doi:10.1111/j.1558-5646.2009.00661.x

GENETICS OF INCIPIENT SPECIATION IN *DROSOPHILA MOJAVENSIS:* II. HOST PLANTS AND MATING STATUS INFLUENCE CUTICULAR HYDROCARBON QTL EXPRESSION AND G × E INTERACTIONS

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Received October 17, 2008
Accepted January 26, 2009

We performed a quantitative trait locus (QTL) analysis of epicuticular hydrocarbon variation in 1650 F_2 males from crosses of Baja California and mainland Mexico populations of *Drosophila mojavensis* cultured on two major host cacti. Principal component (PC) analysis revealed five PCs that accounted for 82% of the total epicuticular hydrocarbon variation. Courtship trials with mainland females were used to characterize hydrocarbon profiles of mated and unmated F_2 males, and logistic regression analysis showed that cactus substrates, two PCs, and a PC by cactus interaction were associated with mating success. Multiple QTLs were detected for each hydrocarbon PC and seven G × E (cactus) interactions were uncovered for the X, second, and fourth chromosomes. Males from the courtship trials and virgins were used, so "exposure to females" was included as a factor in QTL analyses. "Exposed" males expressed significantly different hydrocarbon profiles than virgins for most QTLs, particularly for the two PCs associated with mating success. Ten QTLs showed G × E (exposure) interactions with most resulting from mainland genotypes expressing altered hydrocarbon amounts when exposed to females compared to Baja genotypes. Many cactus × exposure interaction terms detected across QTL and all PCs confirmed that organ pipe-reared males expressed significantly lower hydrocarbon amounts when exposed to females than when reared on agria cactus. Epicuticular hydrocarbon variation in *D. mojavensis* is therefore a multigenic trait with some epistasis, multiple QTLs exhibited pleiotropy, correlated groups of hydrocarbons and cactus substrates determined courtship success, and males altered their hydrocarbon profiles in response to females.

KEY WORDS: **Plant-insect interaction, pleiotropy, quantitative genetics, reproductive isolation, speciation.**

> ". . . isn't it a task of science to detect fundamental similarities concealed by apparent unlikeness? A fundamental but common property of species is the presence of isolating mechanisms" (Dobzhansky 1940, p. 320).

Understanding the initial stages of reproductive divergence that may ultimately lead to speciation has been a recurring theme in evolutionary biology since Dobzhansky (1937) and Mayr (1942, 1963) emphasized differences between pre- and postmating isolating barriers, and proposed how they originated. Genetic divergence caused by adaptation to different environments was recognized as a potent speciation mechanism (Muller 1939; Dobzhansky 1940; Muller 1942). Geneticists, such as

Dobzhansky and Muller, focused their research on diversification at later stages of species divergence and genetic analysis of epistatic postmating isolating mechanisms, i.e., hybrid sterility and inviability, etc. (reviewed in Coyne and Orr 2004). As the role of ecology in the earliest stages of reproductive isolation has become clearer, renewed interest has been placed in genetic studies of ecologically influenced traits associated with premating isolation, thought to evolve sooner, and serve as a stronger barrier than postmating isolation in reducing gene flow between populations (Coyne and Orr 1989; Mallet et al. 1998; Kirkpatrick and Ravigné 2002). Although the proximate and ultimate causes of reproductive isolation have emerged as major emphases in speciation research (Butlin and Ritchie 1994; Panhuis et al. 2001; Coyne and Orr 2004), there remain significant gaps in our understanding of why certain traits associated with premating isolation evolve before others and how the genetic architectures of these traits evolve. A broad and general understanding of the genetics of premating isolating mechanisms has yet to emerge.

Understanding how proximate ecological factors have caused evolutionary diversification requires analysis of traits causing reproductive isolation in natural populations. Studies of parallel diversification in sticklebacks (Schluter and Nagel 1995; Schluter 1996; Rundle et al. 2000), loss of mimicry in butterfly hybrids (Jiggins et al. 2001), adaptation to different hosts in pea aphids (Via 1999; Via et al. 2000) and leaf beetles (Funk 1998; Funk et al. 2002), host shifts in fruit flies (Feder et al. 1994, 2005), selective predation on walking stick morphs (Nosil et al. 2002; Nosil and Crespi 2006), and divergence in bird songs (Ruegg et al. 2006) all point to the intrinsic role of ecology in the speciation process (McKinnon et al. 2004; Rundle and Nosil 2005; Funk et al. 2006). However, the actual phenotypes that mediate premating reproductive isolation have been studied at the genetic level in only a few systems (e.g., Hawthorne and Via 2001; Cresko et al. 2004; Dambroski et al. 2005; Terai et al. 2006).

Laboratory and field genetic analysis of sexual isolation within and among species has revealed descriptions of numbers and the influence of QTLs and genes on male mating success (Mackay et al. 2005), male and female mate discrimination (Moehring et al. 2004; Kronforst et al. 2006), floral differences influencing pollinator success (Bradshaw et al. 1995; Schemske and Bradshaw 1999), courtship songs (Hoikkala et al. 2000; Shaw and Danley 2003; Gleason and Ritchie 2004; Etges et al. 2007), and pheromones (Coyne et al. 1999; Roelofs et al. 2002; Jallon and Wicker-Thomas 2003; Gleason et al. 2005; Sheck et al. 2006). Of these, intraspecific studies are critical to understanding how reproductive divergence evolves, and the ecological context in which genetic changes are assessed should help to inform us of how reproductive isolation is initiated in nature.

Because expression of signal traits involved with variation in mate choice can be influenced by the environments in which they are measured and often result from G × E interactions (Danielson-Francois et al. 2006; Mackay and Anholt 2007; Mills et al. 2007; Rodriguez et al. 2008), cross-environment genetic analysis is also necessary to assess sensitivity of QTL and gene expression, especially in environments like those in the wild. Without specific attention to environmental sensitivity, many laboratory QTL and gene studies may provide misleading insight into the kinds of genetic systems actually involved with reproductive isolation in nature. This problem is especially acute in many *Drosophila* species because the ecological conditions experienced by larvae and adults in nature are not well understood.

ECOLOGY AND REPRODUCTIVE DIVERGENCE IN *D. MOJAVENSIS*

The consequences of host plant use on reproductive isolation in *D. mojavensis* have been investigated because populations adapted to different host cacti in allopatry show low, host plant-dependent sexual isolation between Baja California and mainland populations (Etges 1989, 1990, 1992; Etges and Ahrens 2001). *Drosophila mojavensis* is thought to have originated in Baja California in isolation from its closest relatives due to the northwestward tectonic drift of the peninsula (Gastil et al. 1975), and then diverged into southern California and across the Gulf of California into mainland Sonora, Sinaloa, and Arizona, and in some areas, became sympatric with its closest relative, *D. arizonae* (Heed 1982; Ruiz et al. 1990; Etges et al. 1999). During this transition, *D. mojavensis* switched from its preferred host plant, pitaya agria, *Stenocereus gummosus*, to organ pipe, *S. thurberi*, and sina cactus, *S. alamosensis*, in Sonora and Sinaloa, as well as California barrel cactus, *Ferocactus cylindraceous*, in the Mojave Desert and *Opuntia demissa* (= *phaeacantha*) on Santa Catalina Island off the California coast (Heed and Mangan 1986; Newby and Etges 1998). The Gulf of California is now a major geographical barrier allowing mainland *D. mojavensis* populations to evolve with little apparent gene flow from Baja California (Heed 1978). Mainland populations have undergone considerable evolution including allozyme and inversion frequency shifts (Zouros 1974; Etges et al. 1999), host-related physiological adaptation (Starmer et al. 1977; Etges and Klassen 1989), and cactus-specific changes in egg to adult viability, development time, and thorax size consistent with directional natural selection due to this host plant shift (Etges and Heed 1987; Etges 1989, 1990, 1993; Filchak et al. 2005).

Baja California and mainland Mexico *D. mojavensis* populations are considered incipient species because of low levels of sexual isolation among populations, postmating-prezygotic isolation (Knowles and Markow 2001), but no observed postmating hybrid sterility/inviability (Ruiz et al. 1990). Male courtship songs differ consistently between isolated populations, are genetically differentiated (Etges et al. 2006), and influence courtship

success (Etges et al. 2007). Furthermore, flies reared on fermenting agria tissues show low and often nonsignificant premating isolation in laboratory mating trials whereas organ pipe-reared flies show greater (and significant) levels of premating isolation. These rearing substrate shifts also influence sex-specific epicuticular hydrocarbons that mediate premating isolation (Stennett and Etges 1997; Etges and Ahrens 2001).

EPICUTICULAR HYDROCARBON VARIATION IN *D. MOJAVENSIS*

There is extensive geographic variation between Baja California and mainland populations in epicuticular hydrocarbon (CHC) profiles. Cactus-reared flies from common garden experiments showed CHC differentiation that was coincident with regional differences in life histories, levels of sexual isolation, and courtship songs (Stennett and Etges 1997; Etges and Ahrens 2001; Etges 2002). Many CHCs are sexually dimorphic in *D. mojavensis* and its closest relatives, and composed of *n*-alkanes, methyl-branched alkanes, *n*-alkenes, methyl-branched alkenes, and alkadienes (Toolson et al. 1990; Etges and Jackson 2001) comprising mostly odd-numbered carbon chains ranging in size in from C_{29} to C_{39}. CHC transfer or "perfuming" experiments revealed that CHC differences between Baja California and mainland males were perceived as pheromones by females (Etges and Ahrens 2001). Differences in a small subset of CHC components between mated and unmated males in courtship trials involving Baja California and mainland populations suggested that the number of CHCs with pheromonal activity may be small (Etges and Tripodi 2008).

Here, we investigate the genetic architecture of CHC differences between Baja California and mainland populations of *D. mojavensis* and show that CHCs are influenced by multiple QTLs, some with pleiotropic effects. In the first paper of this series, we demonstrated the role of multiple QTLs and QTL G × E (cactus) interactions on components of male courtship song and mating success in F_2 crosses of sexually isolated populations by assessing QTLs using 21 microsatellite markers located on all five major chromosomes (Etges et al. 2007). From this same set of crosses, we show that male epicuticular hydrocarbon differences are also influenced by growth on agria and organ pipe host cacti experienced during preadult stages, G × E interactions, as well as the presence/absence of females during adulthood influencing QTL detection. By combining courtship song and CHC differences into a regression model, we demonstrate how these multiple elements of mate recognition determine sexual isolation between diverging populations of *D. mojavensis*.

Materials and Methods

Origins of fly stocks and culturing procedures were explained in detail in a previous paper (Etges et al. 2007). In short, a population of *D. mojavensis* was derived from 544 wild-caught adults collected from San Quintin, Baja California in 2003, and a multi-female stock collected in 2002 from Organ Pipe Natl. Monument, Arizona was obtained from T. Markow. All flies were mass reared on banana food (Brazner and Etges 1993) at room temperature, and multiple pair-mated lines were established to create homokaryotypic lines for gene arrangement LP (q^5) on chromosome II and ST on chromosome III. Homokaryotypic lines were established from each population and were cytologically verified: no inversions were segregating. One line from each population was sib-mated for five generations to reduce microsatellite heterozygosity.

A series of mass reciprocal crosses using these lines were then performed over the course of the experiment, and all F_2 flies from each cross were reared on fermenting agria or organ pipe cactus. Five to ten cultures of each cactus were set up at a time in plugged half-pint bottles using established techniques (Etges and Heed 1987; Etges and Ahrens 2001) in an incubator programmed for 27°C during the day and 17°C at night on a 14:10 LD cycle. Egg to adult viability and development times were monitored to insure consistency among cultures. Eclosed adults from each replicate culture were counted daily, separated by sex, and aged to maturity (12–14 days) on banana food in vials in the incubator described above.

We recorded mating success, time to copulation, and courtship songs of each male throughout the experiment (Etges et al. 2007). Each batch of cultured males was split into two groups: those used in courtship trials and song recording, and those that remained virgin, all male groups. For the courtship trials, 10 F_2 males were observed for an hour with 10 mature, laboratory food-reared, mainland females in a 50 mL conical flask plugged with cotton. These females were reared on laboratory food to increase levels of female choice (Brazner and Etges 1993). Time to copulation was recorded, copulating pairs were aspirated out, separated into individual food vials, and male courtship songs were then recorded in the presence of two wingless, sexually mature mainland females (Etges et al. 2007). All unmated males were treated similarly for courtship song recording.

Total epicuticular hydrocarbons were then extracted by immersing each male in hexane for 20 min in a 300 μl glass vial insert (Microliter Analytical Supplies, Suwanee, GA), evaporating off all hexane in a 40°C heating block, and freezing each sample at −20°C until analysis. Individual CHC extracts were redissolved in 5 μl of heptane containing a known amount of docosane (C_{22}) as an internal standard. Each sample was analyzed by capillary gas-liquid chromatography using an automated Shimadzu GC-17A (Shimadzu Scientific Instruments, Columbia, MD) fitted with a 15 m (ID = 0.22 mm) Rtx-5 fused-silica column (Restek Corporation, Bellefont, PA). Injector and detector temperatures were set at 290°C and 345°C, respectively, with the

injector port in split mode (ratio = 3:1), and the column was heated from 200°C to 345°C at 15°C/min holding at 345°C for 4 min.

DNA was then extracted from each male using a Puregene DNA kit (Gentra Systems, Inc., Minneapolis, MN), frozen at −80°C., eluted into 20 μl volume, and shipped to Noor's lab on dry ice for genotyping. DNA samples were gridded into 96-well format, and were genotyped for 21 microsatellite markers. Four of these markers were located near candidate genes (all < 15 kb away) affecting cuticular hydrocarbon profile or courtship song (Dmoj2_2868a near *Slowpoke*, Dmoj2_6540c near *fruitless*, Dmoj2_1603a near *desat1 and desat2* (6781 bp apart), and Dmoj5_1232a near *croaker* (see Etges et al. 2007 for details). PCR was performed in 10 μl reactions containing 0.5–1.0 μl of fly DNA preparation, using the following touchdown cycling protocol: 1 min for 95°C, 3× (95°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec), 3× (95°C for 30 sec, 53°C for 30 sec, 72°C for 30 sec), 30× (95°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec) (Palumbi 1996). Products were visualized on a polyacrylamide gel using a LiCor DNA analyzer (Li-Cor Biosciences, Lincoln, NE). We observed extensive microsatellite polymorphism within the lines, and sometimes there were alleles shared between the two lines at some of the markers used even after inbreeding. As a result, we only scored those individuals that had alleles unambiguously derived from a particular parental line. Genotypes were scored manually and entered into Microsoft Excel.

ANALYSIS OF EPICUTICULAR HYDROCARBON VARIATION AND MATING SUCCESS

Amounts of 31 CHC components (Stennett and Etges 1997; Etges and Ahrens 2001; Etges and Jackson 2001) were quantified in all flies by analysis of peak integrations using Class VP 4.2 software provided by Shimadzu, quantified by using amounts of C_{22} as an internal standard, and expressed as nanograms/fly. All CHC data were $\log_{10}$ transformed to improve normality. First, multivariate analysis of variance (MANOVA) was used to assess F_2 male differences in CHC composition between mated and unmated males with cactus, reciprocal cross type (several Baja × mainland reciprocal crosses were performed throughout the experiment [see Etges et al. 2007]), and all interaction terms. Principal components (PCs) analysis was used to identify different combinations of correlated CHC amounts and canonical discriminant function (CDF) analysis was used to assess CHC differences due to rearing substrate. A binary logit regression model with mating success as a function of cactus substrate, CHC PCs, and all PC × cactus interaction terms was used to detect CHC-related differences in mating success. We combined this model with PCs formed from the five courtship song parameters described in Etges et al. (2007) to assess the relative contributions of cactus, CHCs, and courtship song to mating success in *D. mojavensis*.

QTL ANALYSIS

We used single-marker regressions using PROC GLM (SAS-Institute 2004) for 21 microsatellite loci distributed across all five chromosomes of *D. mojavensis* (Etges et al. 2007) to detect QTL significance associated with CHCs. Linkage analysis revealed that only two loci were linked (Dmoj2010 and Dmoj2030), separated by 29.1 cM, so interval mapping or other derivatives (e.g., CIM, MIM) with QTL Cartographer (Basten et al. 2002) were not possible. Thus, QTL effects revealed here, by regression, are likely to be independent from one another, presumably due to elevated recombination rates in *D. mojavensis* (Ortiz-Barrientos et al. 2006). Overestimating numbers of QTLs due to linkage between markers was possible, but observations that the markers were segregating independently suggested this was not a major concern.

For each PC and locus, analysis of variance (ANOVA) models included cactus, exposure to females (males used in mating trials and song recordings vs. virgins), and replicate reciprocal cross as main effects and all interactions to control for possible variation among reciprocal sublines used throughout the course of the experiment. All F_2 flies from each replicate cross were always cultured in parallel on both agria and organ pipe cactus. As controls, parallel single marker regressions were always performed without the replicate reciprocal cross effect, and in almost all cases, the results were the same. All probabilities were adjusted using sequential Bonferroni corrections (Rice 1989). Least square means were assessed for genotype (additive), cactus, and exposure differences, as well as G × E (cactus), G × E (exposure), and E × E (cactus × exposure) interactions, to scrutinize the expression of mainland vs. Baja QTL genotypes and compare them to known population level differences.

We also assessed epistasis among pairs of loci (as covariates) by multiple regression using only QTLs significant after Bonferroni correction. For each PC, locus × locus interactions were included in the complete ANOVA model described above with each locus as a covariate including cactus, exposure, and all interaction terms. Because of missing values for genotypic data in our final dataset, we replaced missing genotypic values with the mean for each locus. If each fly with any missing values was omitted from the analysis, sample sizes were often fairly small. This is unbiased for the main effects and yielded identical results as the single marker regressions. This was also independent of other main effects, and therefore conservative for finding G × E interaction effects. Additive genetic models for multiple unlinked loci with interaction terms for locus × cactus, locus × exposure, cactus × exposure, and between main locus terms resulted.

Results

A total of 1688 males cultured on both cactus substrates were genotyped at 21 microsatellite loci including those described in

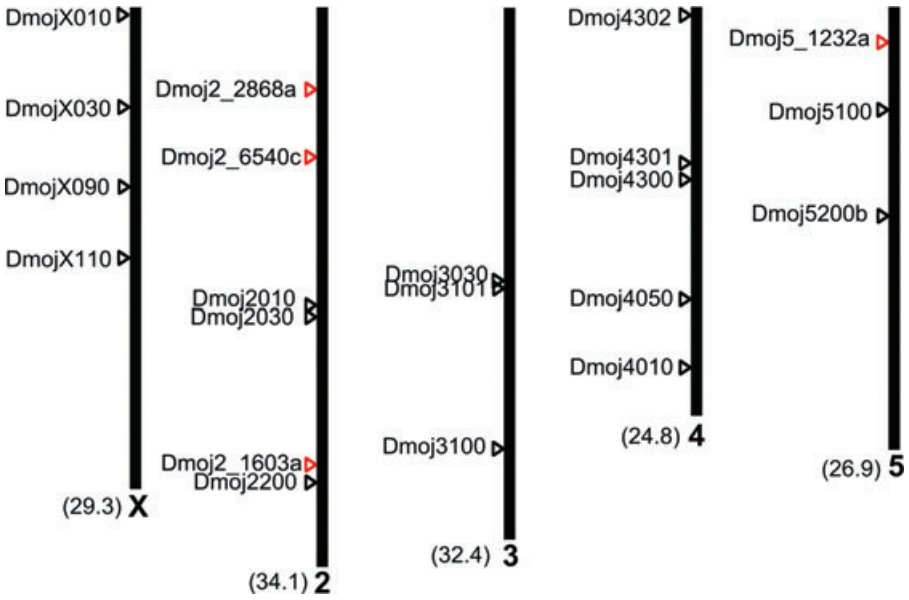

Figure 1. Locations of microsatellite markers in this study using the *D. mojavensis* genome assembly. Physical distances for each chromosome are indicated in parentheses. Gray triangles indicate markers adjacent to candidate genes: Dmoj2_2868a is near *slowpoke*, Dmoj2_6540c is near *fruitless*, Dmoj5_1232a is near *croaker*, and Dmoj2_1603a is near *desat1* and *desat2*. See Etges et al. (2007) for details.

Staten et al. (2004) distributed over the five major chromosomes (Fig. 1). Missing genotypic data were caused by significant allelic sharing in the parental populations present after moderate inbreeding, so not all F_2 genotypes could be unambiguously scored and each locus was first analyzed separately. Some CHC samples were lost due to contamination resulting in a total of 1650 males, comprising 889 from the mating trials and 761 virgin males, referred to as "exposed" to females and "unexposed" males, respectively.

VARIATION IN EPICUTICULAR HYDROCARBONS

The first five PCs for $\log_{10}$ transformed CHC amounts per male accounted for 81.6% of the total variance in the data (Table 1). As PC 5 accounted for just 3% of the total variation, we did not attempt to interpret any smaller components of CHC variation or include them in the QTL analysis. All 31 individual CHC components were positively correlated with PC 1 that accounted for 61.5% of the total CHC variation. Thus, this component reflected overall variation in individual CHC amounts throughout the experiment. The remaining PCs showed considerable structure, with both $\pm$ intermediate loadings for each CHC. Few strong correlations with individual CHCs were observed for PC 2–5 (Table 1), but positive and negative loadings for groups of CHCs covaried along these axes of variation representing different "blends" of CHCs among F_2 male *D. mojavensis*.

Fly age when CHC extracts were prepared could not be completely controlled because the mating trials and courtship song recordings were necessarily performed before CHC extraction. Since *D. mojavensis* males attain sexual maturity at 8–10 days at 25°C (Markow 1982), and our flies were reared in a diurnal temperature regime (weighted 24 h $\bar{X} = 22.8$°C), all males were aged for at least 12–14 days before starting the mating trials, consistent with our previous studies. Ages of F_2 males in the mating trials were equivalent (agria $\bar{X} \pm$ SE; 16.9 ± 0.17 days, $n = 512$: organ pipe $\bar{X} \pm$ SE; 16.9 ± 0.14 days, $n = 390$). However, males from the mating trials and those not exposed to females (virgins) were within a day or two in age when CHC extracts were prepared (agria $\bar{X}$, exposed vs. unexposed; $26.6 < 27.3$ days, $t = 2.15$, $P = 0.032$: organ pipe $\bar{X}$, exposed vs. unexposed; $26.4 < 28.9$ days, $t = 7.33$, $P < 0.0001$).

CHCs AND MATING SUCCESS

CHC profiles were significantly different between mated and unmated males from the mating trials (MANOVA; Wilks' $\lambda = 0.9282$, $F = 2.09$, df $= 31,837$ $P = 0.0005$), and influenced by cactus substrates (Wilks' $\lambda = 0.9188$, $F = 2.39$, df $= 31,837$ $P < 0.0001$), but there was no mating success $\times$ cactus interaction ($P = 0.43$; Table 2). There were significant differences in CHCs in males from different reciprocal crosses cultured throughout the experiment when different cactus cultures were initiated: this was likely influenced by differences among lines used for the crosses and variation in cactus tissues used for culturing flies (Etges 1989; 1993), as well as experimental error. Thus, reciprocal cross was included as a factor in all QTL analyses (Etges et al. 2007).

CDF analyses were then performed separately for agria- and organ pipe-reared flies. Agria-reared, mated males significantly differed in CHC profiles from unmated males (Fig. 2; canonical

Table 1. The 31 epicuticular hydrocarbon components in *D. mojavensis* included in this study—most identified by GCMS; Etges and Jackson (2001), their equivalent chain lengths (ECL) based on relative retention times with known standards, and the loadings of each hydrocarbon component on each of the five PCs based on all adult males in this study reared on both host cacti (n=1650).

Hydrocarbon	ECL[1]	PC 1	PC 2	PC 3	PC 4	PC 5
2-methyloctacosane	$C_{28.65}$	0.1583	0.2449	0.1168	0.3537	−0.2200
2-methyltricontane	$C_{30.65}$	0.1741	0.1910	0.2063	0.2336	−0.3069
7- and 9-hentricontene	$C_{30.78}$	0.2008	−0.1525	−0.0786	0.0663	−0.1650
Unknown	C_{32}	0.1162	−0.1447	0.5165	−0.0291	0.4047
Unknown alkene	C_{33br1}	0.1321	0.1297	−0.2156	0.2265	0.5237
11-and 13-methyldotricontane	C_{33br2}	0.1624	0.3266	0.0579	0.0019	−0.0265
Unknown alkene	C_{33br3}	0.1581	0.3449	−0.0749	−0.0288	0.1866
31-methyldotricont-8-ene	$C_{32.47}$	0.2023	0.2425	−0.0654	−0.0970	0.0304
31-methyldotricont-6-ene	$C_{32.56}$	0.1783	0.1569	0.1652	0.0646	0.1270
8,24-tritricontadiene	$C_{32.63}$	0.1517	0.1532	0.3306	0.2357	−0.2008
7,25-tritricontadiene	$C_{32.70}$	0.1953	−0.0831	−0.1297	0.0355	−0.0184
10-, 12-, and 14-tritricontene	$C_{32.79}$	0.2131	−0.0987	−0.0615	−0.0327	−0.1629
Unknown	$C_{32.86}$	0.1835	−0.1623	0.0477	0.0317	−0.2382
8,26-tetratricontadiene	$C_{34diene1}$	0.1632	−0.1488	0.2034	0.0147	0.0595
6,24- and 6,26-tetracontadiene	$C_{34diene2}$	0.2003	−0.1911	0.1912	−0.0473	0.0676
10-, 12-, and 14 tetretricontene	C_{34ene}	0.1828	−0.2725	0.1570	−0.0480	−0.0389
33-methlytetratricont-10-ene	C_{35alk1}	0.1809	0.3121	−0.0576	−0.1147	0.0829
33-methlytetratricont-8-ene	C_{35alk2}	0.1853	0.2555	−0.0807	−0.2590	−0.0164
Unknown alkene	C_{35alk3}	0.1906	0.1157	0.2779	−0.1778	0.0888
9,25-pentatricontadiene	$C_{34.59}$	0.2130	−0.0934	−0.0958	−0.0440	−0.0412
8,26-pentatricontadiene	$C_{34.66}$	0.2191	−0.0419	−0.0956	−0.1500	−0.0959
7,27-pentatricontadiene	$C_{34.73}$	0.1451	−0.0945	−0.0291	−0.2420	0.1321
Unknown alkene	C_{36a}	0.2035	−0.1593	−0.1607	−0.0866	−0.0915
Unknown alkene	C_{36b}	0.1662	−0.2391	0.1912	0.0132	0.2014
35-methylhexatricont-10-ene	C_{37br}	0.1572	0.0026	−0.1926	0.0979	0.2269
9,27-heptatricontadiene	$C_{36.5}$	0.2050	−0.0619	−0.1699	−0.1263	−0.0120
8,28-heptatricontadiene	$C_{36.6}$	0.2065	−0.0208	−0.1243	−0.2318	−0.0813
14-, 16-, and 12-hexatricontene	$C_{36.7}$	0.2026	−0.0781	−0.1006	−0.1711	−0.0981
Unknown alkene	C_{38}	0.1815	−0.1003	−0.1237	0.2734	−0.0994
Unknown alkene	C_{39}	0.0994	−0.1713	−0.1840	0.4946	0.1362
Unknown alkene	C_{40}	0.1694	−0.0795	−0.1995	0.2352	0.1322
Eigenvalue		19.077	2.661	1.568	1.057	0.945
Percentage of total variance		61.54	8.58	5.06	3.41	3.05

[1]Equivalent chain length as calculated in Stennett and Etges (1997) or other hydrocarbon name if component not yet identified (Etges and Jackson 2001).

means, 0.497 > – 0.248, $F = 1.89$, df = 31,472, $P = 0.0032$). Analysis of least square means from ANOVA for agria-reared flies revealed that mated males were characterized by lower quantities of most CHCs (all $P < 0.05$), particularly 7- and 9-hentricontene, C_{32}, 7,25-tritricontadiene, 6,24- and 6,26-tetracontadiene, and 10-, 12-, and 14- tetretricontene. Only 33-methlytetratricont-8-ene was more abundant in mated males. Mated organ pipe-reared males also tended to have less CHCs than unmated males, but this difference was not significant (Fig. 2; canonical means, – 0.400 < 0.285, $F = 1.30$, df = 31,353, $P = 0.1332$). Interestingly, the proportion of unsuccessful agria-reared males was greater than that of unmated organ pipe-reared males (Fisher's exact test, $P =$ 0.014), suggesting that mainland females in the mating trials were not as receptive toward agria-reared males with low CDF 1 scores. Therefore, agria cactus caused greater variation in CHC profiles than organ pipe cactus, resulting in increased mating success for males with lower amounts of a small group of CHCs.

Binary logit regressions of CHC PCs and all PC × cactus interaction terms with mating success as the dependent variable reaffirmed cactus substrates as a significant predictor of mating success (Table 3). Including age when CHCs were extracted into the model had no effect on these results (results not shown), so the delay in preparing extracts had undetectable effects on CHC profiles. Evaluating all five PCs revealed that variation in PC 2 and

Table 2. MANOVA results for differences in epicuticular hydrocarbons among F_2 males in this study due to mating success (maleperf; 0, 1), cactus rearing substrate, and the reciprocal cross (block effect) used when flies were cultured.

	Wilks' λ	*F* Value	df	Pr>*F*
Maleperf	0.9282	2.09	31,837	0.0005
Cactus	0.9188	2.39	31,837	<0.0001
Cross	0.0409	24.29	155,4146.9	<0.0001
Maleperf × Cactus	0.9633	1.03	31,837	0.4261
Maleperf × Cross	0.7690	1.46	155,4146.9	0.0002
Maleperf × Cactus × Cross	0.8278	1.30	124,3323.2	0.0145

PC 4 was associated with differences in mating success (Table 3, Fig. 3). Organ pipe-reared males maintained higher and more constant mating success ($P = 0.0044$) over a broader range of PC scores for both PC 2 and PC 4 than agria-reared males, except for the highest scores for PC 4 leading to a PC 4 × cactus interaction. Thus PC 2 and PC 4, representing different covarying subsets of F_2 male CHCs, were associated with significant variation in mating success with mainland females, but in a cactus-specific manner in which organ pipe-reared males copulated at higher frequencies than agria-reared males. Only agria-reared males with high positive PC 4 scores tended to be as successful as organ pipe-reared males (Fig. 3B). Overall CHC differences revealed in the CDF analysis obscured this, but did help to explain the higher numbers of unmated agria-reared males relative those reared on organ pipe (Fig. 2).

A handful of CHCs influenced PC 2 and PC 4 that were responsible for variation in mating success, but few individual CHC components were strongly associated with either axis of variation (Table 4). The structure of PC 2 was quite different from that derived from the entire dataset (Table 1) that included virgin males, but PC 4 retained many similar loadings with CHC components in both cases. PC 2 was characterized by negative loadings for the relatively smaller peaks 11- and 13-methyldotricontane,

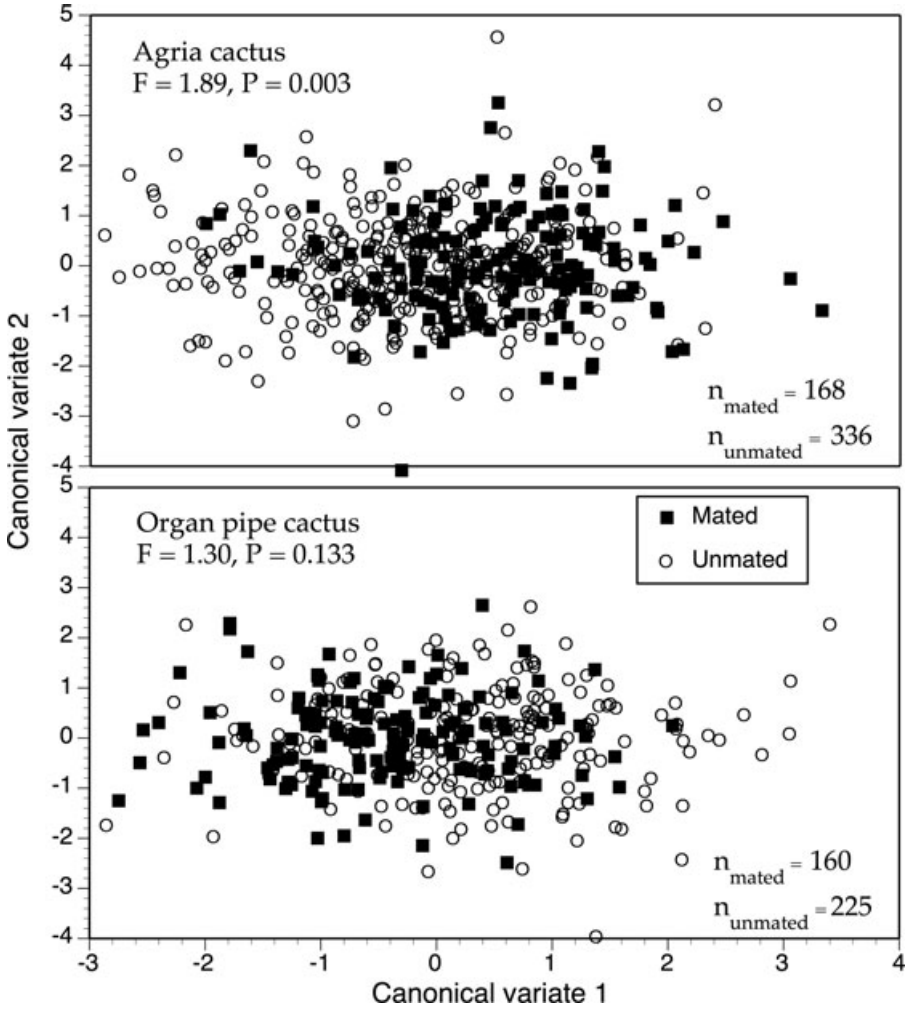

Figure 2. Plots of the first two canonical discriminant functions based on CHC profiles of mated and unsuccessful male *D. mojavensis*. The upper panel shows differences for agria-reared flies and below for organ pipe-cactus reared flies.

Table 3. Logistic regression results for male mating success influenced by cactus rearing substrates, epicuticular hydrocarbon PCs, and their interactions. The overall regression model slope was different from zero (Wald chi-square=32.05, df=11, *P*=0.0007). Significant effects are italicized in bold.

Effect	df	Wald chi-square	*P*
Cactus	1	8.126	***0.0044***
PC 1	1	0.193	0.6604
PC 2	1	4.838	***0.0278***
PC 3	1	0.006	0.9374
PC 4	1	10.787	***0.0010***
PC 5	1	2.032	0.1539
PC 1 × Cactus	1	0.045	0.8312
PC 2 × Cactus	1	1.007	0.3155
PC 3 × Cactus	1	0.013	0.9086
PC 4 × Cactus	1	6.189	***0.0129***
PC 5 × Cactus	1	0.671	0.4129

33-methlytetratricont-10-ene, and 33-methlytetratricont-8-ene, as well as 31-methyldotricont-8-ene, a major CHC component. High positive loadings on PC 2 included all C_{34} CHCs, i.e., 8,26-tetratricontadiene, 6,24- and 6,26-tetracontadiene, and 10-, 12-, and 14-tetretricontene, as well as the uncharacterized C_{32} and C_{36b} components consistent with CHC differences between mated and unmated males in the CDF analysis (see above). Variation in PC 4 scores was associated with positive loadings for 2-methyloctacosane, 2-methyltricontane, 7- and 9-hentricontene, and the three long chain C_{38}, C_{39}, and C_{40} components, and negative loadings for the C_{32} component, two of the smaller C_{35} alkenes, and 8,28-heptatricontadiene (Table 4). These results are concordant with increased mating success of males associated with differences in amounts of C_{34}, C_{37}, and C_{38} CHC components in the CDF analysis (above) and in courtship trials using only the parental populations (Etges and Tripodi 2008). Thus, mating success of F_2 *D. mojavensis* males was determined by variation in small groups of CHCs that characterized each of these PCs, as well as the host cactus they were reared on.

QTL ANALYSIS

Significant differences in CHC amounts were found between the "exposed" and "unexposed" groups of males, so this factor was included in all single-locus QTL models (Table 5). In every case of significance, and for all PCs and QTLs, male least square mean PC scores from the "exposed" group were less than those of the "unexposed" group, or virgin males. Because males from each group were cultured on cactus together, and virgin males were held in small groups in individual vials, "exposed" males differed only in their experience by mating with and being in proximity to mainland females in the mating trials and during song recording. Thus, we treated "exposure" to females as an additional environmental effect.

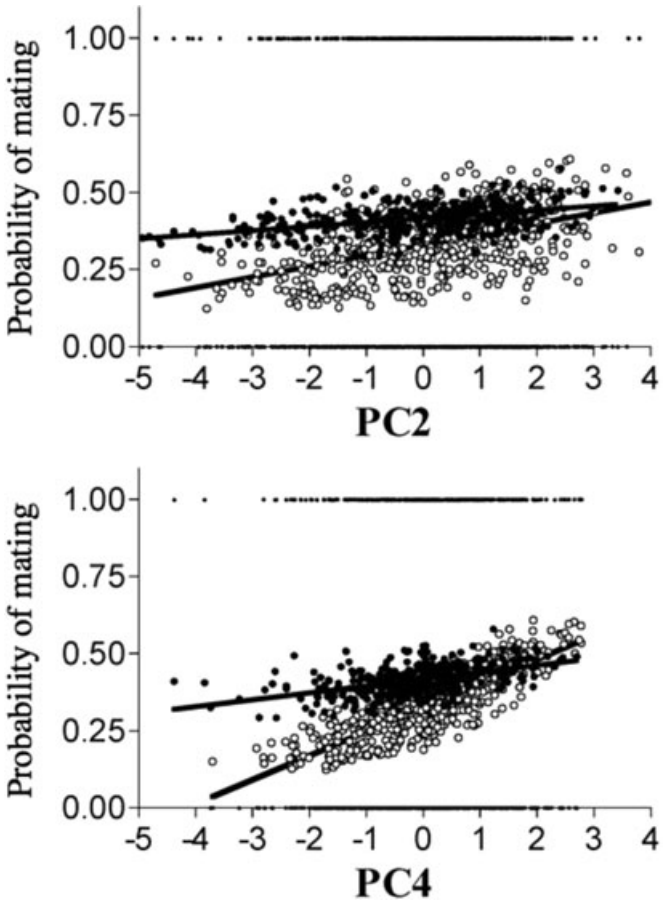

Figure 3. Binary logit regressions of CHC PC 2 and PC 4 on mating success. Filled circles and the top regression lines refer to organ pipe cactus-reared males; open circles and bottom regression lines are agria cactus-reared males.

Main effects of QTLs, cactus, and female exposure

Detected QTLs in the form of significant single-locus effects after sequential Bonferroni correction (Table 5) were found on all chromosomes, but six QTLs influenced multiple PCs. PCs were influenced from four to eight QTLs each, and Baja associated genotypes (BB and/or MB) were associated with significantly greater PC scores for 21 of the 30 detected QTLs (Table 6). All significant X-linked QTLs were consistent with this pattern as were the 5 QTLs influencing PC 3. The region marked by Dmoj2_6540c near *fruitless* influenced PC 1–4, where Baja alleles increased PC scores in three of four cases. Dmoj4300 also influenced four PCs, but Baja allelic effects increased PC scores in two of four cases. The single QTL that influenced mating success, Dmoj2_1603a near *desat1 and desat2* (Etges et al. 2007), also influenced PC 2, 4, and 5. Thus, the main effects of the six detected QTLs with pleiotropic effects in this study influenced PC scores in contrasting ways, and the overall variation in CHCs

Table 4. The structure of PC 2 and PC 4 based on male *D. mojavensis* used in the mating trials (*n*=889). A number of CHCs with high loadings on each PC are indicated in bold.

Hydrocarbon	ECL[1]	PC 2	PC 4
2-methyloctacosane	$C_{28.65}$	−0.1727	**0.3240**
2-methyltricontane	$C_{30.65}$	−0.1520	**0.2304**
7- and 9-hentricontene	$C_{30.78}$	0.1692	**0.1895**
Unknown	C_{32}	**0.2331**	**−0.2218**
Unknown alkene	C_{33br1}	−0.1306	−0.0329
11-and 13-methyldotricontane	C_{33br2}	**−0.3071**	0.0632
Unknown alkene	C_{33br3}	**−0.3434**	−0.1184
31-methyldotricont-8-ene	$C_{32.47}$	**−0.2380**	−0.1018
31-methyldotricont-6-ene	$C_{32.56}$	−0.1040	−0.0332
8,24-tritricontadiene	$C_{32.63}$	−0.1013	0.1319
7,25-tritricontadiene	$C_{32.70}$	0.0910	0.0946
10-, 12-, and 14-tritricontene	$C_{32.79}$	0.1122	0.0771
Unknown	$C_{32.86}$	**0.1886**	**0.1854**
8,26-tetratricontadiene	$C_{34diene1}$	**0.1930**	0.1007
6,24- and 6,26-tetracontadiene	$C_{34diene2}$	**0.2331**	−0.0685
10-, 12-, and 14 tetretricontene	C_{34ene}	**0.3284**	0.0000
33-methlytetratricont-10-ene	C_{35alk1}	**−0.3009**	−0.1558
33-methlytetratricont-8-ene	C_{35alk2}	**−0.2731**	**−0.2394**
Unknown alkene	C_{35alk3}	−0.0616	**−0.2058**
9,25-pentatricontadiene	$C_{34.59}$	0.0735	−0.0078
8,26-pentatricontadiene	$C_{34.66}$	0.0232	−0.0922
7,27-pentatricontadiene	$C_{34.73}$	0.1350	−0.1345
Unknown alkene	C_{36a}	0.1068	−0.0380
Unknown alkene	C_{36b}	**0.2652**	−0.1172
35-methylhexatricont-10-ene	C_{37br}	0.0042	−0.0325
9,27-heptatricontadiene	$C_{36.5}$	0.0454	−0.1169
8,28-heptatricontadiene	$C_{36.6}$	−0.0218	**−0.2008**
14-, 16-, and 12-hexatricontene	$C_{36.7}$	0.0543	−0.1286
Unknown alkene	C_{38}	0.0038	**0.3314**
Unknown alkene	C_{39}	−0.1198	**0.5147**
Unknown alkene	C_{40}	0.0802	**0.1808**
Eigenvalue		2.413	1.746
Percentage of total variance		7.78	4.09

represented by these PCs suggests a multigenic basis for CHC expression in these populations of *D. mojavensis*.

Effects of cactus substrates on CHC variation were most prominent for PC 1 and PC 3 where 12 and 9 QTLs, respectively (Table 5), were associated with significantly greater PC scores for males reared on agria vs. organ pipe cactus (contrasts not shown). However, PC 2, 4, and 5 were influenced by cactus for three, three, and two QTLs, respectively, but in these cases, organ pipe cactus caused higher PC scores than agria (results not shown). Thus, the effects of cactus rearing substrates were specific to different PCs where agria cactus increased PC scores in association with most gene regions. Because all CHC amounts were positively correlated with PC 1 (Table 1), these cactus effects in the QTL analyses suggest that increased CHC amounts due to agria cactus were in part responsible. However, the few QTLs that were significantly influenced by cactus for PC 2 and PC 4 suggest that the number of genomic regions responsible for cactus-related differences in mating success (Fig. 3) may be rather small.

By including virgin males in our QTL analysis, we uncovered a striking effect of exposing males to females in the mating trials and during courtship song recording on CHC abundance and variation (Table 4). Reanalysis of the data showed that least square means of almost all CHC components were greater for virgin males than "exposed" males (results not shown). Not all males from the mating trials could be analyzed for courtship songs (each male was exposed to two mainland females in the recording chamber, about 5 min of song was recorded, and males were then frozen for CHC extraction [Etges et al. 2007]), so we pooled all "exposed" males for QTL analysis. Quantities of a few small CHC components differed between males from the mating trials and those also used for song recording. For almost all QTLs, virgin males had significantly higher least square mean PC scores for PC 4 and PC 5, as well as a handful of X, second, and fourth chromosome QTLs for PC 2. Conversely, all significant differences due to female exposure for PC 1 and PC 3 revealed that virgin males had lower PC scores than exposed males (results not shown). As variation in PC 2 and PC 4 scores was implicated in courtship success in the mating trials, these results suggest that mating status and/or the presence of females are important determinants of male CHC variation.

G × E and E × E interactions for locus, cactus, and female exposure

There were seven significant locus × cactus interactions after Bonferroni correction (Table 4). Those associated with Dmoj4050 could not be evaluated due to missing data and small sample sizes, but the remaining interactions revealed a compelling nonrandom pattern: in all but one case, mainland alleles caused increases in PC scores when males were reared on organ pipe cactus, and/or Baja alleles caused increases in PC scores when reared on agria cactus (Fig. 4). The exception involved DmojX010 that showed both types of interactions for PC 2 and PC 3, respectively. A Dmoj4010 × cactus interaction indicated possible heterosis for organ pipe-reared male PC 3 scores, although both MB and BB genotypes significantly increased PC 3 scores on agria. Thus, most of the G × E(cactus) interactions suggested that mainland and Baja alleles for these QTLs tended to increase different combinations of CHCs when flies were reared on the host cactus typically used in nature. Interestingly, none of these locus × cactus interactions involved the same marker regions as those observed for locus × cactus interactions that influenced mating success or time to copulation (Etges et al. 2007).

The effects of female exposure were also expressed as G × E interactions for 10 of 21 QTLs after Bonferroni correction

Table 5. Single-locus ANOVA results for the first five PCs (PC 1–5) based on variation in 31 epicuticular hydrocarbon components showing the significance of the main effects of locus (as a covariate), cactus, exposure to females, locus by cactus, and locus by exposure (G×E), and cactus by exposure interactions. Significant effects after strict Bonferroni correction are indicated in bold.

		X chromosome				Second chromosome					
Trait	Effect	X010 n=1224	X030 n=814	X090 n=1161	X110 n=1441	2_2868a n=1297	2_6540c n=1249	2010 n=1365	2030 n=1306	2_1603a n=1364	2200 n=1057
PCI	Locus				0.0095	**0.0004**	**<0.0001**	0.0288	0.0484		
	Cactus			**0.0092**	0.0182	**0.0042**	**<0.0001**	0.0204	**0.0040**		**0.0045**
	Exposure	**0.0037**		**0.0019**		**<0.0001**	**<0.0001**	**0.0085**		0.0479	
	Locus × cactus		**0.0086**		0.0209					0.0218	
	Locus × exposure		**0.0005**			**0.0067**	**<0.0001**				
	Cactus × exposure	0.0319	**<0.0001**	**0.0001**	**<0.0001**	0.0315	0.0190	0.0011	**<0.0001**	**<0.0001**	0.0113
PC 2	Locus				**0.0143**		**<0.0001**			**<0.0001**	
	Cactus										
	Exposure			**<0.0001**		**0.0007**	**<0.0001**		0.0426		
	Locus × cactus	**0.0053**									
	Locus × exposure		**0.0004**	**0.0016**			**<0.0001**			**0.0196**	
	Cactus × exposure			0.0095	0.0107	0.0308		0.0084	**<0.0001**	**0.0031**	
PC 3	Locus			**0.0146**		0.0353	**0.0013**				**0.0022**
	Cactus	**0.0011**		0.0109	**0.0004**	**0.0053**		**0.0062**	**0.0083**	**0.0094**	0.0279
	Exposure		**<0.0001**					0.0425			
	Locus × cactus	**0.0084**	0.0252								0.0491
	Locus × exposure		**0.0032**					**0.0006**			
	Cactus × exposure		**0.0044**				**0.0081**				
PC 4	Locus				**<0.0001**		**0.0119**	**<0.0001**	**0.0198**	**0.0094**	**<0.0001**
	Cactus	**0.0108**				0.0234	**<0.0001**				
	Exposure	**<0.0001**	**0.0027**	**<0.0001**	**<0.0001**	**0.0137**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	Locus × cactus					0.0132	**<0.0001**				
	Locus × exposure		0.0394			**0.0002**					
	Cactus × exposure	0.0105	0.0246	0.0347	0.0435		0.0279	**<0.0001**	**0.0002**	0.0019	0.0052
PC 5	Locus	**0.0002**	**0.0002**		**0.0044**					**0.0048**	
	Cactus										
	Exposure	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0002**	**<0.0001**	**<0.0001**
	Locus × cactus										
	Locus × exposure	0.0317					0.0254			0.0340	
	Cactus × exposure	0.0066	0.0339					0.0366		0.0200	

Continued

Table 5. Continued.

Trait	Effect	Third chromosome				Fourth chromosome				Fifth chromosome		
		3030 n=1191	3101 n=352	3100 n=1139	4010 n=1296	4050 n=214	4300 n=876	4301 n=848	4302 n=1020	5_1232a n=1379	5100 n=919	5200b n=955
PC 1	Locus			0.0352	**0.0046**	0.0240	**<0.0001**	0.0192		0.0194	0.0392	
	Cactus	**0.0002**		0.0311	**<0.0001**			**0.0001**	**<0.0001**	**0.0002**	**0.0025**	**<0.0001**
	Exposure				**0.0024**	**0.0161**		**<0.0001**		**0.002**		
	Locus × cactus					**0.0051**			0.0453			0.0146
	Locus × exposure			0.0290		**0.0030**					**0.0017**	**0.0033**
	Cactus × exposure	**<0.0001**		0.0305			**<0.0001**		**0.0004**		**<0.0001**	
PC 2	Locus			**0.0036**		0.0319	**0.0013**	0.0357				
	Cactus		**0.0108**					0.0267	**0.0062**			**<0.0001**
	Exposure	0.0287			**0.0002**	**<0.0001**	0.0302					
	Locus × cactus											**0.0390**
	Locus × exposure			**0.0007**		**<0.0001**		**0.0004**				
	Cactus × exposure	0.0205	**<0.0001**		**0.0005**		**0.0012**	**0.0010**		**0.0025**	0.0227	**<0.0001**
PC 3	Locus				**0.0001**		**0.0017**	**0.0062**				
	Cactus	0.0495			**0.0071**		**0.0090**		0.0171	0.0434	0.0480	**0.0091**
	Exposure										0.0359	
	Locus × cactus							**0.0008**				
	Locus × exposure								0.0112		**0.0014**	
	Cactus × exposure		0.0186					0.0240				
PC 4	Locus	**0.0105**										
	Cactus					**0.0003**						
	Exposure	**<0.0001**		**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0002**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	Locus × cactus					**0.0031**						
	Locus × exposure			0.0267		**0.0024**			0.0161			
	Cactus × exposure										**0.0004**	**0.0003**
PC 5	Locus		0.0281		**<0.0001**		**0.0002**		0.0455		**0.0030**	**0.0026**
	Cactus			**0.0040**		**0.0008**						
	Exposure	**0.0025**	**0.0002**	**<0.0001**	**<0.0001**	**0.0031**	**0.0006**	**<0.0001**	**0.0003**	**<0.0001**	**0.0065**	**0.0004**
	Locus × cactus											
	Locus × exposure					**0.0044**		0.0364			0.0334	
	Cactus × exposure									0.0411		

Table 6. Summary of all genotype differences for significant single-locus QTLs for the first five PCs based on F_2 male cuticular hydrocarbon variation in this study. MM, MB, and BB refer to mainland and Baja microsatellite genotypes, and all contrasts are based on least square means. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.

Marker	PC 1	PC 2	PC 3	PC 4	PC 5
DmojX010					M<B***
DmojX030					M<B***
DmojX090			M<B*		
DmojX110		M<B*		M<B****	M<B**
Dmoj2_2868a	MM<MB,BB***				
Dmoj2_6540c	MM,MB<BB****	MM,MB>BB****	MM<MB,BB**	MM,MB<BB*	
Dmoj2010				MM<MB,BB****	
Dmoj2030				MM<MB,BB*	
Dmoj2_1603a		MM,MB>BB****		MM>MB,BB**	MM,MB<BB**
Dmoj2200			MM,MB<BB**	MM<MB,BB****	
Dmoj3030				MM,MB>BB**	
Dmoj3100		MM<MB<BB**			
Dmoj4010	MM,MB<BB**		MM<MB,BB****		MM,MB>BB****
Dmoj4300	MM>MB>BB****	MM<MB,BB**	MM<MB,BB**		MM,MB>BB***
Dmoj4301			MM<MB,BB**		
Dmoj5100					MM,MB>BB**
Dmoj5200b					MM,MB>BB**

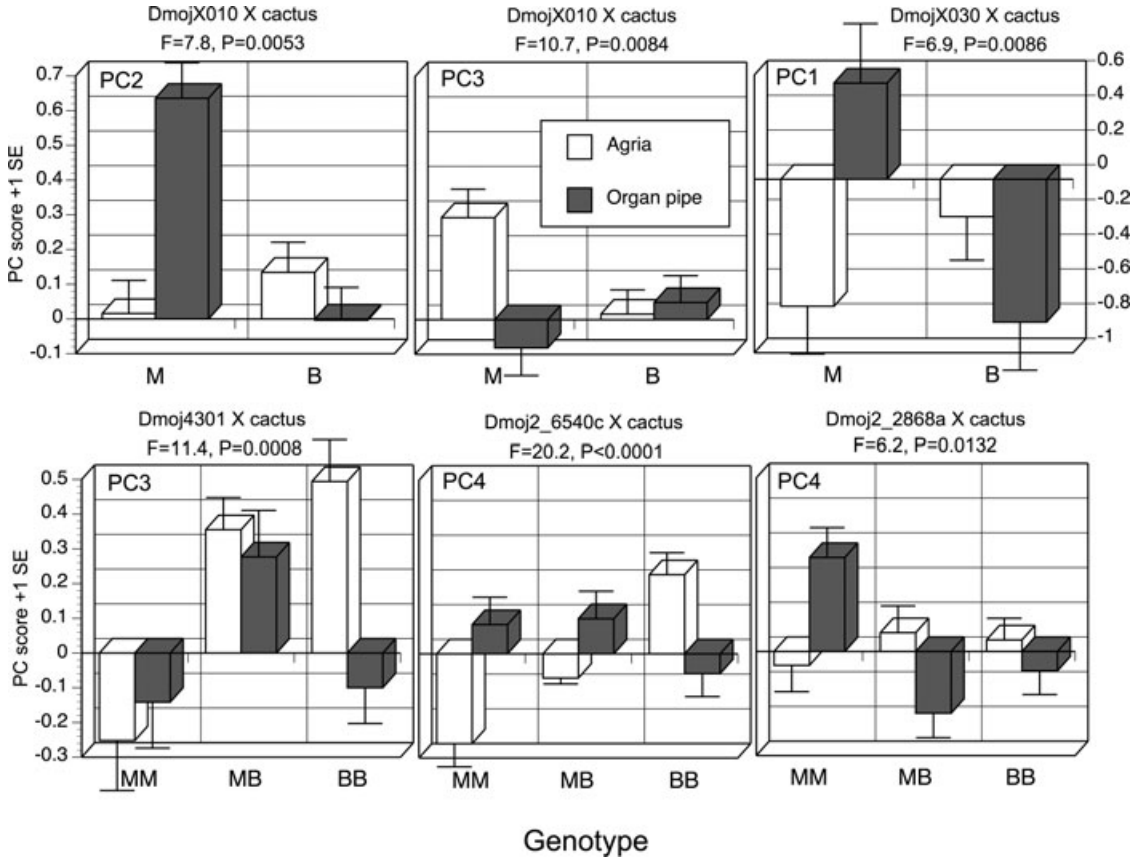

Figure 4. Plots of significant G × E (cactus) interactions for different CHC PCs in this study. MM, MB, and BB (or M and B for X chromosome markers) refer to mainland and Baja genotypes for each microsatellite locus.

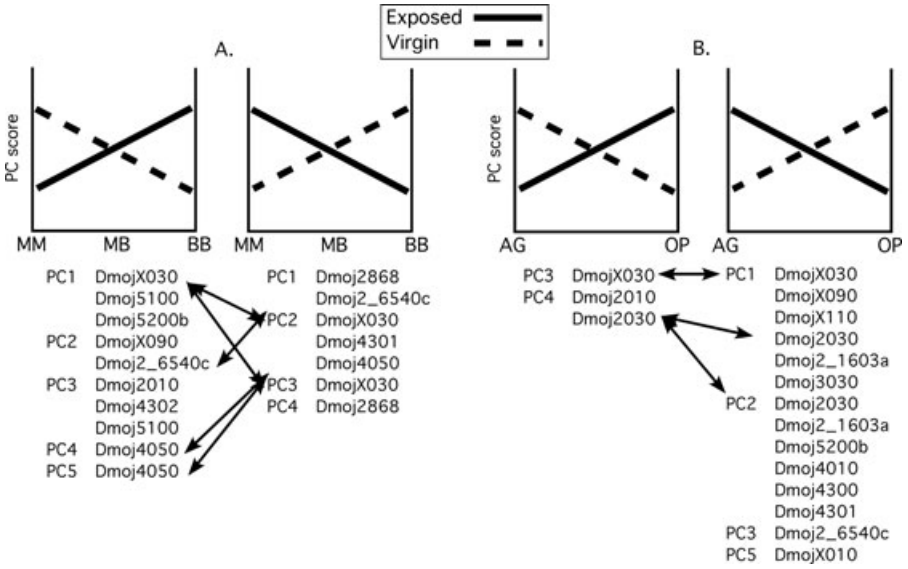

Figure 5. **The nature of significant (A) G × E interactions for exposure to females and (B) E × E interactions for rearing substrate and exposure to females for CHC Cs. All QTLs for which these effects were significant sources of variation after Bonferroni correction are listed. Genotypes are labeled as in Figure 4 and AG and OP refer to agria and organ pipe cactus, respectively.**

(Table 5). Inspection of the least square means for each significant interaction revealed that most of these locus × exposure interactions showed mainland alleles (MM, MB) of males exposed to females were associated with lower PC scores and Baja genotypes of virgin males were associated with higher PC scores (Fig. 5). The remaining significant interaction terms showed the opposite pattern. In a few cases, an interaction was influenced more by genotype-specific differences in exposed versus virgin males or vice versa, but for simplicity, we categorized the interactions into two types (Fig. 5A). Overall, these locus × exposure interactions were influenced by marker regions across all chromosomes and all five CHC PCs, and in three cases, the same gene region was involved in both patterns of locus × exposure interactions as described above, but for different PCs (DmojX030, Dmoj2_6540c, Dmoj4050; Fig. 5A). Although we cannot ascribe these effects to successful versus unsuccessful males in the mating trials because these flies were all subsequently exposed to mainland females for various time periods when songs were recorded, these G × E interactions revealed pervasive genotypic effects on CHC variation due to the presence of females. Thus, the female "environment" had a much more widespread effect on CHC expression across the genome of adult male *D. mojavensis* than cactus rearing substrates for both main and crossed effects.

Effects of E × E interactions between rearing substrates and exposure to females on CHC expression were significant sources of variation for 18 QTLs (Table 5). Inspection of these significant cactus × exposure interactions revealed that 14 of 17 interaction terms resulted from lower PC scores for males reared on organ pipe cactus when exposed to females versus higher or equivalent PC scores for agria-reared, virgin males (Fig. 5B). Six of these interactions influenced PC 1 (Table 5) revealing that amounts of most CHCs were reduced by the interaction of organ pipe cactus rearing and female exposure. Another six interactions involved PC 2, and one for PC 3 and PC 5 (Table 5). The remaining three of 17 interactions, DmojX030 for PC 3, and Dmoj2010 and Dmoj2030 for PC 4, were caused by increased PC scores for agria-reared males exposed to females and decreased PC scores for organ pipe-reared, virgin males (Fig 5B). As Dmoj2010 and Dmoj2030 were the only two markers showing linkage, the cactus × exposure interactions for PC 4 may not be independent. Therefore the pervasive effects caused by rearing on organ pipe cactus (Table 5) were significantly accentuated in "exposed" males, leading to higher overall mating success with mainland females than those reared on agria, particularly for PC 2 and PC 4 (Fig. 3).

Epistasis

Additive locus × locus interactions were infrequent, except for PC 1 where interactions between Dmoj2_2868a and two other QTLs were detected that resulted in a significant multilocus interaction with Dmoj2_6540c and Dmoj4300 (Table 7). As PC 1 encompassed variation in amounts of all CHCs, overall CHC variation in *D. mojavensis* males was influenced by more epistatically interacting regions of the genome than the other covarying groups of CHCs, or PCs. The overall lack of detected epistasis was unexpected given the known number of enzymatic steps required for elongation, desaturation, and translocation of CHCs to the epicuticle (Howard and Blomquist 2005; Legendre et al. 2008).

Table 7. Significance of additive locus × locus interactions for those QTLs found to be significant after Bonferonni correction (Table 5). Each interaction was taken from a complete ANOVA model with all main effects and interaction terms. The three-way interaction for PC 1 was detected in the same fashion. All interaction terms have one degree of freedom. Significant effects are italicized in bold.

Loci	Type III SS	*F*	*P*
PC 1			
Dmoj2_2868a × Dmoj2_6540c	122.057	***7.07***	***0.0079***
Dmoj2_2868a × Dmoj4010	8.747	0.49	0.4834
Dmoj2_2868a × Dmoj4300	93.274	***5.27***	***0.0218***
Dmoj2_6540c × Dmoj4010	28.836	1.64	0.1999
Dmoj2_6540c × Dmoj4300	1.955	0.11	0.7375
Dmoj4010 × Dmoj4300	1.227	0.07	0.7937
Dmoj2_2868a × Dmoj2_6540c × Dmoj4300	153.693	***8.94***	***0.0028***
PC 2			
DmojX110 × Dmoj2_6540c	0.000	0.00	0.9986
DmojX110 × Dmoj2_1603a	0.262	0.10	0.7505
DmojX110 × Dmoj3100	0.756	0.29	0.5892
DmojX110 × Dmoj4300	10.411	***4.01***	***0.0454***
Dmoj2_6540c × Dmoj2_1603a	0.923	0.36	0.5476
Dmoj2_6540c × Dmoj3100	0.000	0.00	0.9896
Dmoj2_6540c × Dmoj4300	0.575	0.22	0.6360
Dmoj2_1603a × Dmoj3100	0.090	0.03	0.8517
Dmoj2_1603a × Dmoj4300	0.716	0.28	0.5982
Dmoj3100 × Dmoj4300	0.386	0.15	0.6996
PC 3			
DmojX090 × Dmoj2_6540c	1.438	0.95	0.3309
DmojX090 × Dmoj2200	8.129	***5.31***	***0.0213***
DmojX090 × Dmoj4010	0.063	0.04	0.8392
DmojX090 × Dmoj4300	2.416	1.58	0.2092
DmojX090 × Dmoj4301	0.680	0.44	0.5049
Dmoj2_6540c × Dmoj2200	0.028	0.02	0.8927
Dmoj2_6540c × Dmoj4010	3.663	2.43	0.1194
Dmoj2_6540c × Dmoj4300	1.886	1.25	0.2642
Dmoj2_6540c × Dmoj4301	3.701	2.46	0.1173
Dmoj2200 × Dmoj4010	2.446	1.60	0.2058
Dmoj2200 × Dmoj4300	0.259	0.17	0.6807
Dmoj2200 × Dmoj4301	0.765	0.50	0.4797
Dmoj4010 × Dmoj4300	0.760	0.50	0.4796
Dmoj4010 × Dmoj4301	1.272	0.84	0.3602
Dmoj4300 × Dmoj4301	0.410	0.27	0.6040
PC 4			
DmojX110 × Dmoj2010	0.346	0.38	0.5384
DmojX110 × Dmoj2_1603a	2.073	2.24	0.1349
DmojX110 × Dmoj2200	2.119	2.33	0.1275
DmojX110 × Dmoj3030	5.443	***5.92***	***0.0151***
Dmoj2010 × Dmoj2_1603a	0.465	0.50	0.4805
Dmoj2010 × Dmoj2200	0.432	0.47	0.4948
Dmoj2010 × Dmoj3030	0.093	0.10	0.7514
Dmoj2_1603a × Dmoj2200	3.285	3.51	0.0610
Dmoj2_1603a × Dmoj3030	0.523	0.56	0.4557
Dmoj2200 × Dmoj3030	0.047	0.05	0.8231
PC 5			
DmojX010 × DmojX030	5.563	***6.34***	***0.0119***
DmojX010 × DmojX110	1.120	1.27	0.2605
DmojX010 × Dmoj2_1603a	0.836	0.95	0.3306
DmojX010 × Dmoj4010	0.001	0.00	0.9732
DmojX010 × Dmoj4300	0.816	0.94	0.3336
DmojX010 × Dmoj5100	0.090	0.10	0.7483
DmojX010 × Dmoj5200b	1.058	1.20	0.2735
DmojX030 × DmojX110	0.171	0.19	0.6618
DmojX030 × Dmoj2_1603a	0.782	0.88	0.3489
DmojX030 × Dmoj4010	0.006	0.01	0.9337
DmojX030 × Dmoj4300	0.593	0.67	0.4125
DmojX030 × Dmoj5100	0.013	0.01	0.9045
DmojX030 × Dmoj5200b	2.077	2.34	0.1264
Dmoj2_1603a × Dmoj4010	1.893	2.15	0.1426
Dmoj2_1603a × Dmoj4300	1.002	1.14	0.2860
Dmoj2_1603a × Dmoj5100	8.275	***9.39***	***0.0022***
Dmoj2_1603a × Dmoj5200b	0.994	1.11	0.2916
Dmoj4010 × Dmoj4300	5.118	***5.87***	***0.0155***
Dmoj4010 × Dmoj5100	0.581	0.67	0.4148
Dmoj4010 × Dmoj5200b	0.493	0.56	0.4555
Dmoj4300 × Dmoj5100	0.549	0.63	0.4291
Dmoj4300 × Dmoj5200b	0.541	0.61	0.4338
Dmoj5100 × Dmoj5200b	1.225	1.39	0.2388

Courtship songs and CHCs determine mating success

A PC analysis was performed on five courtship song components from Etges et al. (2007) to combine song and CHC PCs in the same logistic regression model with mating success as the dependent variable. The first song PC was clearly associated with burst frequency, PC 2 encompassed variation in interpulse interval (IPI) variation, and PC 3 was determined by differences in burst duration (Table 8). PC 4 and PC 5 were associated with IPI, song burst number, and duration, respectively. Variation in all these song components was significantly associated with differences in mating success except for short interpulse intervals (S-IPI) (Etges et al. 2007). Together with cactus substrates and all five CHC PCs, logit regression of five courtship song PCs and all PC × cactus interaction terms showed that variation in song PC 1 and PC 2 was associated with differences in mating success, as well as CHC PC 4 (Table 9). Not all variation in song and CHCs was independent, however. After Bonferonni correction, song PC 1 and CHC PC 5 were negatively correlated in agria-reared flies ($r = 0.308$, $P < 0.0001$, $n = 207$), and song PC 3 and CHC PC 1 and song PC 2 and CHC PC 4 were negatively correlated in organ pipe flies

Table 8. Loadings of five elements of male courtship songs from a PCs analysis for males reared on agria and organ pipe cactus. Song bursts and two kinds of interpulse intervals are described in the text and in Etges et al. (2007).

	PC 1	PC 2	PC 3	PC 4	PC 5
No. Bursts	−0.7023	−0.0408	−0.0133	0.0917	0.7046
Burst duration	0.0831	0.2830	0.9470	0.0665	0.1085
Interburst interval	0.6979	−0.0024	−0.1366	−0.0594	0.7006
L-IPI	0.0333	0.6799	−0.2519	0.6875	−0.0217
S-IPI	−0.1084	0.6752	−0.1445	−0.7148	0.0214
Percent of total variance	0.3606	0.2418	0.1973	0.1593	0.0410

($r = -0.226$, $P = 0.0014$ and $r = -0.253$, $P = 0.0003$, respectively, both $n = 201$). Thus, numbers of song bursts and IPI, and a small group of CHCs predicted courtship success in cactus-reared *D. mojavensis* males.

Discussion

CHC differences between Baja California and mainland male *D. mojavensis* were influenced by many regions of the genome that were influenced by both larval-rearing environments and exposure to females as adults. Pervasive quantitative and qualitative geographic variation in CHCs was previously described, mostly between populations and sexes in these two geographical regions, as well as a population from the Mojave Desert (Stennett and Etges 1997; Etges and Ahrens 2001). Several "diagnostic" alkadienes are absent or in very low amounts in Baja California populations but are large peaks in mainland populations that suggested a simple genetic basis for these CHC differences, i.e., 8,24-tritricontadiene, 9,25-pentatricontadiene, and 9,27-heptatricontadiene, but these differences did not covary in the F_2 males assayed here (Table 1), and were not significantly associated with courtship success (Fig. 3, Table 4). Thus, the genetic basis of the large phenotypic differences in these CHCs evident from common garden experiments (Etges and Ahrens 2001) was multigenic with several QTLs expressing pleiotropic effects for multiple CHCs, consistent with other genetic analyses of CHC variation (Foley et al. 2007). Further analysis of the complex genetic basis of pheromonal variation among these geographically isolated populations will require isolating the effects of individual genes causing CHC-related sexual isolation and knowledge of CHC biochemical pathways in *D. mojavensis*. In this regard, associations of markers Dmoj2_6540c near *fruitless* and Dmoj2_1603a near *desat1* and *desat2* with CHC profile suggest these candidate genes deserve further scrutiny. Differences in the relatively small group of less prominent CHCs that characterized mated versus unmated males (Fig. 3, Table 4) and those involved in sexual isolation among populations (Etges and Tripodi 2008) suggest that the genetic architecture of the CHCs relevant to mating success and sexual isolation may be somewhat simpler.

Table 9. Logistic regression results for the first five PCs of epicuticular hydrocarbon and courtship song variation on male mating success, including the effect of host cactus and PC × cactus interaction terms. Significant effects are italicized in bold.

Source	df	Type III SS	*F* Value	Pr>*F*
CACT	1	0.5018	2.18	0.1405
HCPC 1	1	0.3329	1.45	0.2297
HCPC 2	1	0.0516	0.22	0.6359
HCPC 3	1	0.0559	0.24	0.6224
HCPC 4	1	0.9159	3.98	***0.0467***
HCPC 5	1	0.3749	1.63	0.2025
SONGPC 1	1	3.0270	13.16	***0.0003***
SONGPC 2	1	2.0073	8.73	***0.0033***
SONGPC 3	1	0.0359	0.16	0.6927
SONGPC 4	1	0.4518	1.96	0.1619
SONGPC 5	1	0.2038	0.89	0.3471
HCPC 1 × CACT	1	0.1746	0.76	0.3841
HCPC 2 × CACT	1	0.0548	0.24	0.6256
HCPC 3 × CACT	1	0.0161	0.07	0.7913
HCPC 4 × CACT	1	0.0007	0.00	0.9567
HCPC 5 × CACT	1	0.1345	0.58	0.4450
SONGPC 1 × CACT	1	0.1369	0.60	0.4409
SONGPC 2 × CACT	1	0.0207	0.09	0.7642
SONGPC 3 × CACT	1	0.0675	0.29	0.5882
SONGPC 4 × CACT	1	0.2683	1.17	0.2808
SONGPC 5 × CACT	1	0.0247	0.11	0.7436

For all significant QTLs, additive effects of Baja and mainland genotypes on different sources of CHC variation represented by PC scores were not random (Table 6). All X-linked QTLs were characterized by significantly larger PC scores associated with Baja alleles, as well as most autosomal QTLs. No marker region showed consistent pleiotropy where either Baja or mainland allelic effects were consistently associated with larger or smaller effects on different PCs. Interestingly, male mating success was associated with a single marker, Dmoj1603a near *desat1* and *desat2* (Etges et al. 2007) where mainland alleles significantly increased copulation success with mainland females, i.e.,

MM, MB > BB, $F = 5.12$, $P = 0.024$. This same genomic region was associated with similar mainland allele increases in PC 2 and PC 4 scores (Table 6) that were also associated with increases in mating success (Fig. 3). Thus the region around *desat1* and *desat2* should be further scrutinized for variation influencing these CHC differences.

We observed that expression of these genetic effects was largely a function of the environments experienced during the life cycle, both during preadult stages due to rearing substrates and in adults due to exposure to females. The influences of mating status (Polerstock et al. 2002) and the "social environment" (Petfield et al. 2005; Svetec and Ferveur 2005) on CHC variation all underscore the dynamic nature of these pheromone systems (Kent et al. 2007; Krupp et al. 2008) in response to the environments in which they are expressed. For 15 QTLs across the genome, cactus substrates shifted CHC amounts where agria significantly increased PC 1 and PC 2 scores versus organ pipe cactus, but for seven QTLs, organ pipe significantly increased PC 2, 4, and 5 scores over those for agria-reared males. A combination of these effects in the form of significant E × E interactions further shifted CHC amounts along different axes of variation. A majority, 14 of 17, of the E × E interactions that influenced QTL expression were due to decreases in PC scores in organ pipe-reared males exposed to females versus agria-reared, virgin males. Because most of these interactions influenced PC 1 and PC 2 (Fig. 5), this result suggests that organ pipe cactus reduces CHC amounts relative to agria, although for PC 2, both agria- and organ pipe-reared males with higher PC 2 scores enjoyed increased mating success (Fig. 3). Thus, characterizing CHC variation in virgin adults may give a very different perspective on sex-specific CHC profiles than those derived from adults reared in mixed sex groups. As these differences in CHCs are involved in sexual isolation among allopatric populations diverging in different environments, the strength of detected QTL effects and the environmental influences on them have provided an initial glimpse into the genetic basis of incipient speciation in this system.

MATING SUCCESS AND CHC VARIATION

The CHCs of mated and unmated males differed significantly, and mating success was generally greater for males reared on organ pipe cactus (Fig. 3). As both PC 2 and PC 4 scores were positively associated with increased mating success, and there was a PC 4 × cactus interaction (Table 3), covarying groups of CHCs determined mating success in the final stages of courtship (Table 4). Identification of the CHCs that are perceived as pheromones by females will ultimately require experimentation with single CHC components, but several of the same CHC components associated with courtship success have been identified in different studies. Markow and Toolson (1990) hypothesized that the ratio of male C_{35}/C_{37} alkadienes determined mating success, but they did not discriminate between the two positional isomers of these molecules, 9,25- and 8,26-pentatricontadiene and 9,27- and 8,28-heptatricontadiene, and this result was not repeatable in cactus-reared flies (Stennett and Etges 1997). In courtship trials with the same populations used in the present study, discriminant function analysis revealed that C_{34} (8,26-tetratricontadiene and 10-, 12-, and 14 tetretricontene) and C_{37} (8,28-heptatricontadiene and 14-, 16-, and 12-hexatricontene) CHCs were among the best discriminating components between mates and unmated males (Etges and Tripodi 2008). These C_{34} and C_{37} isomers were among the CHCs contributing to the variation in PC 2 and PC 4 (Table 4), along with several others, that were significantly associated with differences in mating success (Fig. 3). The commonality of these results in these three independent studies suggests that these C_{34} and C_{37} CHCs are associated with male mating success. Further, the observation that the most abundant CHC components, the two C_{35} alkadienes that can comprise ca 50% of adult CHCs but do not seem to be involved in mating decisions, suggests that CHC profiles can be highly geographically differentiated, but such variation may have little or no role in determining mate choice. Certainly, the role of these CHCs in courtship success needs to be expanded to other populations.

ECOLOGY AND SPECIATION

The myriad roles of how ecology can impact the expression of genes associated with mate choice and sexual isolation is becoming better understood. The strength of local selective forces that may result in genetic differentiation and behavioral isolation will be balanced by rates of gene flow among diverging populations, but gene flow alone may not prevent reproductive isolation (Feder et al. 1994, 2005; Mallet 1995; Mallet et al. 1998). Because allopatric populations of *D. mojavensis* have diverged in association with the use of different host plants that have driven the evolution of adaptive life-history differences, as well as courtship song and CHC differences, divergence may have been rapid with little or no gene flow to slow isolation. Certainly, the degree of genetic divergence in these signal traits that has evolved since *D. mojavensis* invaded mainland Mexico from Baja California suggests that complex genetic traits like CHC expression can evolve rapidly, and in some cases, have resulted in some qualitative differences in CHC phenotypes and strong region-specific sexual dimorphism (Etges and Ahrens 2001).

Unfortunately we cannot directly test the hypothesis of ecological speciation (e.g., Funk 1998; Via et al. 2000; Nosil et al. 2002; Rundle et al. 2003) because populations of *D. mojavensis* tend to use one host in different parts of the species range even though host cacti are broadly sympatric in some areas (Heed 1978). Nevertheless, understanding the nature of sexual isolation between allopatric populations has required information on host use (Etges 1992), and clearly the QTLs detected for time to

copulation, components of courtship songs (Etges et al. 2007), and CHC variation associated with mating success were significantly influenced by both the main effects of rearing substrates and G × E interactions with them. The consequences for signal trait evolution in the presence of G × Es have been discussed, including the role of host plant use on maintenance of genetic variation of these traits and the slowed efficacy of sexual selection in depleting genetic variation (Etges et al. 2007). Thus, genetic analysis of reproductive isolation in *D. mojavensis* could not have been fully evaluated without in-depth, a priori knowledge of their ecology.

Because both courtship song and CHC differences play a role in sexual isolation between Baja California and mainland populations of *D. mojavensis* (Table 9), further high-resolution mapping of both traits will be necessary to extend the QTL results presented here. Even though these QTL results were based on a series of reciprocal crosses involving a Baja California and a mainland population, any conclusions are likely to be general to other populations in these regions given the strong interregional differentiation in CHC profiles (Etges and Ahrens 2001). More populations of *D. mojavensis* should be studied, however, to examine the generality of the influences of G × E interactions on sexual isolation. The evolution of isolating mechanisms in diverging populations of *D. mojavensis* should provide a detailed portrait of how new species arise.

ACKNOWLEDGMENTS

We thank G. Almeida and A. Tripodi for excellent technical assistance, W. Starmer and P. Ganter for fresh yeast stocks, J. Foster for a replacement *E. cacticida* culture, and J. Wolf and two anonymous reviewers for comments on the manuscript. R. McNew provided essential statistical advice, and H. Wistrand, J. Mutic, and L. Jaureguy assisted with field collections of cactus and flies. M. Ashburner provided office space in the Department of Genetics and hospitality during much of the data analysis and writing phases of this article. We also thank the Secretaria de Medio Ambiente y Recursos Naturales in Mexico City for issuing a CITES permit, and M. A. Armella for helping us obtain it. Funding was provided by a Fulbright College Cambridge Faculty Fellowship and National Science Foundation grants DEB-0211125 to WJE and DEB-0211007, 0509780, & 0549893 to MAFN, and by the National Environment Research Council, U.K., to MGR.

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Associate Editor: J. Wolf