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EPICUTICULAR HYDROCARBON VARIATION IN *Drosophila mojavensis* CLUSTER SPECIES[1]

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Abstract—Epicuticular hydrocarbon variation was investigated among the three species of the *Drosophila mojavensis* cluster (*D. mojavensis*, *D. arizonae*, and *D. navojoa*) within the large *D. repleta* group. Because these hydrocarbons serve as contact pheromones in adult *D. mojavensis*, the chemical characteristics and differences in hydrocarbon profiles in populations of these three sibling species were further investigated. Twenty-seven hydrocarbon components with chain lengths ranging from C_{28} to C_{40}, including *n*-alkanes, methyl-branched alkanes, *n*-alkenes, methyl-branched alkenes, and alkadienes were observed. Hydrocarbon profiles among the three species reared on different cactus hosts were easily aligned with previously identified components in *D. mojavensis*. Male and female *D. navojoa* possessed a 31-methyldotricont-6-ene absent in both *D. arizonae* and *D. mojavensis*, while lacking the 8,24-tritricontadiene present in these two species. *D. navojoa* adults had far less 2-methyloctacosane than these sibling species, but the significance of this difference was obscured by the degree of variation among populations in amounts of this hydrocarbon. Mainland and Baja California populations of *D. mojavensis* were fixed for differences in the amounts 8,24-tritricontadiene, 9,25-pentatricontadiene, and 9,27-heptatricontadiene, consistent with all previous studies. Amounts of 18 of the 27 hydrocarbon components were greater in flies reared on *Opuntia* cactus. Canonical discriminant function analysis resolved all three species into distinct, nonoverlapping groups, suggesting that epicuticular hydrocarbon profiles are species-specific in the *D. mojavensis* cluster. Based on the amounts of interpopulation variation in hydrocarbon profiles in these three species, we hypothesize

[1]This is paper VI in the series "Premating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*."

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that epicuticular hydrocarbon differences may evolve early during the formation of new species.

Key Words—Species recognition, cuticular hydrocarbons, *Drosophila*, cactus, speciation.

INTRODUCTION

In closely related animal species that no longer share a common fertilization system, species recognition systems may be preserved by strong stabilizing selection (Paterson, 1993) and perhaps enhanced by reinforcement of these barriers if there is continuing gene exchange (Dobzhansky, 1951; Coyne and Orr, 1989; Noor, 1995, 1999). In order to understand more fully the origin of new species, the conditions that cause particular isolating mechanisms to arise must be identified (Masters, 2000), and the progression of changes in courtship cues during the evolution of reproductive isolation across species groups must be understood. Mate recognition in many *Drosophila* species involves a stereotyped series of behavioral cues exchanged between males and females. Courtship is elicited by males and may involve behavioral, acoustic, and chemical cues that females use in the evaluation of prospective mates. In the initial stages of reproductive isolation, how does selection shape divergence in mating systems prior to complete isolation? Do mate recognition systems function within populations in the same ways that they function between populations and species? If sexual selection is unrelated to sexual isolation (Boake et al., 1997; Carson, 2000) and if we are to understand how species originate in general, comparative studies of within- and between-species mating systems may offer insight into the sequential evolution of recognition signals.

A common form of chemical communication in *Drosophila* species involves contact pheromones made of epicuticular hydrocarbons biosynthesized in the pupal stage and early in adult life (Ferveur et al., 1997). Specific components of cuticular hydrocarbons of females elicit male courtship behaviors in *Drosophila melanogaster* (Antony and Jallon, 1982). (*Z*,*Z*)-7,11-Heptacosadiene elicits courtship from *D. melanogaster* males (Antony and Jallon, 1982). (*Z*)-11-Pentacosene (Oguma et al., 1992a) along with (*Z*,*Z*)-5,13-pentacosadiene, (*Z*,*Z*)-5,15-pentacosadiene, and (*Z*,*Z*)-7,15-heptacosadiene elicits courtship from *D. virilis* males (Oguma et al., 1992b). The multimethylene interrupted alkadiene, (*Z*,*Z*)-5,25-hentricontadiene, elicited courtship from male *D. pallidosa* (Nemoto et al., 1994). All of these hydrocarbons have long chains and are thought to act as contact pheromones, probably by stimulating chemoreceptors on the male foreleg or proboscis (Jallon, 1984; Oguma et al., 1992b; Nemoto et al., 1994).

In addition to the exchange of behavioral cues during courtship including courtship songs (Spieth, 1974; Ewing and Miyan, 1986), epicuticular hydrocarbons have been implicated as determinants of mate choice in *D. mojavensis* (Markow

and Toolson, 1990; Toolson et al., 1990; Stennett and Etges, 1997). Hydrocarbon transfer experiments (cf. Coyne et al., 1994) have demonstrated that epicuticular hydrocarbons are involved in mate recognition with mainland and Baja *D. mojavensis* (Etges and Ahrens, 2001). Previous analysis of the epicuticular hydrocarbon profiles of *D. arizonae* and *D. mojavensis* revealed a high degree of similarity between species, some large differences in particular hydrocarbon components among populations of *D. mojavensis*, and significant effects of larval rearing substrates, particularly laboratory food versus cactus, on amounts of epicuticular hydrocarbons (Stennett and Etges, 1997). Detailed analysis of multiple populations of *D. mojavensis* from different parts of the species range revealed consistent geographical differentiation in amounts of C_{33}, C_{35}, and C_{37} alkadiene components, and gender–specific amounts of 16 different hydrocarbons. Further, significant sex $\times$ region interactions for eight of these hydrocarbons showing sexual dimorphism were statistically significant, indicating region-specific male–female hydrocarbon differences (Etges and Ahrens, 2001). In the present study, we characterized the chemical nature of these epicuticular hydrocarbons for all three *D. mojavensis* cluster species so that we can begin to understand the role of these chemical cues in between-species mate recognition.

Phylogeny and Natural History of D. mojavensis *Cluster Species*. The *D. mojavensis* cluster is part of the *D. mulleri* species subgroup inferred from the sharing of chromosomal gene arrangements and phylogenetic analyses of nuclear and mitochondrial gene regions (Wasserman, 1992; Durando et al., 2000). The *mulleri* cluster (*D. aldrichi*, *D. mulleri*, *D. wheeleri*, and *D. nigrodumosa*) is the sister group to the *D. mojavensis* cluster (Durando et al., 2000). Although *D. huaylasi* from Peru is closely related to the *D. mojavensis* cluster and has sometimes been included within it (Durando et al., 2000), we do not include it here because hybridization data, gene arrangements, and differences in male genitalia clearly point to a closer affiliation with the *mulleri* subgroup (Fontdevila et al., 1990). *D. navojoa* is ancestral to both *D. mojavensis* and *D. arizonae*: the latter two species share a common intermediate ancestor (Figure 1). The range of *D. navojoa* extends from the lowlands along the Pacific coast of southern Mexico and north to southern Sonora. *D. arizonae* is widespread from Guatemala and southern Mexico to Arizona and New Mexico, and is sympatric with *D. mojavensis* and *D. navojoa* in southern Sonora. The range of *D. mojavensis* is restricted to the Sonoran desert, Sinaloan thornscrub, and adjacent areas including the Colorado/Mojave deserts in southern California (Heed, 1982). Ecologically, *D. navojoa* is restricted to the more ancestral *Opuntia* breeding habit, similar to most other members of the *mulleri* subgroup, whereas both *D. mojavensis* and *D. arizonae* use a variety of hosts including a number of more derived columnar cacti (Ruiz et al., 1990).

The goals of this study were to characterize the epicuticular hydrocarbons of the three *D. mojavensis* cluster species in order to understand more about

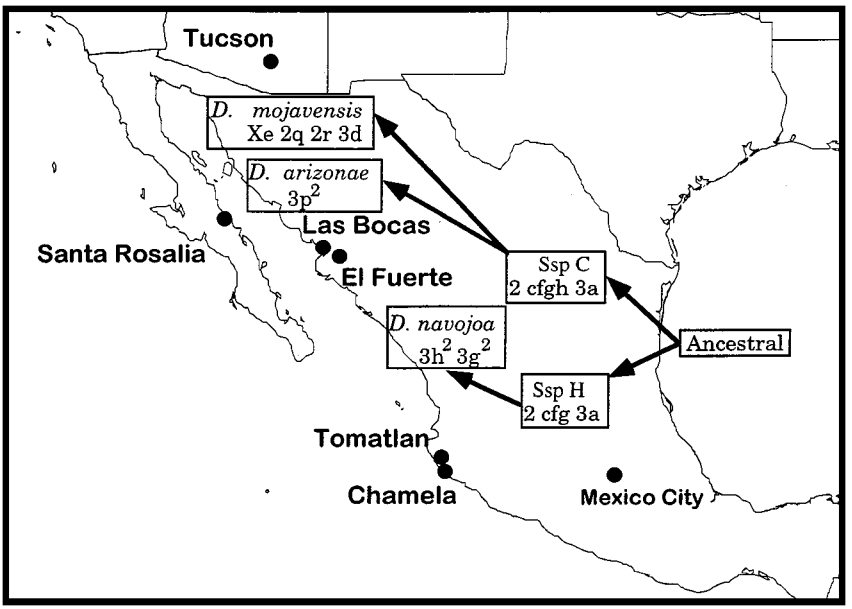

FIG. 1. Map of Mexico and the southwestern United State showing the locations of the populations sampled for this study and a phylogeny of the three *D. mojavensis* cluster species based on chromosomal gene arrangements (Wasserman, 1992).

the magnitude of species-specific mate recognition systems. We assessed variation between two populations of each species reared on fermenting *Opuntia* and *Stenocereus gummosus*, pitaya agria, tissues in order to estimate the degree of intraspecific and substrate-induced variation in hydrocarbon profiles previously documented in *D. mojavensis*. In this study, we used fermenting *Opuntia ficus-indica* and the more chemically specialized agria cactus rearing substrates to characterize further the consequences of this ecological transition of feeding and breeding sites on both expression of adult epicuticular hydrocarbons and components of fitness.

METHODS AND MATERIALS

Origin of Stocks. A stock of *D. arizonae* from Tucson, Arizona, was founded in November 1995 by aspirating approx. 35 adults from the fermenting fruits of *Opuntia ficus-indica*. Another stock originated from seven adults that were baited in a *Stenocereus thurberi–S. alamosensis–Opuntia wilcoxii* forest near Las Bocas, Sonora in April 1996 (Figure 1). Both *D. navojoa* stocks were collected by baiting

and rearing from *Opuntia* pads in March 1997. Eight females and 10 males were collected west of Tomatlan, Jalisco, in a dry forest where *S. standleyi*, *Pachycereus pecten-aboriginum*, *Cephalocereus purpusii*, and an arborescent *Opuntia* species were common. At several locations within the Chamela Biological Station reserve in Jalisco, 34 female and 29 male *D. navojoa* were collected from giant *Opuntia excelsa* plants. The *D. mojavensis* stocks originated from collections made in 1996 in Santa Rosalia, Baja California, and El Fuerte, Sinaloa. The Baja stock was founded from 468 adults that emerged from a *S. gummosus* rot and the Sinaloa stock was initiated with 185 adults from baits and flies that emerged from a *S. thurberi* rot. All flies were reared in the laboratory in large numbers on banana food (Brazner and Etges, 1993) prior to cactus rearing and hydrocarbon analysis.

Chemical Analysis of D. arizonae and D. navojoa Hydrocarbons. The four *D. arizonae* and *D. navojoa* stocks were reared for one generation on banana food in 8-dr shell vials in an incubator programmed on a 14L:10D cycle at 27°C during the day and 17°C at night. All emerged adults were separated by sex and aged for at least 10 days on banana food at room temperature. Epicuticular hydrocarbons were extracted from adults ($N = 549$–902) of each group in Biosil mini-columns. Each column consisted of a Pasteur pipet that contained packed glass wool and Biosil (silica gel, Sigma S-4133) washed several times with HPLC grade hexane. Flies were then added, washed in 8 ml of hexane, and the hydrocarbons were collected in hexane-rinsed vials. After the hexane was evaporated with nitrogen, each sample was sealed and stored at −20°C. Each sample extract was characterized by gas chromatography–mass spectrometry (GC-MS) of the most abundant components composed of methyl-branched hydrocarbons, alkenes, and alkadienes. The samples were analyzed by capillary gas–liquid chromatography by using a Hewlett Packard 5890 GC fitted with a 12-m HP-1 fused silica column. The GC was programmed from 150°C to 300°C at 10°C/min and held at 300°C for 5 min. The temperature of the injector and detector (Hewlett Packard 5971 mass selective detector) was 280°C. The internal standard was 100 ng/fly of octacosane (C_{28}). The unsaturated epicuticular hydrocarbons were derivatized with dimethyl disulfide, and the resulting thiomethyl derivatives were analyzed by GC-MS to identify the positions of the double bonds (Toolson et al., 1990).

Cactus Rearing Experiment. All populations of *D. arizonae*, *D. mojavensis*, and *D. navojoa* were cultured on fermenting cactus tissues in 1997 in order to assess the degree to which rearing substrates affected adult epicuticular hydrocarbon composition. Several hundred adults were collected from each population that had been cultured as described above. Eggs were collected from aged adults and washed in deionized water, 70% ethanol, and again in sterile deionized water. Eggs were counted out in groups of 200, transferred to a 1-cm^2 piece of sterilized filter paper, and placed on fermenting cactus. Cactus cultures were set up in plugged 8-oz bottles with 75 g of aquarium gravel at the bottom covered with a 5.5-cm-diameter piece of filter paper. Bottles were then autoclaved, and after 60 g

of either agria or *O. ficus-indica* tissues were in place, autoclaved again for 10 min. After cooling to room temperature, each culture was inoculated with 0.5 ml of a pectolytic bacterium, *Erwinia cacticida* (Alcorn et al., 1991), and 1.0 ml of a mixture of seven species of yeast common in natural agria rots (Starmer, 1982): *Pichia cactophila*, *P. mexicana*, *P. amethionina* var. *amethionina*, *Cryptococcus cereanus*, *Candida valida*, *C. ingens*, and *C. sonorensis*. Three replicate cultures of each cactus type were started for each of the two populations for each species and cultured in an incubator programmed as above. All unhatched eggs were counted to allow calculation of egg to adult viability. Eclosed adults from each replicate culture were counted daily allowing determination of egg-to-adult development time, separated by sex, and aged on banana food in vials at room temperature.

Aged adults were then transferred to hexane rinsed vials and stored at −20°C until hydrocarbon extracts were prepared by using groups of adults (usually 20–30) as described above. Each hydrocarbon sample was redissolved in hexane (2.5 μl/fly) containing 385 ng of docosane (C_{22}) per microliter as an internal standard. One microliter of each sample was analyzed by capillary gas–liquid chromatography with a Shimadzu G14 fitted with a 30-m DB-1 fused silica column. Injector and detector temperatures were set at 345°C with the injector port in split mode. Running temperatures started at 200°C and increased to 345° at 10°/min, with a hold at 345°C for 7 min (Stennett and Etges, 1997).

Statistical Analyses. Development time was measured in days, and viability was calculated as the number of eclosed adults divided by the number of counted eggs that hatched. Variation in egg to adult development and viability was assessed by ANOVA with PROC GLM in SAS (SAS Institute, 1989). Viability data were arcsin transformed, and development time data were $\log_{10}$ transformed prior to analysis.

Hydrocarbon amounts were estimated by analysis of peak integrations using EZCHROM software (ver. 2.1) provided by Shimadzu. Each sample amount was normalized by the measured amount of the internal standard. Replicate groups of flies were analyzed together. All data were expressed as nanograms per fly of cuticular hydrocarbons and were analyzed with population, rearing substrate, and sex as main effects, and for all interactions between main effects. Population, replicates, and all interactions with population were considered random effects. The TEST command was used in the RANDOM statement to generate the appropriate F ratios and adjusted degrees of freedom by using Satterwaite's approximation (SAS Institute, 1989). Within-species ANOVAs were also evaluated to more closely assess some of the higher order interaction terms. Canonical discriminant function analysis (PROC CANDISC) (SAS Institute, 1989) was performed on the replicate means of each population ignoring sex and cactus differences for all hydrocarbons analyzed. This procedure forms linear combinations of the hydrocarbons with the highest multiple correlation with the populations and maximizes the univariate F ratios. Each canonical variable was obtained by finding the linear combination

least correlated with the previous canonical variable: the first three canonical variables were used to plot the variation in hydrocarbons among populations.

RESULTS

Chemical Descriptions of Hydrocarbons. The major epicuticular hydrocarbons are alkanes, 2-methylalkanes, alkenes, methyl-branched alkenes, and multimethylene interrupted alkadienes (Table 1). The location of the double bonds in the alkenes were at odd-numbered carbons for the hentricontenes (7-hentricontene and 9-hentricontene) but were at even-numbered carbons for the longer-chained alkenes (e.g., 10-tritricontene, 10-tetratricontene, and 14-hexatricontene). Locations of the double bonds in the methyl-branched alkenes were also at even-numbered carbons. The multimethylene interrupted alkadienes had an odd number

TABLE 1. KEY MASS SPECTRA PEAKS IN IDENTIFICATION OF EPICUTICULAR HYDROCARBONS OF *D. arizonae*, *D. mojavensis*, AND *D. navojoa*

		m/*z*		
ECL[a]	Hydrocarbon	Untreated	Dimethyl disulfide derivative	Hydrogenated
28.00	*n*-octacosane	394		
28.65	2-methyloctacosane	365, 393		
30.65	2-methyltricontane	393, 421		
30.78	7-and 9-hentricontene	434	173, 355, 528	
33 Br2	11-and 13-methyldotricontane	168, 322 196, 294		
32.47	31-methyldotricont-8-ene	462	159, 397, 556	421, 449
32.56	31-methyldotricont-6-ene	462	131, 425, 556	421, 449
32.63	8,24-tritricontadiene	460	159, 173, 381, 395	
32.70	7,25-tritricontadiene	460	145, 159, 395, 409	
32.79	10-, 12-, and 14-tritricontene	462	187, 369; 215, 341; 243,313	464
34 diene	8,26-tetratricontadiene	474	159, 409	
34 diene	6,26- and 6,24-tetratricontadiene	474	131, 381, 409	
34 ene	10-, 12-, and 14-tetratricontene	476	187, 383; 215, 355; 243, 327	478
35 ene 1	33-methyltetratricont-10-ene	490	187, 397, 584	449, 477
35 ene 2	33-methyltetratricont-8-ene	490	159, 425, 584	449, 477
34.59	9,25-pentatricontadiene	488	173, 187, 395, 409	
34.66	8,26-pentatricontadiene	488	159, 173, 409, 423	
34.66	7,27-pentatricontadiene	488	145, 159, 423, 437	
37 ene	35-methylhexatricont-10-ene	518	187, 425	520
36.5	9,27-heptatricontadiene	516	173, 187, 423, 437	
36.7	14-, 16-, and12-hexatricontene	518	243, 369; 271, 341; 215, 397	520

[a] Equivalent chain length calculated as in Stennett and Etges (1997).

of carbons to the double bond from one end, and an even number of carbons from the other end to the double bond. In the tetratricontadienes (34 dienes), the double bonds were an even number of carbons from both ends.

Epicuticular Hydrocarbon Differences Among Species. A total of 27 hydrocarbon peaks were scored in each sample with chain lengths ranging from C_{28} to C_{40}. The number of observed peaks and their retention times were similar among the three species with only a few notable qualitative differences. The most ancestral species, *D. navojoa*, possessed 31-methyldotricont-6-ene, which was not observed in the other two species with an equivalent chain length of $C_{32.56}$. 8,24-Tritricontadiene was present in high quantities in *D. arizonae* and mainland *D. mojavensis* but was absent in *D. navojoa*. The Baja California population of *D. mojavensis* population from Santa Rosalia was characterized by the near absence of the $C_{32.63}$, $C_{35.59}$, and $C_{36.5}$ alkadienes that are major peaks in mainland populations, such as El Fuerte, consistent with all previous studies (Stennett and Etges, 1997; Etges and Ahrens, 2001). *D. navojoa* was also characterized by far lower amounts of 2-methyloctacosane ($C_{28.65}$) than *D. arizonae* and *D. mojavensis* (± 1 SD); $35.4 \pm 11.9 < 123.1 \pm 32.4 < 175.3 \pm 71.6$ ng/fly, respectively. This difference was not significant in the mixed model nested ANOVA (Appendix 1) because the mean square error term, populations nested within species, was so large. This was also the case for $C_{32.63}$ (absent in *D. navojoa*), $C_{34.59}$, and $C_{36.5}$, indicating that significant geographic variation in a variety of hydrocarbon components has obscured the levels of statistical significance of the differences between species.

Population Differences. Within-species ANOVAs were performed with populations to consider random effects in order to assess more directly hydrocarbon differences among populations and some of the higher order interaction terms. The degree of geographic variation observed between the Baja California and mainland population of *D. mojavensis* in hydrocarbon amounts (Table 2) was consistent with earlier results (Stennett and Etges, 1997). These same two populations were part of a larger study of epicuticular hydrocarbon variation among six populations from Baja California and five mainland Mexico populations (Etges and Ahrens, 2001). Thus, we can directly compare the magnitude of interpopulation variation in epicuticular hydrocarbons with that of the other two species. Here 15 of the 27 hydrocarbon components varied geographically: these were the same components that contributed to the overall geographic differences between Baja California and mainland populations of *D. mojavensis*. A greater proportion, 22/27, differed between the Tucson, Arizona, and southern Sonoran populations of *D. arizonae*. Just 11 of these components varied among the two populations of *D. navojoa*; only four of these are major peaks: C_{28} alkane, 2-methyloctacosane, 7,25-tricontadiene, and 8,26-pentatricontadiene (Table 2). Such intraspecific variation in *D. navojoa* was surprising given that the two populations studied were only 45 km apart (Figure 1).

Together, these 27 hydrocarbon components significantly discriminated among each of the six populations. The first three canonical variables accounted

TABLE 2. *F* VALUES FROM WITHIN-SPECIES ANOVAS FOR 27 HYDROCARBON COMPONENTS[a]

Hydrocarbon component	C_{RT}		*D. arizonae*	*D. mojavensis*	*D. navojoa*
n-Octacosane	C_{28}	(P)	31.11***	25.62***	60.59***
		(C)	7.15*	1.83	13.77**
2-Methyloctacosane	$C_{28.65}$	(P)	23.00***	178.83***	11.07**
		(C)	6.17*	2.90	0.23
2-Methyltricontane	$C_{30.65}$	(P)	34.92***	0.03	0.0
		(C)	0.30	6.29*	0.12
7- and 9-Hentricontene	$C_{30.78}$	(P)	22.79***	1.36	1.36
		(C)	20.19***	14.96**	1.12
Unknown	C_{33Br1}	(P)	NA	92.30***	NA
		(C)	NA	0.22	NA
11- and 13-Methyldotricontane	C_{33Br2}	(P)	27.07***	74.06***	1.15
		(C)	13.45**	0.77	5.25
31-Methyldotricont-8-ene	$C_{32.47}$	(P)	30.77***	7.34*	0.26
		(C)	14.46**	5.84	2.72
31-Methyldotricont-6-ene	$C_{32.56}$	(P)	NA	NA	1.28
		(C)	NA	NA	0.22
8,24-Tritricontadiene	$C_{32.63}$	(P)	37.28***	70.13***	NA
		(C)	31.91**	7.31*	NA
7,25-Tricontadiene	$C_{32.70}$	(P)	11.26**	0.24	10.05**
		(C)	20.03***	11.12**	0.85
10-, 12-, and 14-Tritricontene	$C_{32.79}$	(P)	39.37***	12.10**	2.91
		(C)	4.77*	23.96***	0.08
8,26-Tetratricontadiene	C_{34} diene	(P)	26.30***	39.14***	6.95*
		(C)	14.46**	24.40***	7.42*
6,26- and	C_{34} alkene	(P)	3.92	0.05	19.98***
6,24-Tetratricontadiene		(C)	24.42***	22.40***	29.37***
10-, 12- and14-Tetratricontene	C_{34} ene	(P)	0.90	19.36***	20.00***
		(C)	13.85**	13.64**	53.58***
33-Methyltetratricont-10-ene	C_{35} alkene1	(P)	3.31	30.82***	0.44
		(C)	2.68	0.26	3.04
33-Methyltetratricont-8-ene	C_{35} alkene 2	(P)	17.46**	1.52	0.19
		(C)	2.10	2.82	6.20*
9,25-Pentatricontadiene	$C_{34.59}$	(P)	44.29***	85.68***	4.66
		(C)	23.66***	19.49***	0.01
8,26-Pentatricontadiene and	$C_{34.66}$	(P)	40.94***	13.23**	11.03*
7,27-Pentatricontadiene		(C)	22.07***	7.17*	0.83
Unknown branched alkene	C_{36a}	(P)	14.28**	12.28*	12.13*
		(C)	21.38***	6.05*	27.44***
Unknown branched alkene	C_{36b}	(P)	8.55*	3.86	25.93***
		(C)	4.37	34.89***	42.30***
35-Methylhexatricont-10-ene	C_{37} alkene	(P)	8.42*	40.17***	11.36**
		(C)	18.10***	3.30	5.95
9,27-Heptatricontadiene	$C_{36.5}$	(P)	48.50***	54.94***	4.93
		(C)	21.34***	11.11**	0.58

TABLE 2. CONTINUED

Hydrocarbon component	C_{RT}		*D. arizonae*	*D. mojavensis*	*D. navojoa*
Unknown alkadiene	$C_{36.6}$	(P)	43.81***	6.42	2.58
		(C)	12.48**	22.51***	0.08
14-, 16-, and 12-Hexatricontene	$C_{36.7}$	(P)	41.35***	4.42	3.31
		(C)	21.38***	9.89**	0.61
Unknown alkene	C_{38}	(P)	16.83**	0.94	15.65**
		(C)	5.42	6.00	13.44***
Unknown	C_{39}	(P)	1.04	4.80	6.63*
		(C)	1.08	7.78**	17.54***
Unknown	C_{40}	(P)	28.43**	0.46	6.67*
		(C)	2.11	6.52	10.34**
Total hydrocarbons		(P)	44.13***	1.10	5.35
		(C)	23.54***	12.50**	0.25

[a] For each component, F values and significance levels are listed for differences between populations (P) over that for differences between cactus hosts (C). Significance of all P values was adjusted using the sequential Bonferroni procedure across species.
C_{RT} refers to the hydrocarbon component's retention time or carbon chain length as described in Stennett and Etges (1997) or in this paper. NA: not applicable because hydrocarbon component is absent for this species. $^{*}P < 0.05, ^{**}P < 0.01, ^{***}P < 0.001$; significance tests are based on a stepwise Bonferroni test with initial $P = 0.05$ using the number of P values in each row to correct for the number of simultaneous tests.

for 93.9% of the total hydrocarbon variation (Figure 2). All pairwise squared Mahalanobis distances between populations were significant ($p < 0.0001$), as were the overall multivariate differences among populations (Wilks $\lambda = 0.00012742$, $F = 20.56$, $p < 0.0001$). The first five canonical correlations were all significantly greater than zero ($p < 0.0001$). Thus, significant geographic variation exists in the epicuticular hydrocarbon profiles of the populations of these three species.

Sex Differences. Amounts of 2-methyloctacosane, 2-methyltricontane, the C_{38} alkene, and the C_{40} alkene differed between males and females of all three species (Appendix 1). However, 17 hydrocarbon components differed between males and females in a population-specific manner as indicated by the significant sex × population within-species interaction terms. Such interactions imply that male–female differences in amounts of these hydrocarbon components differ among populations of these three species. These interactions were also observed in the larger analysis of epicuticular hydrocarbon variation between Baja California and mainland populations of *D. mojavensis* (Etges and Ahrens, 2001), so local variation in sexual dimorphism of hydrocarbon profiles is not restricted to this species.

Substrate Differences. Although not statistically significant in all cases ($P < 0.05$; Appendix 1), hydrocarbon amounts generally differed due to cactus-rearing substrates for 18 of the epicuticular hydrocarbons assayed. In every case, flies reared on *Opuntia* cactus had increased amounts of cuticular hydrocarbons as

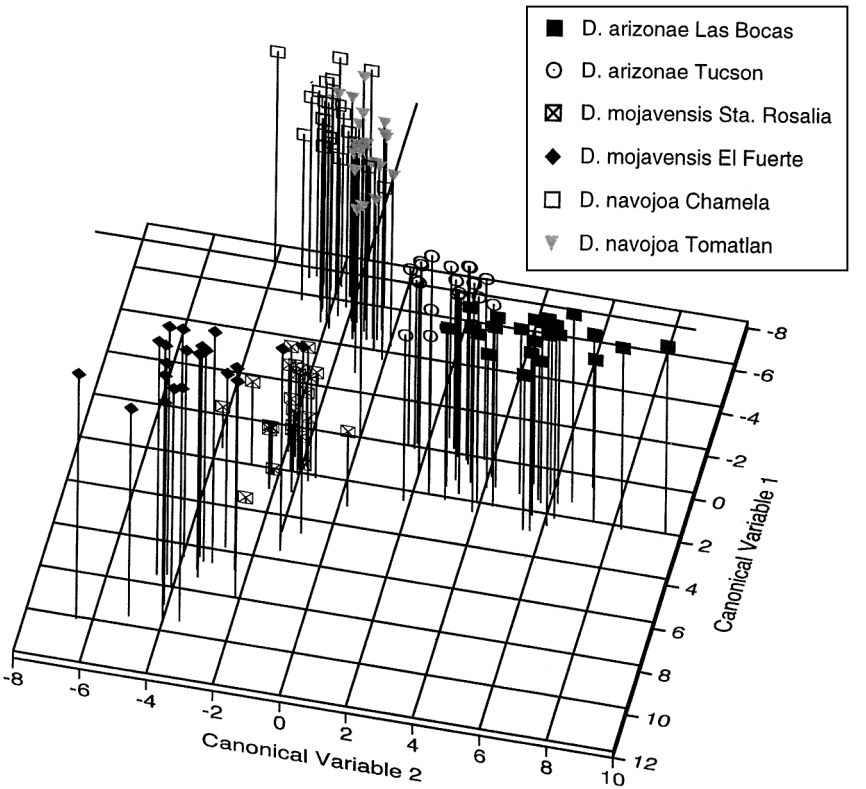

FIG. 2. Three dimensional plot of the populations of *D. mojavensis*, *D. arizonae*, and *D. navojoa* based on the first three canonical discriminant variables (CDVs) formed from the 27 hydrocarbon components observed in this study. CDV 1 accounted for 53.6%, CDV 2 accounted for 26.0%, and CDV 3 accounted for 14.3% of total variance, respectively. Sex and host cactus were ignored to emphasize populations and species differences. All Mahalanobis distances between populations were significant ($P < 0.0001$).

compared to agria-reared flies (Figure 3). Results of the within-species ANOVAs (Table 2) also suggested that the effect of rearing flies on agria versus *Opuntia* was usually statistically significant in one or two of these species for a given hydrocarbon component. Thus, many of the cases of marginal significance in the nested ANOVAs were due to this result. Only the two C_{34} alkenes and one of the C_{36} alkenes differed in amounts between agria and *Opuntia*-reared flies in all three species (Table 2), and the nested ANOVA suggested that 2-methyloctacosane, 2-methyltricontane, and 14-, 16-, and 12-hexatricontene also varied among species (Appendix 1). The 2-methyloctacosane, 7- and 9-hentricontene, 31-methyldotricont-8-ene, and $C_{36.6}$ alkadiene components varied in a species–specific manner as

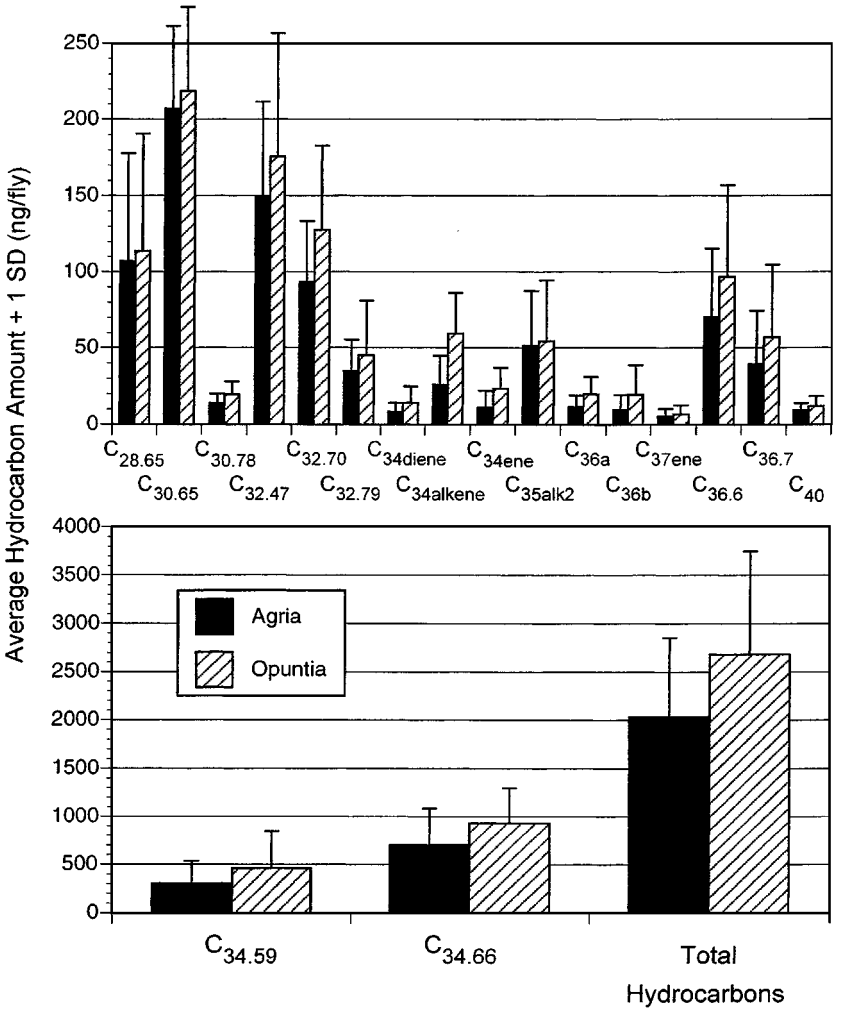

FIG. 3. Averages (+1 SD) of *D. arizonae*, *D. mojavensis*, and *D. navojoa* hydrocarbon amounts for the 18 components that differed between rearing substrates. In all cases, hydrocarbon amounts were greater in flies reared in *Opuntia* than in agria tissues. Individual components are referred to by their equivalent chain lengths or other names (Appendix 1); see Table 2 for chemical names.

shown by the significance of the cactus × species interaction terms. These cactus substrates also influenced the total amounts of hydrocarbons per fly (Figure 3) for *D. arizonae* and *D. mojavensis*, but not for *D. navojoa* (Table 2). Overall, fermenting *Opuntia* cactus tissues must contain more available precursors for synthesizing

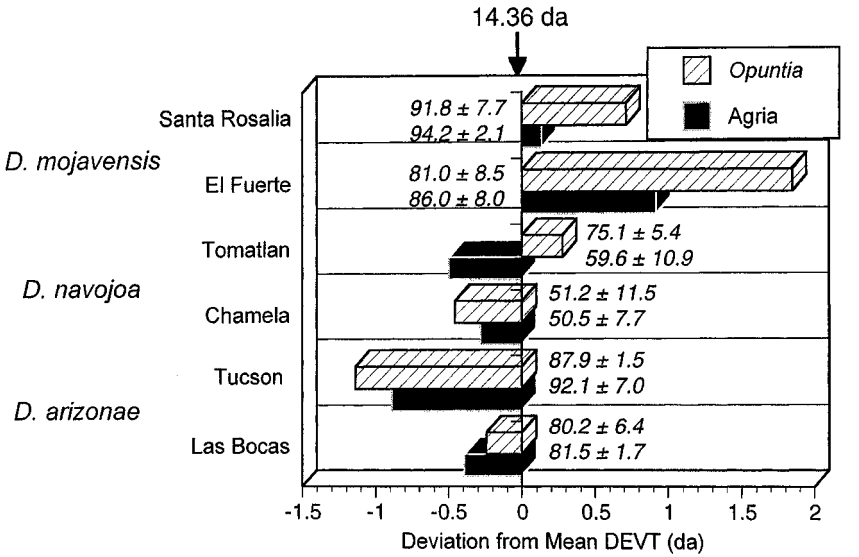

FIG. 4. Deviations in egg to adult development time from the overall mean (indicated above the graph) for the six populations of *D. mojavensis* cluster species in this study illustrating the significant population × cactus interaction term from the ANOVA. Mean egg to adult viability (±1 SD) is indicated in italics adjacent to each type of cactus substrate for each population.

the majority of adult epicuticular hydrocarbons in these three *Drosophila* species than do agria tissues.

Life History Differences. Differences in egg-to-adult development time among populations were expressed in a substrate-specific manner (Figure 4). Although overall differences in development time were only marginally significant among species ($F = 5.42$, $P = 0.10$) in the nested design, females emerged earlier than males in all three species ($F = 94.52$, $P = 0.002$). the interaction between cactus hosts and populations nested within species was also significant ($F = 3.84$, $P = 0.022$). Only *D. mojavensis* populations consistently expressed shorter development times on their primary host plant, agria cactus. The correlation between development time and total hydrocarbons per fly was not significant ($r = 0.52$, $P > 0.05$, $N = 12$), so variation in hydrocarbon amounts was unrelated to the length of preadult development time. Egg to adult viability was lower in the two populations of *D. navojoa* ($F = 39.87$, $P < 0.0001$), in part due to the lower viabilities observed on agria versus *Opuntia*-reared flies (Figure 4). Overall, these life history differences are consistent with the known patterns of host plant use in nature (Ruiz and Heed, 1988) and suggest that any host cactus-induced variation in hydrocarbon amounts should also be expressed in natural populations.

DISCUSSION

The overall chemical similarity of the epicuticular hydrocarbon components among the three *D. mojavensis* cluster species suggests that the biosynthetic pathways for hydrocarbon production and deposition have not yet widely diverged. However, significant quantitative differences in hydrocarbon amounts among populations (Figure 3) indicate that the chemical signatures of these hydrocarbons have evolved within and between species. Whether individual or groups of these epicuticular hydrocarbons serve as within- and/or between-species signaling systems remains to be determined. In *D. mojavensis*, transferring male mainland-specific hydrocarbons to Baja males in "perfuming" experiments significantly enhanced the mating success of these perfumed Baja males with mainland females in comparison with controls. Thus, these cuticular hydrocarbons are part of the mate recognition system in this species (Etges and Ahrens, 2001). Certainly, the role of these chemical cues must be evaluated in the context of other components of the mating systems of these species expressed in an environment-specific manner, including mating behavior (Etges, 1992; Brazner and Etges, 1993; Stennett and Etges, 1997) and courtship songs.

The influences of rearing substrates on adult epicuticular hydrocarbon profiles suggests that an extensive understanding of the ecology and distribution of natural populations is necessary if we are to identify the mechanisms responsible for shaping mate recognition divergence. For a number of *Drosophila* species, preadult rearing environments are significant determinants of both genetic and phenotypic variation in fitness characters (Etges and Heed, 1987; Ruiz and Heed, 1988; Etges and Klassen, 1989; Etges, 1990, 1993; Fanara et al., 1999) as well as mating behavior (Ehrman, 1990; Brazner and Etges, 1993; Kim et al., 1996; Kim and Ehrman, 1998) and epicuticular hydrocarbon variation. Thus, the use of discrete resources in nature, such as fermenting cactus rots, is a key determinant of variation in fitness characters and intraspecific mate recognition systems. The frequency and intensity of courtship interactions should be determined largely by the abundance of adults, the number of different species present, and the male mating propensity at feeding and breeding sites. There can be considerable overlap of species feeding on rots of any of the major host cacti in the Sonoran Desert (Fellows and Heed, 1972), although host plant specificity, resulting from the effects of stem chemistry on larval growth and development (Fogleman and Heed, 1989; Fogleman and Abril, 1990) and interspecific larval competition (Heed and Mangan, 1986), is the general rule.

Since *D. navojoa* is restricted to *Opuntia* cacti and its range only overlaps that of *D. mojavensis* in a small area of southern Sonora and northern Sinaloa (Heed, 1982), these two species probably do not encounter each other frequently in nature. However, Markow and Maveety (1985) documented higher premating

isolation among sympatric populations than allopatric populations of each species and concluded that reproductive character displacement was responsible. Although they included no statistical analysis of their data concerning the significance of the differences in premating isolation between sympatric and allopatric populations, further analysis of their data supports their contention. Their estimates of premating isolation using the Joint I statistic (Stalker, 1942) were greater in sympatric than allopatric populations (two-group comparison; $\chi^2 = 20.56$, 1 *df*, $P < 0.0001$) (Sauer and Williams, 1989). Thus, there is geographic variation in levels of premating isolation between *D. mojavensis* and *D. navojoa*, even though they do not regularly share host plants.

The ecology of *D. mojavensis* is perhaps the best known as it uses different host cacti throughout its range (Heed and Mangan, 1986; Etges et al., 1999). In Baja California, pitaya agria, *S. gummosus*, is the preferred host even though several secondary hosts used elsewhere are sympatric with agria such as organ pipe cactus, *S. thurberi*, and California barrel cactus, *Ferocactus cylindraceous*. In mainland Sonora, Sinaloa, and Arizona, organ pipe cactus is the major host except for where a small patch of agria grows in coastal Sonora and occasional use of sina cactus, *S. alamosensis*, in southern Sonora and coastal Sinaloa (Markow et al., 1983; Ruiz and Heed, 1988). In the Mojave/Colorado deserts of southern California, *D. mojavensis* use California barrel cactus and have been found on Santa Catalina Island near Los Angeles using the fruits and pads of *Opuntia demissa*. The more widespread *D. arizonae* has been reared out of sina, saguaro, *Carnegiea gigantea*, and more rarely from *S. gummosus* in coastal Sonora and the Cape region in Baja California along with *D. mojavensis*. To the north in Arizona and New Mexico outside of agricultural areas, *D. arizonae* breeds in *Opuntia* pads and fruits and feeds on a variety of cacti (Fellows and Heed, 1972; Heed, 1982). In southern Mexico, *D. arizonae* has been collected from fermenting *Myrtillocactus geometrizans* and *S. pruinosus* arms in Chiapas, as well as *Opuntia* pads north of Pachuca, Hidalgo (Etges, unpublished data).

Reproductive character displacement has been described in mainland Mexico and Arizona populations *D. mojavensis* due to sympatry with *D. arizonae* (Wasserman and Koepfer, 1977). Mainland populations of *D. mojavensis* are considered derived from those in Baja California where *D. arizonae* is absent except for a few small demes outside of the desert in the more subtropical thornscrub in the Cape region of Baja California. Johnson (1980) hypothesized that *D. mojavensis* colonized mainland Mexico from Baja California by switching to a secondary host plant, organ pipe cactus, and secondarily became sympatric with *D. arizonae*. Both species occasionally use sina cactus in southern Sonora (Markow et al., 1983; Ruiz and Heed, 1988), and *D. arizonae* has been reared out of agria in low frequencies in coastal Sonora following the summer monsoons, but then disappears through December and January (Etges and Heed, unpublished data). The presence of

D. arizonae on the mainland was hypothesized to have caused a shift in patterns of mate preference in *D. mojavensis*, so that now these populations exhibit behavioral isolation with the more ancestral *D. mojavensis* populations from Baja California (Zouros and d'Entremont, 1980). However, the species range of sina cactus is small relative to the range sizes of both species limiting the overall degree of host plant sharing. Furthermore, rearing *D. mojavensis* on agria cactus reduces premating isolation among populations to nonsignificant levels in comparison to laboratory food and organ pipe cactus (Etges, 1992, 1998; Brazner and Etges, 1993). In similar laboratory trials, rearing *D. mojavensis* and *D. arizonae* on agria cactus also reduced premating isolation from that observed with laboratory-food-reared flies (Yule's $V \pm 1$ SE, $0.811 \pm 0.076 > 0.643 \pm 0.008$, ($\chi^2 = 4.83$, 1 *df*, $P = 0.028$) (Etges, unpublished data), and alters amounts of a number of cuticular hydrocarbon components (Table 2). Thus, the documented sharing of host plants is crucial to the understanding of the evolution of sexual isolation within *D. mojavensis* and between *D. mojavensis* and *D. arizonae*. In the group as a whole, ecological isolation is perhaps the main factor contributing to species isolation.

There have been no studies of sexual isolation between *D. arizonae* and *D. navojoa*, but the degree of genetic differentiation and postmating isolation between them (Ruiz et al., 1990) suggests sexual isolation should be at least as strong as that between *D. mojavensis* and *D. navojoa* (Markow and Maveety, 1985). The role of epicuticular hydrocarbon variation in sexual isolation among *D. mojavensis* cluster species has yet to be studied. Given the degree of geographic variation within *D. mojavensis* cluster species in epicuticular hydrocarbons, it is reasonable to infer that epicuticular hydrocarbons may differentiate prior to species divergence. This is consistent with the broad-scale variation between Baja California and mainland populations of *D. mojavensis* that is responsible for premating isolation between populations (Stennett and Etges, 1997; Etges and Ahrens, 2001). Species-specific mate recognition and sexual isolation may then be more influenced by behavioral and mating song differences (Wasserman and Koepfer, 1977; Byrne, 1999) when species are drawn to the same cacti. Further within-species data concerning mating song variability as well as determination of the role of epicuticular hydrocarbons in species mate recognition is badly needed.

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APPENDIX 1. NESTED ANOVA RESULTS FOR MAJOR HYDROCARBON COMPONENTS IN TWO POPULATIONS OF *D. mojavensis*, *D. Arizonae*, AND *D. navojoa* REARED ON AGRIA AND *Opuntia* CACTI[a]

Source	*df*	Type III SS	Mean square ratio	*F* value	*P*	Type III SS	*F* value	*P*
			A. *n*-Octacosane (C_{28})			B. 2-Methyloctacosane ($C_{28.65}$)		
1. Species	2	66.16	1/2	0.07	NS	466440.30	4.53	NS
2. Population(species)	3	1448.57	2/5	7.62	0.076	154543.83	45.87	0.032
3. Cactus	1	244.14	3/5	3.66	NS	3654.64	71.13	0.003
4. Cactus × species	2	9.98	4/5	0.07	NS	2271.73	22.26	0.015
5. Cactus × population(species)	3	200.23	5/6	1.17	NS	151.94	0.01	NS
6. Replicates	24	1394.31	6/12	12.11	0.0001	12086.19	1.25	NS
7. Sex	1	0.78	7/11	0.55	NS	46229.80	31.28	0.011
8. Sex × species	2	21.51	8/11	7.64	0.066	7184.62	2.43	NS
9. Sex × cactus	1	0.73	9/12	0.15	NS	815.95	2.02	NS
10. Sex × cactus × species	2	8.69	10/12	0.91	NS	1179.00	1.46	NS
11. Sex × population(species)	3	4.21	11/12	0.29	NS	4439.56	3.67	0.015
12. Error	95	455.90				38300.52		
			C. 2-Methyltricontane ($C_{30.65}$)			D. 7- and 9-Hentricontene ($C_{30.78}$)		
1. Species	2	77253.42		2.65	NS	834.04	1.55	NS
2. Population(species)	3	43813.11		33.28	NS	807.81	1.07	NS
3. Cactus	1	5636.83		9.99	0.049	979.90	5.87	0.094
4. Cactus × species	2	3142.43		2.79	NS	318.65	22.26	0.015
5. Cactus × population(species)	3	1685.56		0.43	NS	501.69	2.68	0.069
6. Replicates	24	30855.63		0.72	NS	1518.05	2.72	0.0003
7. Sex	1	62546.87		37.44	0.009	210.31	1.96	NS
8. Sex × species	2	6082.50		1.82	NS	262.92	1.22	NS
9. Sex × cactus	1	654.90		0.37	NS	29.56	1.27	NS
10. Sex × cactus × species	2	11518.45		3.21	0.045	84.64	1.82	NS
11. Sex × population(species)	3	5011.01		0.93	NS	322.95	4.62	0.005
12. Error	95	170412.19				2213.05		

(*Continued*)

APPENDIX 1. CONTINUED

Source	*df*	Type III SS	Mean square ratio	*F* value	*P*	Type III SS	*F* value	*P*
		E. 31-Methyldotricont-8-ene ($C_{32.47}$)				F. 31-Methyldotricont-6-ene ($C_{32.56}$)		
1. Species	2	186456.47		2.78	NS	106356.06	438.34	0.0002
2. Population(species)	3	100815.85		3.14	NS	364.02	0.37	NS
3. Cactus	1	23395.77		8.34	0.063	19.47	0.05	NS
4. Cactus × species	2	34078.00		6.07	0.089	40.21	0.05	NS
5. Cactus × population(species)	3	8422.37		0.62	NS	1193.24	4.05	0.018
6. Replicates	24	110327.70		2.95	0.0001	2357.38	0.97	NS
7. Sex	1	71211.10		7.53	0.071	567.10	20.98	0.019
8. Sex × species	2	8506.10		0.45	NS	1168.77	21.67	0.016
9. Sex × cactus	1	1.08		0.00	NS	180.04	1.78	NS
10. Sex × cactus × species	2	3583.57		1.15	NS	185.53	1.84	NS
11. Sex × population(species)	3	28422.40		6.08	0.0008	80.68	0.27	NS
12. Error	95	148019.13				9602.00		
		G. 8,24-Tritricontadiene ($C_{32.63}$)				H. 7,25-Tritricontadiene ($C_{32.70}$)		
1. Species	2	498175.25		6.54	0.081	67692.13	4.64	NS
2. Population(species)	3	114307.36		3.29	NS	21923.18	1.04	NS
3. Cactus	1	32666.90		3.96	NS	38913.29	8.74	0.059
4. Cactus × species	2	33112.95		2.01	NS	15157.24	1.70	NS
5. Cactus × population(species)	3	24789.43		4.19	0.016	13385.06	1.48	NS
6. Replicates	24	48079.49		3.23	0.0001	73626.64	2.94	0.0001
7. Sex	1	6255.15		1.59	NS	5131.89	1.43	NS
8. Sex × species	2	3242.69		0.41	NS	6136.23	0.85	NS
9. Sex × cactus	1	331.25		0.53	NS	283.44	0.27	NS
10. Sex × cactus × species	2	434.06		0.35	NS	1549.09	0.74	NS
11. Sex × population(species)	3	11849.44		6.36	0.0006	10812.35	3.45	0.020
12. Error	95	58970.44				99169.17		

		I. 10-, 12-, and 14-Tritricontene ($C_{32.79}$)			J. 8,26-Tetratricontadiene (C_{34} diene)		
1. Species	2	27818.86	6.56	0.080	4216.17	4.47	NS
2. Population(species)	3	6365.78	0.45	NS	1414.92	1.93	NS
3. Cactus	1	3636.22	9.68	0.053	1093.09	6.20	0.088
4. Cactus × species	2	4403.26	5.86	0.092	537.05	1.52	NS
5. Cactus × population(species)	3	1128.59	1.36	NS	529.67	4.18	0.016
6. Replicates	24	6729.44	1.93	0.013	1030.80	2.96	0.0001
7. Sex	1	17123.73	3.82	NS	2.75	0.03	NS
8. Sex × species	2	17042.86	1.90	NS	7.08	0.04	NS
9. Sex × cactus	1	340.45	2.34	NS	6.58	0.45	NS
10. Sex × cactus × species	2	2508.13	8.63	0.0004	1.11	0.04	NS
11. Sex × population(species)	3	13485.57	30.94	0.0001	246.13	5.65	0.001
12. Error	95	13800.18			1380.69		
		K. 6,26- and 6,24-Tetratricontadiene (C_{34} alkene)			L. 10-, 12-, and 14-Tetretricontene (C_{34} ene)		
1. Species	2	5499.28	2.76	NS	295.35	0.14	NS
2. Population(species)	3	2992.63	0.31	NS	3162.55	1.49	NS
3. Cactus	1	20317.30	11.60	0.042	4837.71	8.50	0.062
4. Cactus × species	2	2390.59	0.68	NS	74.97	0.07	NS
5. Cactus × population(species)	3	5262.46	2.44	0.089	1709.73	2.46	0.087
6. Replicates	24	17549.61	2.77	0.0002	5648.90	3.38	0.0001
7. Sex	1	3414.26	2.00	NS	1551.16	7.52	0.071
8. Sex × species	2	1293.31	0.38	NS	242.45	0.59	NS
9. Sex × cactus	1	334.62	1.27	NS	136.37	1.96	NS
10. Sex × cactus × species	2	1587.81	3.00	0.054	218.56	1.57	NS
11. Sex × population(species)	3	5128.15	6.47	0.0005	619.94	2.97	0.036
12. Error	95	25107.80			6613.49		

(*Continued*)

APPENDIX 1. CONTINUED

Source	*df*	Type III SS	Mean square ratio	*F* value	*P*	Type III SS	*F* value	*P*
			M. 33-Methyltetratricont-10-ene (C_{35} alkene 1)			N. 33-Methyltetratricont-8-ene (C_{35} alkene 2)		
1. Species	2	29918.67		5.84	0.092	24751.34	3.66	NS
2. Population(species)	3	7692.63		1.70	NS	10155.61	1.90	NS
3. Cactus	1	97.68		1.05	NS	1445.43	2.98	0.003
4. Cactus × species	2	1125.77		6.09	0.087	14078.26	14.52	0.028
5. Cactus × population(species)	3	276.98		0.49	NS	1452.49	0.34	NS
6. Replicates	24	4542.96		0.82	NS	34574.21	1.47	0.096
7. Sex	1	6152.50		3.74	NS	5770.59	2.53	NS
8. Sex × species	2	2684.38		0.81	NS	1236.94	0.27	NS
9. Sex × cactus	1	686.96		2.98	0.088	4002.40	4.09	0.046
10. Sex × cactus × species	2	1331.97		2.89	0.061	3017.67	1.54	NS
11. Sex × population(species)	3	4948.71		7.16	0.0002	6846.91	2.33	0.079
12. Error	95	21901.10				92872.53		
			O. 9,25-Pentatricontadiene ($C_{34.59}$)			P. 8,26-Pentatricontadiene ($C_{34.66}$) and 7,27-Pentatricontadiene		
1. Species	2	5193134.70		2.33	NS	587654.86	0.18	NS
2. Population(species)	3	3351930.21		4.37	0.070	4848975.72	4.95	0.079
3. Cactus	1	855615.28		5.80	0.095	1691134.64	7.39	0.073
4. Cactus × species	2	533897.68		1.81	NS	499093.03	1.09	NS
5. Cactus × population(species)	3	443598.48		2.90	0.056	687547.88	1.14	NS
6. Replicates	24	1243459.08		2.80	0.0002	4904409.26	2.88	0.0001
7. Sex	1	126983.98		1.00	NS	27083.52	0.16	NS
8. Sex × species	2	47671.09		0.19	NS	39518.40	0.12	NS
9. Sex × cactus	1	9626.20		0.52	NS	6160.70	0.09	NS
10. Sex × cactus × species	2	62454.61		1.69	NS	283728.06	2.00	NS
11. Sex × population(species)	3	380112.01		6.84	0.0003	505519.50	2.38	0.075
12. Error	95	1759166.67				6735046.70		

			Q. Unknown alkene (C_{36} a)			R. Unknown alkene (C_{36} b)	
1. Species	2	1091.40	0.95	NS	11392.48	18.62	0.020
2. Population(species)	3	1727.91	1.20	NS	918.74	0.22	NS
3. Cactus	1	2156.25	5.81	0.095	3506.97	8.76	0.059
4. Cactus × species	2	87.28	0.12	NS	3206.82	4.00	NS
5. Cactus × population(species)	3	1115.42	4.46	0.012	1203.45	2.85	0.057
6. Replicates	24	2025.21	2.02	0.009	3422.58	2.57	0.0006
7. Sex	1	1148.69	7.64	0.07	24.89	0.02	NS
8. Sex × species	2	185.64	0.62	NS	536.90	0.25	NS
9. Sex × Cactus	1	279.75	6.69	0.011	102.06	1.84	NS
10. Sex × Cactus × species	2	252.38	3.02	0.054	728.48	6.56	0.002
11. Sex × population(species)	3	451.94	3.60	0.016	3235.43	19.42	0.0001
12. Error	95	3973.97			5275.30		
			S. 35-Methylhexatricont-10-ene (C_{37} ene)			T. 9,27-Heptatricontadiene ($C_{36.5}$)	
1. Species	2	1689.49	3.08	NS	66717.28	1.30	NS
2. Population(species)	3	822.83	2.38	NS	77250.84	1.11	NS
3. Cactus	1	91.21	23.08	0.017	10210.65	3.29	NS
4. Cactus × species	2	15.50	1.96	NS	7439.22	1.20	NS
5. Cactus × population(species)	3	11.83	0.55	NS	9315.69	7.05	0.0013
6. Replicates	24	171.95	0.95	NS	10516.01	0.81	NS
7. Sex	1	84.65	0.71	NS	20970.68	1.01	NS
8. Sex × species	2	78.95	0.33	NS	23414.31	0.56	NS
9. Sex × cactus	1	0.90	0.12	NS	1265.47	2.34	NS
10. Sex × cactus × species	2	2.41	0.16	NS	4761.07	4.40	0.015
11. Sex × population(species)	3	358.60	15.78	0.0001	62370.22	38.41	0.0001
12. Error	95	719.64			51418.34		
			U. Unknown alkadiene ($C_{36.6}$)			V. 14-, 16-, and 12-Hexatricontene ($C_{36.7}$)	
1. Species	2	75406.38	4.19	NS	90032.95	11.92	0.037
2. Population(species)	3	27042.92	0.44	NS	11339.85	0.63	NS
3. Cactus	1	23554.63	15.66	0.029	10287.00	15.36	0.029
4. Cactus × species	2	17626.42	5.86	0.092	7406.80	5.53	0.098
5. Cactus × population(species)	3	4515.40	1.07	NS	2010.28	0.61	NS
6. Replicates	24	33974.88	1.70	0.038	26522.15	2.22	0.004

(*Continued*)

APPENDIX 1. CONTINUED

Source	*df*	Type III SS	Mean square ratio	*F* value	*P*	Type III SS	*F* value	*P*
7. Sex	1	33606.64		1.69	NS	9022.62	1.56	NS
8. Sex × species	2	31350.25		0.79	NS	13946.76	1.20	NS
9. Sex × cactus	1	69.21		0.08	NS	148.10	0.30	NS
10. Sex × cactus × species	2	10801.58		6.47	0.0023	3553.79	3.56	0.032
11. Sex × population(species)	3	59888.07		23.91	0.0001	17415.24	11.65	0.0001
12. Error	95	79311.84				47354.13		
			W. Unknown alkene (C_{38})				X. Unknown (C_{39})	
1. Species	2	1253.94		1.26	NS	126.05	2.97	NS
2. Population(species)	3	1499.74		1.88	NS	63.77	0.88	NS
3. Cactus	1	1039.83		4.65	NS	107.23	5.36	NS
4. Cactus × species	2	19.16		0.04	NS	15.50	0.39	NS
5. Cactus × population(species)	3	671.88		2.49	0.084	60.09	3.33	0.035
6. Replicates	24	2189.03		2.22	0.003	144.04	0.92	NS
7. Sex	1	1689.51		20.40	0.020	0.01	0.00	NS
8. Sex × species	2	1427.34		8.61	0.057	48.33	2.28	NS
9. Sex × cactus	1	138.23		3.37	0.07	24.90	3.82	0.053
10. Sex × cactus × species	2	19.91		0.24	NS	4.59	0.35	NS
11. Sex × population(species)	3	248.68		2.02	NS	31.80	1.63	NS
12. Error	95	3901.38				618.41		
			Y. Unknown (C_{40})				Z. Total hydrocarbons	
1. Species	2	3.14		0.01	NS	13691361.37	1.03	NS
2. Population(species)	3	607.86		4.65	NS	20042887.39	1.73	NS
3. Cactus	1	300.93		9.72	0.052	14287083.41	9.08	0.057
4. Cactus × species	2	21.29		0.34	NS	5836835.07	1.85	NS
5. Cactus × population(species)	3	93.01		1.36	NS	4727332.59	1.59	NS
6. Replicates	24	552.01		1.42	NS	24082067.69	2.57	0.0006
7. Sex	1	696.11		24.22	0.016	4072518.47	1.52	NS
8. Sex × species	2	322.22		5.69	0.096	1902737.72	0.36	NS
9. Sex × cactus	1	108.94		6.74	0.011	54525.83	0.14	NS
10. Sex × cactus × Species	2	12.90		0.40	NS	2057103.82	2.63	0.077
11. Sex × population(species)	3	86.29		1.78	NS	8031515.40	6.85	0.0003
12. Error	95	1534.81				37105155.0		

[a] Approximate mean square ratios for the calculation of *F* values are indicated the first table (A) for C_{28} *n*-alkane. These same mean square ratios were used for each nested ANOVA.

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