoff between the proportion of acts a male performs wing vibrations and ovipositor licking versus ignoring and simply following the female during courtship. See text for number of isofemale lines studied per location. Multiple regression of longitude and latitude on proportion data is shown. Partial  $R^2_{\text{long.}} = 0.022$  and  $R^2_{\text{lat.}} = 0.011$  are not significant ( $P = 0.22$  and  $P = 0.63$ , respectively). Inset graph shows a geographical correlogram of the first three Principal components of courtship behavior (see Table 2 for detail; PASSAGE 1.1; M. Rosenberg 2004). PC1 and PC3 are significant correlograms at  $P < 0.05$  while PC2 does not deviate from random spatial distribution. Black symbols represent significant Moran's I values at the designated significance level (each value for each distance class) and white symbols represent non-significant Moran's I values.

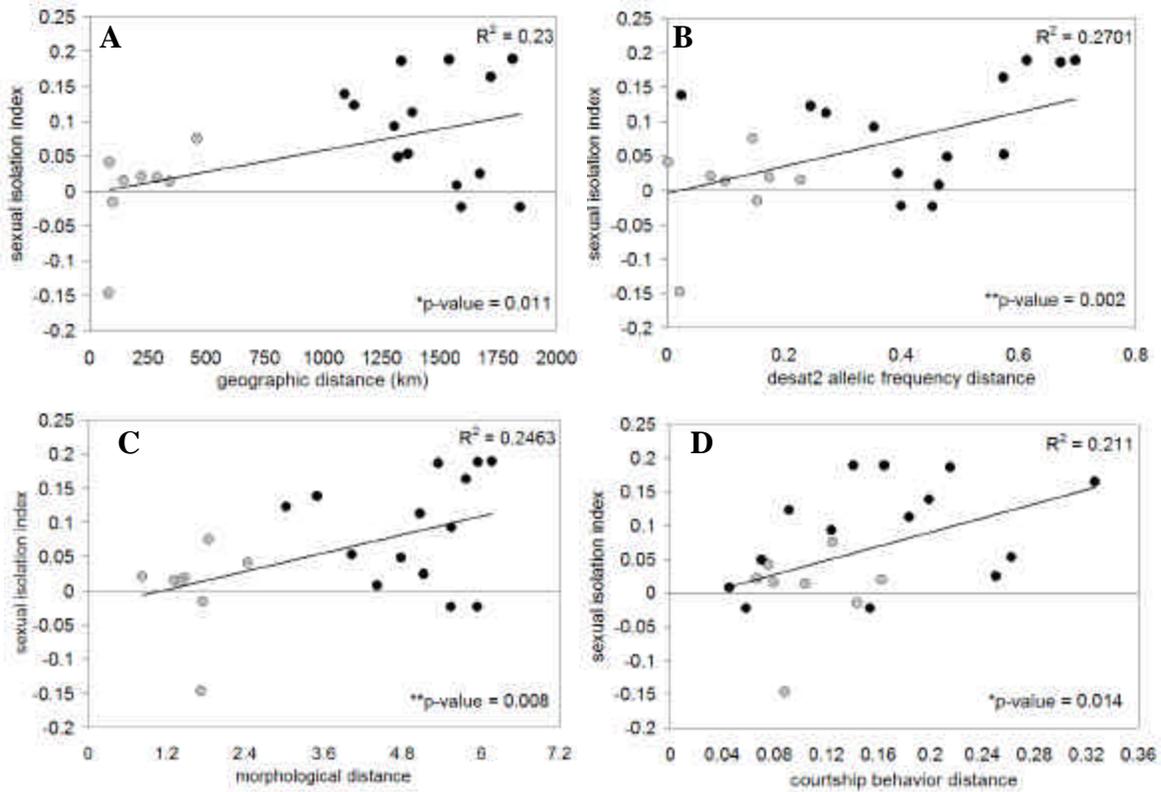


Figure 7. Geographical relationship between sexual isolation and distances based on geography or measured variables between pairwise populations that were tested for mate choice. Gray symbols represent within-region comparisons (either within US or within Caribbean) and black symbols represent between-region comparisons. See Yukilevich and True (2008) for standard deviation of individual sexual isolation indexes. Since our sexual isolation matrix is incomplete, we determined  $P$ -values and significance using the ‘sparse’ Mantel test of matrix correlations (see *Materials and Methods*). Note that even though the correlation coefficient ( $R$ ) was tested in Mantel tests, we show its  $R^2$  in plots.

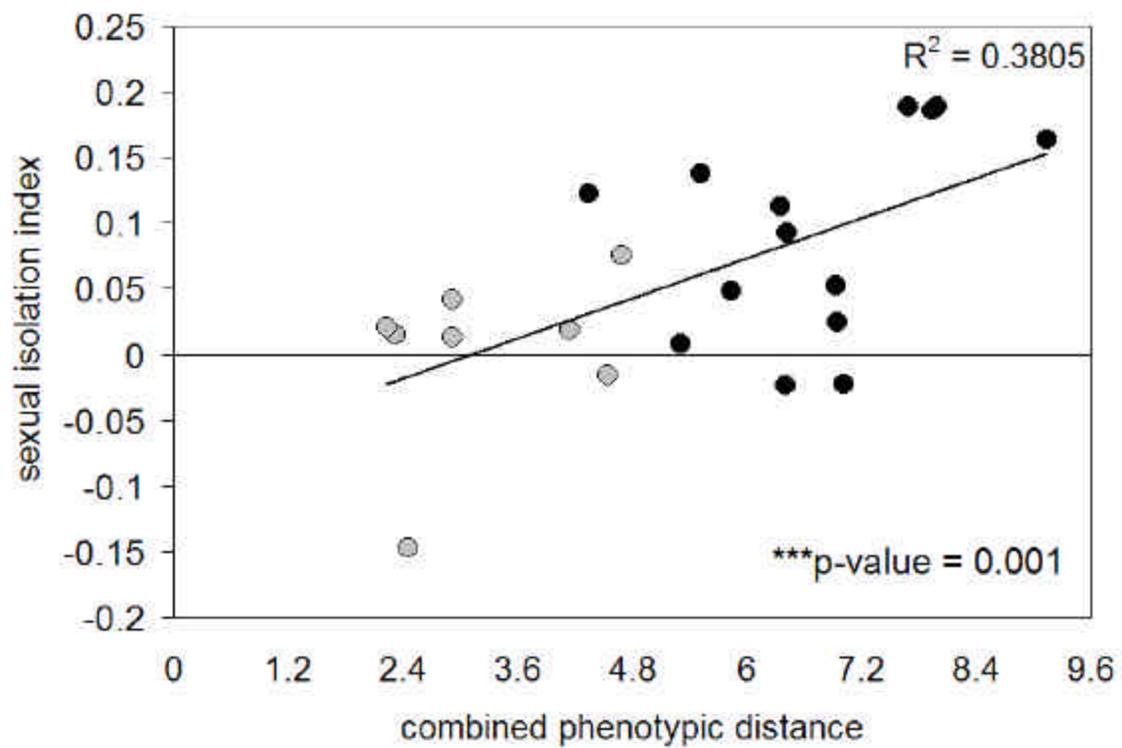


Figure 8. Geographical relationship between a combined distance matrix based on standardized values of *desat-2* locus allele frequency, six morphological traits and six courtship behavior elements and sexual isolation index. Grey symbols represent within-region comparisons (within US or within Bahamas) and black symbols represent between-region comparisons (see Fig. 7 for further details).

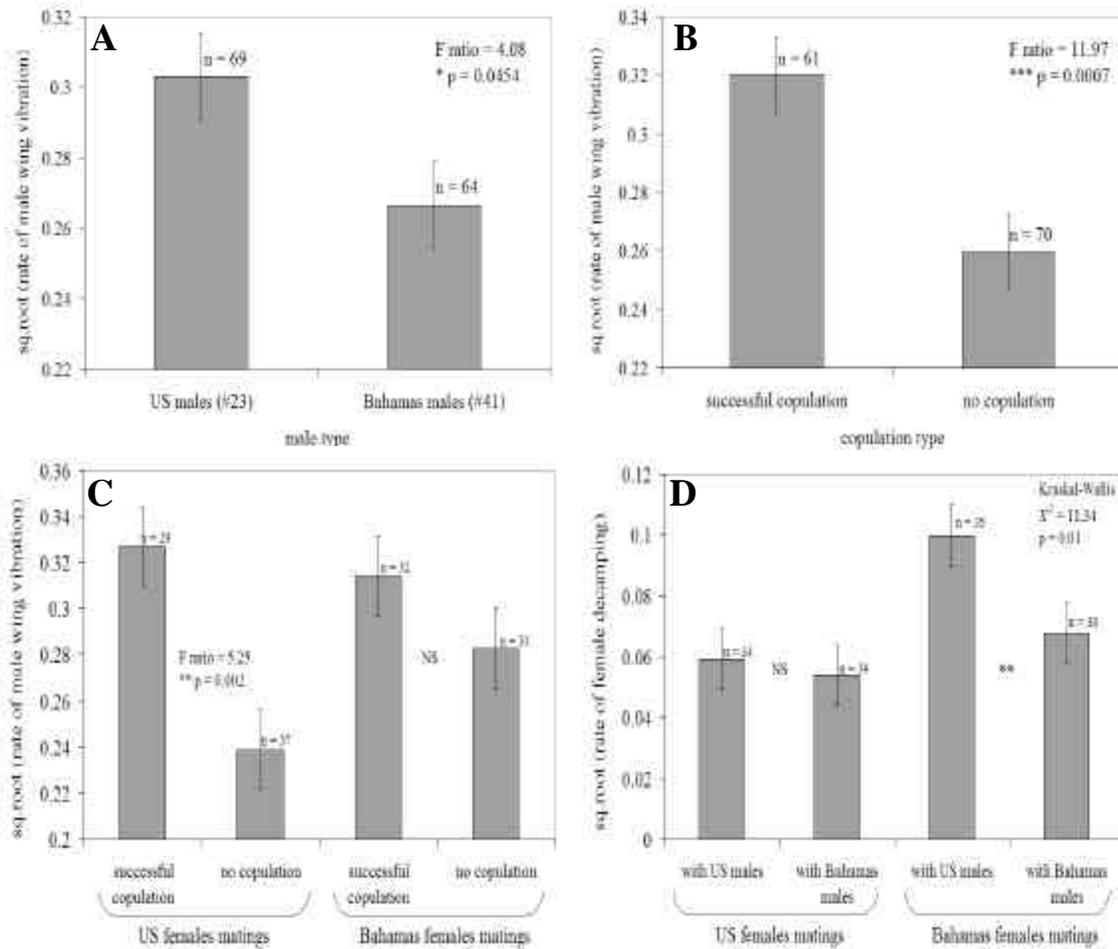


Figure 9. No-choice mating tests results between a US population (Columbus, Mississippi #23) and a Bahamas island population (Port Nelson, Rum Cay #41). All rates were determined by the number of acts divided by the total courtship time (in seconds) from initiation of male courtship to copulation or 10 mins. (note the rate of male wing vibration was measured differently than in Fig. 6; see *Materials and Methods*). Wing vibration rates were then square-root transformed to achieve normality. A) The average rate of wing vibrations displayed by males during courtship: A) from US location #23 and Bahamas location #41 towards females from both locations, B) in all successful matings versus unsuccessful matings, and C) in successful versus unsuccessful matings by US or Bahamas females. D) The average rate of decamping by females from courting males from US location #23 and Bahamas location #41 in homotypic and heterotypic mating tests. Note that the square-root transformation of decamping rates did not achieve normality. Kruskal-Wallis test was used to test significance. Error bars designate standard errors and ‘n’ is the sample size per category.

Table 1. Principal Component Analysis based on five morphological traits

| <b>PC's</b>                    | <b>1</b> | <b>2</b> | <b>3</b> | <b>4</b> | <b>5</b> |
|--------------------------------|----------|----------|----------|----------|----------|
| EigenValue                     | 3.08     | 0.692    | 0.581    | 0.377    | 0.27     |
| Percent                        | 61.61    | 13.84    | 11.61    | 7.54     | 5.40     |
| Cum Percent                    | 61.61    | 75.45    | 87.06    | 94.60    | 100.00   |
| <b>Eigenvectors (loadings)</b> |          |          |          |          |          |
| thorax length                  | 0.437    | 0.011    | -0.729   | 0.526    | -0.045   |
| thorax width                   | 0.467    | 0.227    | -0.297   | -0.801   | 0.036    |
| head width                     | 0.385    | 0.761    | 0.441    | 0.277    | 0.023    |
| wing length                    | 0.472    | -0.409   | 0.326    | 0.007    | -0.710   |
| wing width                     | 0.469    | -0.449   | 0.283    | 0.073    | 0.702    |

Note: Analyses were performed in JMP software and all eigenvalues were based on correlation matrices of variables with standard variance. Each individual replicate constituted a data point in the PCA. Distribution of variables were not necessarily normalized since multiple populations are included in analyses.

Table 2. Principal Component Analysis of Male Courtship Behavior

| <b>PC's</b>                    | <b>1</b> | <b>2</b> | <b>3</b> | <b>4</b> | <b>5</b> | <b>6</b> |
|--------------------------------|----------|----------|----------|----------|----------|----------|
| EigenValue                     | 2.537    | 1.220    | 0.970    | 0.861    | 0.408    | 0.005    |
| Percent                        | 42.284   | 20.328   | 16.173   | 14.341   | 6.793    | 0.081    |
| Cum Percent                    | 42.284   | 62.612   | 78.785   | 93.126   | 99.919   | 100.000  |
| <b>Eigenvectors (loadings)</b> |          |          |          |          |          |          |
| attemp copulation              | 0.282    | -0.058   | 0.493    | 0.806    | -0.016   | 0.159    |
| ovipositor licking             | 0.496    | -0.163   | -0.310   | 0.140    | 0.252    | 0.528    |
| wing scissoring                | 0.240    | 0.174    | 0.748    | -0.007   | 0.783    | 0.134    |
| vibrations+extensions          | 0.553    | 0.067    | -0.288   | -0.555   | 0.150    | 0.149    |
| ignore female                  | -0.369   | 0.702    | -0.096   | -0.079   | 0.061    | 0.603    |
| following+standing             | -0.417   | -0.666   | 0.097    | -0.130   | -0.545   | 0.541    |

Note: see Table 1 for details on analysis.

Table 3. Description of different design matrices used to test explicit geographical patterns of differentiation in phenotype space

| <b>Design Matrices</b> | <b>Hypotheses</b>                 | <b>Regional Splits*</b>                                 |
|------------------------|-----------------------------------|---|
| <i>Design1</i>         | Divergent Northern US             | Northern US vs. All                                     |
| <i>Design2</i>         | Florida-Islands Connection        | Northern+Middle US vs. Florida+Cuba+Bahamas+Haiti       |
| <i>Design3</i>         | Mainland and Cuba vs Islands      | US+Cuba vs. Bahamas+Haiti                               |
| <i>Design4</i>         | Mainland vs All Islands           | US vs. Cuba+Bahamas+Haiti                               |
| <i>Design5</i>         | Divergent Family Islands          | US+Cuba+Northern Bahamas vs. Middle+South Bahamas       |
| <i>Design6</i>         | Divergent South Bahamas and Haiti | US+Cuba+Northern+Middle Bahamas vs. South Bahamas+Haiti |

\*see Appendix Fig. A2 for exact geographical locality delimitations of regional splits

Table 4. Partial matrix correlations between six design matrices and phenotypic distance matrices (holding geographical distance constant)

|               | <i>Design 1</i>       | <i>Design 2</i>       | <i>Design 3</i>       | <i>Design 4</i>       | <i>Design 5</i>       | <i>Design 6</i>      |
|---------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|----------------------|
| <i>desat2</i> | -0.1087 <sup>ns</sup> | -0.0444 <sup>ns</sup> | -0.2782 <sup>ns</sup> | -0.1041 <sup>ns</sup> | <b>0.2252**</b>       | 0.1281 <sup>ns</sup> |
| morphology    | <b>0.2456*</b>        | 0.1785*               | 0.039 <sup>ns</sup>   | -0.0344 <sup>ns</sup> | -0.1102 <sup>ns</sup> | 0.0209 <sup>ns</sup> |
| courtship     | -0.1259 <sup>ns</sup> | -0.0532 <sup>ns</sup> | <b>0.2266**</b>       | 0.1109 <sup>ns</sup>  | 0.1538*               | 0.0191 <sup>ns</sup> |

\* p < 0.05

\*\*p < 0.01

\*\*\*p < 0.001

Table 5. Matrix correlations of *desat-2* locus, morphology and courtship behavior to geographical distance and partial matrix correlations to each other (holding geographical distance constant)

|               | <i>geography</i> | <i>desat2</i>        | <i>morphology</i>    |
|---------------|------------------|----------------------|----------------------|
| <i>desat2</i> | 0.6229***        |                      |                      |
| morphology    | 0.8472***        | 0.1991**             |                      |
| courtship     | 0.3177***        | 0.0660 <sup>ns</sup> | 0.1126 <sup>ns</sup> |

\*\*p < 0.01

\*\*\*p < 0.001

## Appendix

Table A1. Location information and the number of isofemale lines collected and maintained in the lab.

| ID # | State/Island, Town                             | # of Lines | Date Collected | Longitude | Latitude |
|------|--|------------|----------------|-----------|----------|
| 4    | Florida, Tampa Bay (downtown)                  | 13         | Jun-04         | -82.27    | 27.56    |
| 9,10 | Florida, Crosscity-Chiefland                   | 6          | Jun-04         | -83.07    | 29.38    |
| 11   | Florida, Lamont                                | 6          | Jun-04         | -83.48    | 30.22    |
| 12   | Florida, Tallahassee (downtown)                | 15         | Jun-04         | -84.16    | 30.26    |
| 13   | Georgia, Thomasville                           | 20         | Jun-04         | -83.58    | 30.5     |
| 14   | Georgia, Valdosta                              | 16         | Jun-04         | -83.16    | 30.49    |
| 15   | Georgia, Blakeley                              | 20         | Jun-04         | -84.56    | 31.22    |
| 16   | Alabama, Ozark                                 | 20         | Jun-04         | -85.38    | 31.27    |
| 17   | Alabama, Greenville                            | 19         | Jun-04         | -86.38    | 31.49    |
| 18   | Alabama, Montgomery (downtown)                 | 23         | Jun-04         | -86.18    | 32.22    |
| 19   | Alabama, Clanton                               | 20         | Jun-04         | -86.37    | 32.5     |
| 20   | Alabama, Selva                                 | 19         | Jun-04         | -86.53    | 33.25    |
| 21   | Alabama, Birmingham (downtown)                 | 16         | Jun-04         | -86.48    | 33.31    |
| 22   | Alabama, Tuscaloosa                            | 18         | Jun-04         | -87.34    | 33.12    |
| 23   | Mississippi, Columbus                          | 15         | Jun-04         | -88.25    | 33.29    |
| 24   | Mississippi, Meridian                          | 7          | Jun-04         | -88.42    | 32.21    |
| 25   | Alabama, Atmore                                | 14         | Jun-04         | -87.29    | 31.01    |
| 28   | Florida, Sabastian                             | 6          | Jun-04         | -80.28    | 27.48    |
| 29   | Eluthera (south), Governor's Harbor            | 14         | Jul-04         | -76.18    | 25.15    |
| 30   | Eluthera (north), Gregory Town                 | 20         | Jul-04         | -76.33    | 25.23    |
| 31   | Abaco, Marsh Harbor                            | 20         | Jul-04         | -77.03    | 26.32    |
| 32   | Andros (north), Andros Town                    | 22         | Jul-04         | -77.46    | 24.42    |
| 33   | Grand Bahamas (west), Freeport                 | 18         | Jul-04         | -78.38    | 26.3     |
| 34   | Grand Bahamas (east), McLean's Town            | 20         | Jul-04         | -77.56    | 26.38    |
| 35   | Andros (south), High Rock                      | 20         | Jul-04         | -77.33    | 25.07    |
| 36   | Exumas, George Town                            | 20         | Jul-04         | -75.47    | 23.31    |
| 37   | Long Island, Deadman's Cay                     | 17         | Jul-04         | -75.06    | 23.1     |
| 38   | Cat Island, New Bight                          | 22         | Jul-04         | -75.25    | 24.18    |
| 39   | Bimini Island, Alice Town                      | 20         | Jul-04         | -79.17    | 25.43    |
| 40   | Berry Islands, Bullock's Harbor                | 20         | Jul-04         | -77.51    | 25.45    |
| 41   | Rum Cay, Port Nelson                           | 22         | Jul-04         | -74.5     | 23.38    |
| 42   | San Salvador, Cockburn Town                    | 20         | Jul-04         | -74.31    | 24.03    |
| 43   | Mayaguana, Mayaguana                           | 16         | Jul-04         | -72.54    | 22.22    |
| 44   | Crooked Island, Colonel Hill                   | 20         | Jul-04         | -74.1     | 22.44    |
| 45   | Acklins Island, Spring Point                   | 16         | Jul-04         | -73.59    | 22.26    |
| 46   | Cuba, Havana                                   | 17         | Jul-04         | -82.23    | 23.06    |
| 47   | New Providence, Nassau                         | 22         | Jul-04         | -77.2     | 25.03    |
| 48   | Lesser Antelles, St. Lucia (Soufriere)         | 23         | Dec-05         | -60.58    | 13.54    |
| 52   | Haiti, Port-Au-Prince (near downtown)          |            | Jun-06         | -72.2     | 18.32    |
| 49   | West Africa, West Cammeroon (Mbalang-Djalingo) | 31         | 2004           | 10.05     | -5.23    |
| 50   | Southeast Africa, Zimbabwe (Sengwa)            | 13         | 1990           | 28.34     | -16.5    |
| 51   | Southeast Africa, Zimbabwe (Lake Kariba)       | 20         | 1994           | 27.59     | -17      |
| 53   | Guinea   | 12         | 2004           | 10.52     | -10.47   |
| 54   | Niger  | 20         | 2004           | 7.12      | -15.23   |
| 55   | Uganda   | 20         | 2004           | 32.12     | -0.48    |
| 56   | Malawi   | 20         | 2004           | 33.35     | -13.19   |

Note: All lines were collected by R. Yukilevich, except lines from Haiti (collected by S. Tumuluri) and from Africa (obtained from J. Pool and C. Acquadro).

Table A2. Full correlations between phenotypic distance measures.

|               | <i>desat2</i> | morphology |
|---------------|---------------|------------|
| <i>desat2</i> |               |            |
| morphology    | 0.5139***     |            |
| courtship     | 0.2180**      | 0.3259***  |

\*\* $p < 0.01$ \*\*\* $p < 0.001$

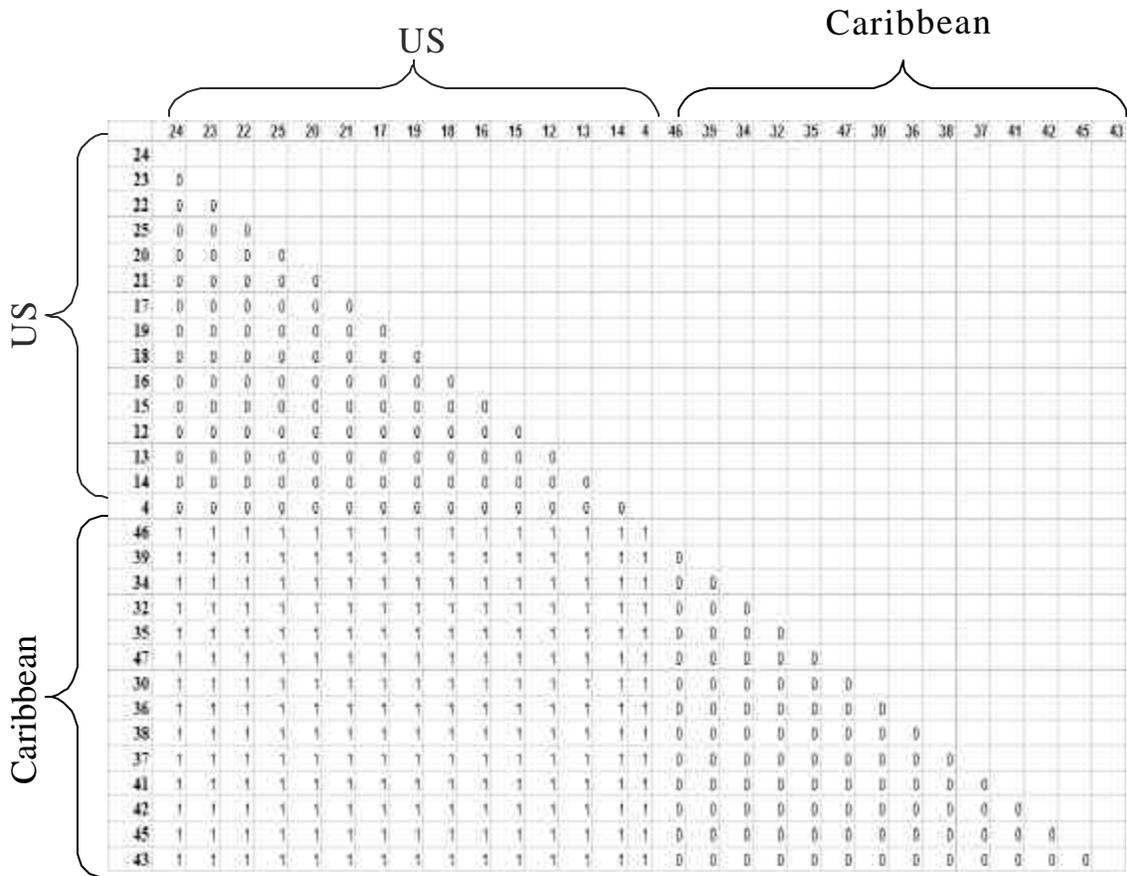


Figure A1. Example of a binary design matrix #4 (Mainland vs. All Islands) where all localities that are grouped together (in this case either within US region or within Caribbean region) are assigned a value of 0 ('less differentiation') while all localities between groups (in this case between region locality pairs) are assigned a value of 1 ('more differentiation'). Other design matrices in Table 3 vary with respect to which locality pairs are assigned values of 1 and 0, depending on the geographical hypothesis.

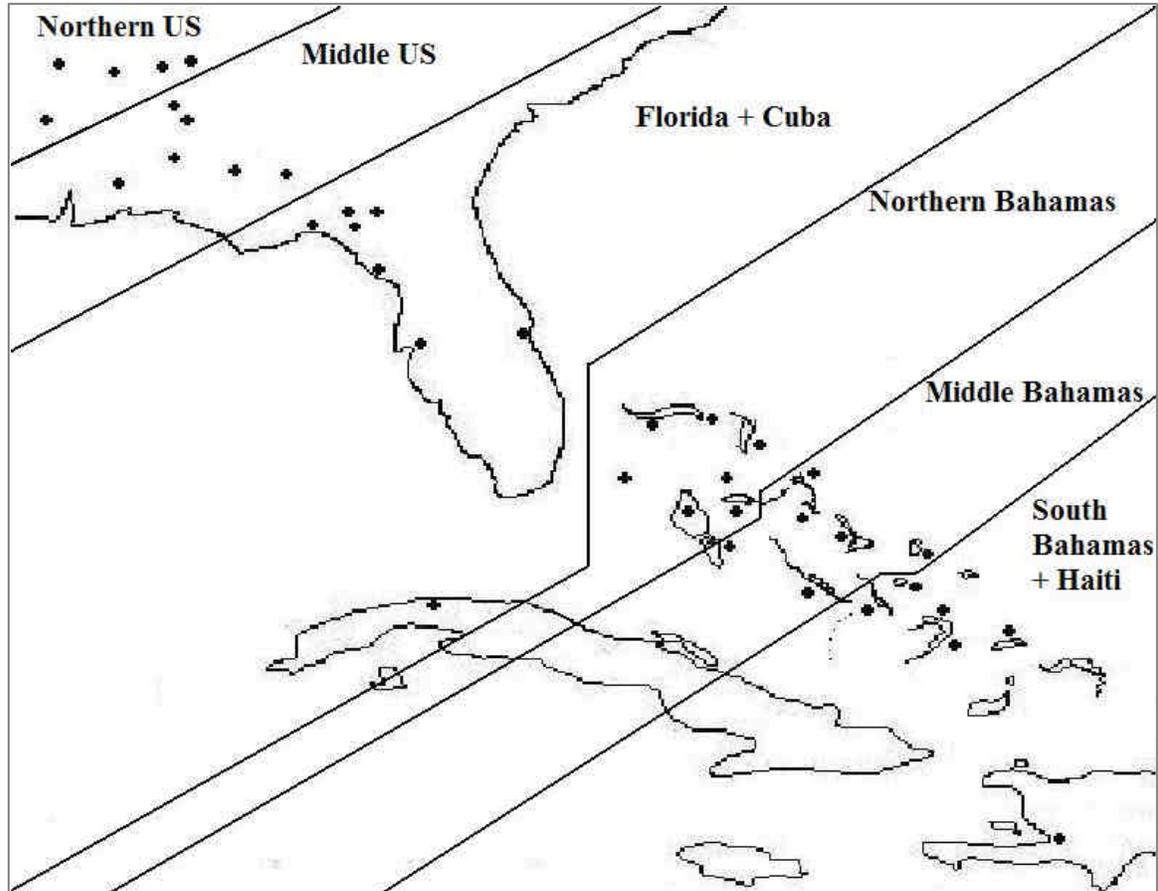


Figure A2. Map of arbitrary regional splits used to create geographical design matrices in Table 3 to test for specific spatial hypotheses about *desat-2* locus, morphology and courtship behavior variation.